การศึกษาผลของการใช้กรดไลโปอิกในเซลล์เยื่อบุเป็นระยะสั้นในแคลเซียมคาร์บอเนตระดับนาโนเมตร
ต่อการเปลี่ยนแปลงและพัฒนาของเซลล์ต้นกำเนิดจากไขมันและผลกระทบต่อหน้าที่ของเซลล์ไขมัน

นางสาวสโรชา เชิดโฉม

บทคัดย่อและเพิ่มเติมข้อมูลวิธีการศึกษา, วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

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Lipoic acid-incorporated calcium carbonate nanoparticles for controlling of adipose derived stem cells (ADSCs) differentiation and adipocyte function
Thesis Title: Lipoic acid-incorporated calcium carbonate nanoparticles for controlling adipose derived stem cells (ADSCs) differentiation and adipocyte function

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Field of Study: Medical Science

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CaCO₃ NPs has received a growing interest due to its potential properties as a safe drug carrier. The LA-CaCO₃ NPs were synthesized by a chemical precipitation method and characterized using transmission electron microscopy, thermogravimetric analysis, and zeta potential measurement. We used LA-CaCO₃ NPs to evaluate their therapeutic effects in lipid accumulation, lipolysis, adipogenesis, and adipokines expression in ADSCs and differentiated adipocytes. The average particle sizes were observed to be 10-20 nanometers. Thermogravimetric analysis results confirmed the percentages of LA in CaCO₃ NPs. The zeta potential showed a negative charge -30.9 mV. Our findings indicated that LA-CaCO₃ NPs were apparently more effective in lipid accumulation and lipolysis than LA alone. Additionally, LA-CaCO₃ NPs were also inhibit adipogenesis in ADSCs as well as adipokine expression. LA-CaCO₃ NPs directly reduced lipid accumulation by enhancing of lipolysis activity. Adipogenesis was also suppressed by LA-CaCO₃ NPs and eventually decreases in adipokine expression. The LA-CaCO₃ NPs with beneficial therapeutic effects could provide a promising approach to target adipose tissue for obesity interventions.
SAROCHA CHERDCHOM: Lipoic acid-incorporated calcium carbonate nanoparticles for controlling of adipose derived stem cells (ADSCs) differentiation and adipocyte function. ADVISOR: ASST. PROF. AMORNPUN SEREEMASPUN, M.D., Ph.D, CO-ADVISOR: ASST. PROF. ROJRIT ROJANATHANES, Ph.D., PROF. PORNANONG ARAMWIT, Pharm.D., Ph.D.

In this project, we have aimed to develop calcium carbonate nanoparticles (Drug carrier) that are safe and effective. The purpose of this research is to develop calcium carbonate nanoparticles loaded with lipoic acid for the study of their ability to reduce lipids, stimulate lipid breakdown, change and differentiate ADSCs, and influence the function of adipocytes. The results of this study show that calcium carbonate nanoparticles loaded with lipoic acid are effective in reducing lipid accumulation and stimulating lipid breakdown. In addition, they are effective in inhibiting the change and differentiation of ADSCs and inhibiting adipocyte cell cytokine release. These properties of calcium carbonate nanoparticles loaded with lipoic acid may lead to the development of calcium carbonate nanoparticles loaded with lipoic acid for the treatment of obese patients with excessive fat accumulation in the future.
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The most of all, my deepest is especially expressed my family who supports me in everything, for their encouragement, believing and continuous support all my success.
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Obesity is a rising global problem in human. It is an underlying risk factor of many common and severe human diseases, including type 2 diabetes, cardiovascular diseases and hypertension[1, 2]. Obesity in human is generally caused by excessive caloric intake accompanied by physical inactivity. Once the caloric intake is more than necessary, the excess fat is accumulated in the form of fatty acids and triglyceride in adipose tissue. Adipose tissue plays a key role in development of obesity and obesity-related disorders. Adipocyte-related cell lineage is originated from stem cells known as adipose-derived stem cells (ADSCs), which differentiated into adipocytes. Three major types of the adipocytes, including white, brown, and beige adipocytes, were found to have distinct functions. The major functions of white adipocyte are energy storage in the form of triglycerides and fatty acid release over the periods of starvation by lipolysis. On the other hand, brown and beige adipocytes have the capacity to use fatty acids and glucose through uncoupled respiration and heat production[3-5]. This thermogenesis process in brown and beige adipocytes also appears during prolonged energy restriction, resulting in a reduction of adiposity (6). Furthermore, adipose tissue is known to produce and secrete many bioactive polypeptides known as adipokines including adiponectin, leptin, and resistin. These adipokines play an influential role in energy homeostasis and development of diseases associated with obesity (7). Therefore, modulation of adipogenic differentiation of ADSCs into brown or beige adipocytes and increase of lipolysis attribute as interesting targets for therapeutic intervention in obesity.

The recommended therapy of obesity is non-pharmacological treatment such as physical exercise and lifestyle changes. Nevertheless, these methods for lifestyle modification is often counterproductive and unsuccessful because the success of
exercise in reducing weight loss vary between individuals. The effective pharmacological agents such as orlistat and sibutramine are alternatives for treatment of obesity, but usage of the drugs results in occurrence of some side effects and drug tolerance. (8, 9) Among the alternative medicines for anti-obesity, 1,2-dithiolane-3-pentanoic acid or thiocytic acid, also known as α-lipoic acid (LA), exhibits very potent antioxidant activity (10). It is found in many natural resources and abundant in spinach, cabbage, broccoli, and tomato (11). Interestingly, LA is a compound with beneficial effects on obesity. Previous studies have described functions of LA in anti-obesity by reducing body weight gain in rodents and human, (12, 13) stimulating lipolysis in adipocyte, (14) and inhibiting of adipogenesis in 3T3-L1 cells (15). However, LA is unstable and poor aqueous solubility, leading to low bioavailability (16). Improving a novel drug delivery system is determined to be crucial for enhancing therapeutic efficacy in obesity.

In recent years, there have been widespread interests in the use of nanotechnology as a drug delivery system for enhancing therapeutic efficacy. Among various nanoparticles used as drug carriers, CaCO₃ nanoparticles (CaCO₃ NPs) are introduced and it has attracted considerable attention due to a potential drug carrier for their innovative properties such as good bioavailability, low toxicity, and slow biodegradability (17). From a positive viewpoint, the good sustained-release performance and high stability of CaCO₃ NPs expose new avenues of a proper drug delivery carrier in this study (17).

Our study was conducted to evaluate the properties of CaCO₃ NPs, including its use as a vehicle for delivery of LA in ADSCs. We developed the LA-incorporated CaCO₃ NPs (LA-CaCO₃ NPs) using chemical precipitation and evaluated the efficacy of LA-CaCO₃ NPs by lipolytic activity, adipogenesis, and adipokines expression. Here, we show that LA-CaCO₃ NPs exert potent potential benefits for lipid reduction in ADSCs, compared to anti-obesity properties of LA alone.
1. Obesity

Obesity is a worldwide epidemic. The incidence of obesity (and/or being overweight) is dramatically increasing globally in both children and adults. It has been considered a public health issue due to its association with many diseases such as insulin resistance, diabetes, metabolic syndrome, and increased cardiovascular morbidity and mortality (1, 2). Obesity also has serious social and psychological consequences such as low self-esteem and clinical depression and affects all ages and socioeconomic groups (18). Many practical methods used in clinical practice to assess body fat (Table 1). Heights and weights were obtained using standardized methods (19). The World Health Organization (WHO) definition is a person with a BMI of 30 or more is generally considered obese. A person with a BMI equal to or more than 25 is considered overweight. It is defined as a person’s weight in kilograms divided by the square of his height in meters (kg/m²). A WHO expert committee has proposed the classification of overweight and obesity that applies to both men and women and to all adult age groups (Table 2). The behavioral and environmental changes such as poor dietary habits and sedentary lifestyle contribute the development of obesity (2). However, the excess accumulation of lipid within adipose tissue is a fundamental to the development of obesity by alteration of adipose tissue metabolic and endocrine function.
Table 1 Defining obesity (18).

<table>
<thead>
<tr>
<th>Method</th>
<th>Definition</th>
<th>Advantages/limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMI</strong></td>
<td>Weight in kilograms divided by square of the height in meters.</td>
<td>BMI correlated strongly with densitometry measurements of fat mass; main limitation is that it does not distinguish fat mass from lean mass.</td>
</tr>
<tr>
<td><strong>Waist circumference</strong></td>
<td>Measured (in centimeters) at midpoint between lower border of ribs and upper border of the pelvis.</td>
<td>Waist circumference and waist-to-hip ratio provide measures for assessing upper body fat deposition; neither provide precise estimates of intra-abdominal (visceral) fat.</td>
</tr>
<tr>
<td><strong>Skinfold thickness</strong></td>
<td>Measurement of skinfold thickness (in centimeters) with callipers provides a more precise assessment if taken at multiple sites.</td>
<td>Measurements are subject to considerable variation between observers require accurate callipers and do not provide any information on abdominal and intramuscular fat.</td>
</tr>
<tr>
<td><strong>Bioimpedance</strong></td>
<td>Based on the principle that lean mass conducts current better than fat mass because it is primarily an electrolyte solution; measurement of resistance to a weak current (impedance) applied across extremities provides an estimate of body fat using an empirically derived equation.</td>
<td>Devices are simple and practical but neither measure fat nor predict biological outcomes more accurately than simpler anthropometric measurements.</td>
</tr>
</tbody>
</table>
Table 2 Cut-off points proposed by a WHO expert committee for the classification of overweight (18).

<table>
<thead>
<tr>
<th>BMI* (kg m$^{-2}$)</th>
<th>Classification</th>
<th>Popular description</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;18.5</td>
<td>Underweight</td>
<td>Thin</td>
</tr>
<tr>
<td>18.5-24.9</td>
<td>-</td>
<td>‘Healthy’, ‘normal’, ‘acceptable’</td>
</tr>
<tr>
<td>25.0–29.9</td>
<td>class I obesity</td>
<td>Overweight</td>
</tr>
<tr>
<td>30.0–39.9</td>
<td>class II obesity</td>
<td>Obesity</td>
</tr>
<tr>
<td>I≥40.0</td>
<td>class III obesity</td>
<td>Morbid obesity</td>
</tr>
</tbody>
</table>

1.1 Adipose tissue

Adipose tissue plays an essential role in regulating energy balance through its metabolic, cellular and endocrine functions. It is an organ that typically functions as the body’s energy storage in the form of triglyceride and mobilizing energy in the form of fatty acids. There are homeostatic mechanisms in adipose tissue to balance lipid storage and mobilization because fatty acids and their metabolic products can be toxic in peripheral organs. Lipotoxicity and the accumulation of saturated fat in peripheral tissues are an important step in the development of metabolic syndrome.

Adipose tissue is included of many cell types including the precursor cells of the adipocyte, preadipocytes, adipocytes, vascular endothelial cells, and immune cells. However, adipocytes are abundant in adipose tissue that differentiates from adipocyte progenitors/adipose-derived stem cell (ADSCs) by adipogenesis (Figure 1). Adipogenesis, the differentiation of fibroblast-like mesenchymal stem cells (MSCs) into adipocytes, plays an essential role in regulation of whole body energy metabolism. At the cellular level, adipogenesis is generally described as a two-step process: a commitment step,
wherein committed adipocyte progenitors (or preadipocytes) are generated from multipotent MSCs, and a differentiation step, wherein pre-adipocytes acquire the features of mature, functional adipocytes (20). The generation of adipocytes by adipogenesis contributes development of obesity (21).

There are three types of adipocytes including white, beige, and brown adipocytes with different phenotype, function, and regulation (Table 3). The major adipocytes in the body consist of white adipocytes. It is predominant form found in adults (brown adipose tissue is principally found in neonates) and contribute to metabolic disease in obesity (22). White adipocytes also develop in multiple anatomical sites, and there are known metabolic and endocrine differences between subcutaneous and visceral adipocytes (23). The function of white adipocytes is store excess energy as lipids to regulate energy homeostasis and fatty acid release over periods of starvation by lipolysis. Lipolysis in white adipocytes is a process leading to release of fatty acids and glycerol to the plasma. Additionally, white adipocytes are also an important target for the action of human therapeutics such as the thiazolidinedione (TZD) antidiabetic drugs being the most notable example (24).

In contrast, the function of brown adipocytes is to transfer fatty acids from food and generate heat (thermogenesis), particularly in human neonates. There is abundant mitochondrial content in brown adipocytes because it require a large amount of ATP to maintain processes such as lipolysis, β-oxidation of fatty acids, and fatty acid synthesis. Moreover, brown adipocytes contain a specialized mitochondrial protein called UCP1 to generate heat (25). Cold exposure can stimulate lipolysis, which activates sympathetic nervous system signaling in brown adipocytes. The free fatty acids from lipolysis are used to generate heat using the UCP1 protein. Recent study has shown that brown adipocytes are dispersed throughout the human adipose tissue and are
metabolically active (26). Modulation of brown adipocyte activity has been proposed as a potential strategy in obesity and its associated diseases.

Interestingly, white adipocytes have ability to switch between energy storage and expenditure. White adipocytes phenotype can shift into brown-like adipocytes phenotype in terms of features such as morphology, gene expression pattern, and mitochondrial respiratory activity (27). This process is called the “browning” of white adipocytes. The brown-like adipocytes appearing in white adipocytes are often called “inducible, beige, or brite adipocytes.” The appearance of these inducible brown adipocytes in white adipocytes may also involve trans-differentiation processes of white-to-brown adipocytes (23). The beige adipocytes are derived from precursor cells that are different from classical brown adipocytes and are closer to the white adipocytes lineage (27, 28). These beige adipocytes appear a white adipocyte-like phenotype, such as large lipid droplets and the lack of UCP1 expression, under basal conditions. Nevertheless, in response to certain stimuli, beige adipocytes transform into a brown adipocyte-like phenotype, such as multilocular/small lipid droplets and UCP1 expression (29, 30). The activation of brown/beige adipocytes in humans may contribute to an important strategy for treating obesity.
Figure 1 Differentiation into white, beige, or brown adipocytes. Modified from (28)
Table 3 Differences amongst the three types of adipocytes (31).

<table>
<thead>
<tr>
<th></th>
<th><strong>White adipocytes</strong></th>
<th><strong>Beige adipocytes</strong></th>
<th><strong>Brown adipocytes</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location</strong></td>
<td>Inguinal (sWAT), mesenteric, retroperitoneal, perigonadal, omental (vWAT)</td>
<td>Within inguinal WAT, other sWAT?</td>
<td>Interscapular, perirenal, axillary, paravertebral</td>
</tr>
<tr>
<td><strong>Morphology</strong></td>
<td>Unilocular/large lipid droplets</td>
<td>Unilocular large/multiple small lipid droplets</td>
<td>Within inguinal WAT, other sWAT?</td>
</tr>
<tr>
<td><strong>Function</strong></td>
<td>Storage of energy as triglycerides</td>
<td>Adaptive thermogenesis</td>
<td>Heat production</td>
</tr>
<tr>
<td><strong>Mitochondria</strong></td>
<td>(+)</td>
<td>Upon stimulation (++)</td>
<td>(+++)</td>
</tr>
<tr>
<td><strong>UCP1</strong></td>
<td>Nearly undetectable</td>
<td>Upon stimulation (++)</td>
<td>(+++)</td>
</tr>
<tr>
<td><strong>Obesity</strong></td>
<td>Positive effect</td>
<td>Negative effect</td>
<td>Negative effect</td>
</tr>
</tbody>
</table>

**Abbreviation**: WAT, white adipose tissue; vWAT, visceral white adipose tissue; sWAT, subcutaneous white adipose tissue; UCP1, uncoupling protein 1
### 1.1.1 Specific cell surface marker in adipocyte population

Adipose tissue exists in three forms, white, beige and brown adipocytes, which differ in terms of morphology, function, abundance and regulation. Specific cell surface marker is used to distinguish adipocyte population in adipose tissue. A panel of molecular markers has been identified white, beige, and brown adipocytes (32-34). These include leptin, HOXC8, and HOXC9 for white adipocytes; TBX1 and TMEM26 for beige adipocytes; and UCP-1, CIDEA, and PRDM16 for brown adipocytes. These markers have been used to characterize adipocyte subtypes. Nevertheless, some of these markers such as PRDM16 are also expressed in non-adipose tissues (33). Consequently, specific surface markers would be very helpful in identifying and targeting different adipocyte subpopulations. For this reason, Ussar and colleagues have identified specific cell surface markers for adipocyte population using a combination of 

in silico, in vitro, and in vivo studies. These results show that ASC-1, PAT2, and P2RX5 are newly identified cell-surface proteins. These proteins localized the plasma membrane of adipocytes that can define the heterogeneity and intrinsic differences of adipose tissue depots. ASC-1 is a white adipocyte–specific cell surface protein, with no expression in beige and brown adipocytes. PAT2 are cell surface markers express in beige adipocytes and P2RX5 is specific in brown adipocytes (35).

Ussar and colleagues have identified specific cell surface markers for adipocyte population. It arises regarding the physiological role of these markers in adipocyte biology. The specific cell surface markers including ASC-1, PAT2, and P2RX5 are induced upon adipocyte differentiation but nearly absent in mouse and human preadipocytes, suggesting a role in adipocyte formation and/or physiology. Moreover, expression of PAT2 and P2RX5 was lower in BAT of diabetic (db/db) mice, which is in line with the “whitening” of brown adipose
tissue. Contrary, whereas $P2RX5$ expression was induced in WAT upon cold exposure (also known as “browning” of WAT), ASC-1 (amino acid transporter) was down-regulated.

PAT2 and P2RX5 are both membrane transporters (amino acid transporter and ion channel, respectively), they may be associated with intracellular signaling pathways involved in activation of brown adipocytes. A main activation route of brown adipocytes is the $\beta_3$-adrenergic pathway, resulting in increased levels of intracellular cyclic cAMP, activation of protein kinase A, and subsequent phosphorylation of downstream targets. Whether the $\beta_3$-adrenergic pathway and/or other intracellular pathways are coupled to PAT2 and P2RX5 membrane transporters remains to be evaluated.

1.1.2 Regulation of lipolysis and thermogenesis in adipocytes

Adipose tissue is richly innervated by sympathetic and parasympathetic fibers. It requires large amounts of ATP to maintain their functions. The main roles of adipose tissue are lipogenesis and lipolysis (36). The lipogenesis is to store excess dietary fat in the form of triglycerides. On the other hand, lipolysis is hydrolysis of triglycerides to fatty acids and glycerol in adipocytes (Figure 2). These processes are importance for the control of lipid and carbohydrate metabolism (37).

When nutrient excess, energy uptake by white adipocytes is regulated by insulin’s anabolic role, mediating glucose and lipid uptake, lipogenesis and inhibition of lipolysis. On the other hand, energy deficits caused by energy shortages (for example, fasting) or increased energy expenditure (for example, cold exposure) are regulated by the sympathetic nervous system (SNS) (38, 39). The tone of the sympathetic innervation exerts master control on lipolysis by the release of noradrenaline (also known as norepinephrine) (40).
Physiologically, cold exposure stimulates sympathetic nervous system crosstalk between white, beige, and brown adipocytes with respect to nutrient release, partitioning and utilization (41). Upon cold exposure, the neurotransmitter noradrenaline stimulates $\beta_3$-adrenergic pathway, resulting in the lipolysis in adipocytes. Increased sympathetic tone to white adipose tissue activates lipolysis resulting in intracellular lipid stores are released to provide nutrients for other organs.

The lipolysis consists of at least three major enzymes including adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL). The primary cleavage of triacylglycerol to diacylglycerols is performed by adipose triglyceride lipase (ATGL). Hormone-sensitive lipase (HSL) is the major diglyceride lipase in adipocytes, and monoglyceride lipase (MGL) completes the process by generating glycerol and free fatty acids. The free fatty acids are the main fuel for UCP1 mediated brown adipose tissue thermogenesis (42). Increased sympathetic tone to brown adipose tissue also rapidly induce intracellular lipolysis and eventually lead to free fatty acids release from lipid droplets to locally activate UCP1 and to serve as substrates for oxidation (41). Importantly, the substrate uptake machinery is also upregulated, allowing lipids released by white adipocytes to be taken up by brown adipocytes (42). Sustained SNS activation will also result in increased thermogenic capacity due to increased thermogenic gene expression and, in cases of chronic activation, due to proliferation and differentiation of brown adipocyte precursors (42).
Moreover, white adipocytes also recruit and activate beige adipocytes by increased SNS tone, which might contribute to increased total thermogenic capacity (43, 44). The function of beige adipocytes is an energy burning that has yet to be clarified in rodents. It is difficult to experimentally distinguish the thermogenic contribution of beige adipocytes from that of brown adipocytes. Because there are several factors including cold exposure, which activate and recruit beige adipocytes as well as brown adipocytes.

Additionally, lipolysis has been driven by β-adrenergic pathway in the adipocyte, but other inducers exist and may have physiological relevance. For example, the transcription factor FOXC2 sensitizes adipocytes to adrenergic stimulation by modulating the expression and activity of adrenergic signaling molecules (45-47). Moreover, BMP family is essential for brown adipose tissue development and energy balance (48). BMP8B induces the phosphorylation of downstream adrenergic targets such as hormone-sensitive lipase and p38 MAPK signalling through Smad1, 5 and 8 (49). BMP7 can also drive brown adipogenesis and recruit beige adipocytes by activating p38 MAPK (50). However, there are several alternative mechanisms that activate brown and beige adipocytes independently of the SNS have been studied.
The sympathetic nervous system triggers intracellular signaling events that lead to lipolysis (black arrows). Noradrenaline (NA) released by sympathetic nerve endings binds to β3-adrenoreceptors (β-ARs), which couple to GαG-proteins that activate adenylyl cyclase (AC), increasing the cellular concentration of cAMP. This activates protein kinase A (PKA), which phosphorylates lipid droplet proteins including hormone-sensitive lipase (HSL) and perilipin, allowing activation of adipose triglyceride lipase (ATGL). The net result is the hydrolysis of triglycerides stored in the lipid droplet to free fatty acids (FFAs), which are released. This response can be attenuated (red arrows) by the activation of phosphodiesterases (PDE), which hydrolyse cAMP. In the case of recruitment and activation of beige adipocytes and the activation of brown adipocytes, this signalling pathway (blue arrows) also leads to increased expression of UCP1 and other thermogenic genes through the p38 MAPK cascade. FFAs released by lipolysis activate existing UCP1 in the mitochondria, and their oxidation produces heat.
1.2 Adipose depots

Although the total adipose tissue is important for the development of insulin resistance, it is considered that some fat depots are more linked to risk factors for many diseases. There is different adipose tissue between subcutaneous and visceral adipose tissue. Whole body adipose tissue has generally subdivided in two main components: subcutaneous and visceral adipose tissue.

1.2.1 Subcutaneous adipose tissue

Subcutaneous adipose tissue is the most easily identifiable because it is stored directly under the skin. Subcutaneous adipose tissue is less metabolically active than visceral adipose tissue, it may have better short-term and long-term storage capacity. Thus, this depot is important to accumulate triglyceride in periods of excess energy intake and supply the organism with free fatty acids in periods of fasting, starvation, or exercise. Another suggested a role of subcutaneous adipose tissue is to be a buffer during intake of dietary lipids, thus protecting other tissues from lipotoxic effects (37). New smaller adipocytes act as a powerful buffer, which absorb free fatty acid and triglyceride after caloric intake. As adipocytes grow larger, they become dysfunctional capacity. Large adipocytes are insulin resistant, hyper lipolytic and resistant to anti-lipolytic effect of insulin. Subcutaneous adipose tissue contains the small adipocytes, which are more insulin-sensitive and high absorb for free fatty acids and triglyceride, preventing their deposition in non-adipose tissue. On the other hand, visceral adipose tissue contains greater number of large adipocytes.
1.2.2 Visceral adipose tissue

Visceral adipose tissue is of great interest. The excess visceral adipocytes accumulation of obesity is associated with many risk factors in obesity-related disorders. It generally has indistinctly included omental, mesenteric as well as extraperitoneal adipose tissue. Mesenteric and omental adipose tissue are drained by the portal vein, a feature which has been at the center of some hypotheses linking visceral adipose tissue accumulation and metabolic disease (52, 53). Anatomical position, molecular biology and physiology in visceral adipose tissue quite different among the subcutaneous adipose tissue. Visceral adipose tissue has been shown to correlate with the development of insulin resistance compared with subcutaneous adipose tissue (54). Additionally, subcutaneous and visceral adipose tissues seem to display depot-specific responsiveness to lipolytic activity. Lipolysis in visceral adipose tissue are more responsive to adrenergic agonist stimulation than subcutaneous adipocytes (55). Lipolysis is markedly increased in visceral adipocytes as compared to subcutaneous adipocytes in obese males, due to $\beta_3$-adrenoceptor is higher in visceral adipose tissue (56). In obesity, visceral adipose tissue is more sensitive to weight loss than subcutaneous adipose tissue (57). Furthermore, excess visceral adipocytes accumulation results in altered release of adipokines (7).
1.3 Adipokines

Adipose tissue is also recognized as an important endocrine organ, which secretes several peptides and other factors, which are known as adipokines (58). Adipokines have been linked to adipose tissue dysfunction and metabolic complications of obesity.

1.3.1 Adiponectin

Adiponectin, an adipose-tissue derived hormone, are associated with a decreased risk for development of obesity, cardiovascular disease, and type 2 diabetes (59). Adiponectin is mainly secreted by adipocytes and abundantly present in the circulation. There are at least 3 forms in blood circulation including low molecular weight, middle molecular weight, and high molecular weight. The high molecular weight adiponectin is considered the most metabolically active form (60). Adiponectin receptors are expressed in many tissues such as muscle, liver tissue, and mainly adipocytes. The functions of adiponectin have been shown to play important roles in the modulation of glucose and lipid metabolism. It is commonly anti-inflammatory by inflammatory cytokine suppression such as TNF-α (61). Serum levels of adiponectin are reduced in obese and insulin-resistant human subjects and animal models (62-65). Contrary, diet-induced weight loss induces adiponectin synthesis, parallel by an increase in plasma levels of adiponectin. Interestingly, adiponectin is also antidiabetic adipokine that enhances insulin sensitivity (66, 67).
1.3.2 Leptin

Leptin, a 16kDa product of obese (Ob) gene, is produced mainly by adipocytes. The functions of leptin regulate food intake, glucose and lipid metabolism and energy expenditure via the central nervous system (68). Biological activities of leptin are mediated through binding to the transmembrane receptor (ObR), a member of the class I cytokine receptor superfamily, that includes six isoforms different in the length of their intracellular tails. The binding of leptin induces the dimerization of receptor molecules and leads to the activation a broad array of multiple intracellular downstream signal pathways (69). Plasma levels of leptin highly correlate with body mass index (BMI). Most obese individuals have an increased food intake despite high circulating leptin levels, which is mentioned to as leptin resistance (70). The leptin levels are decreased while weight loss due to food restriction is associated with a decrease in plasma leptin in mice and humans (71). Additionally, leptin plays a role in controlling the immune system and inflammatory response. It stimulates proliferation of immune cells, by inducing the production of inflammatory cytokines (72, 73). There is strongly association between leptin levels and insulin sensitivity in human (74).
1.3.3 Resistin

Resistin is another pro-inflammatory adipokines that derived from both adipocytes and immune cells, including macrophages and monocytes (75). Resistin is a member of the cysteine-rich family of resistin-like molecules (RELMs) that are related to the activation of inflammation. Resistin plays a role in the pathogenesis of obesity and insulin resistance in humans, both of which appear to contribute to the development of type 2 diabetes. Resistin levels are increased in diet-induced obesity as well as in genetic models of obesity and insulin resistance. Administration of anti-resistin antibody improves blood sugar and insulin action in mice with diet-induced obesity (76). Moreover, resistin has been shown to associate Type 2 diabetic patients (77). The mechanisms of resistin in obesity and obesity-related disorders remain unresolved. Interestingly, new targets for pharmacological intervention to decrease the level of resistin will be identified, thereby providing a novel therapeutic strategy for the treatment of obesity.
2. Lipoic acid (LA)

Lipoic acid (LA), also known as 1,2-dithiolane-3-pentanoic acid or thiocetic acid, has one chiral center and therefore exists in both R- and S-enantiomeric forms (Figure 3). The R-LA is essential as a mitochondrial α-ketoacid dehydrogenases, and thus serves a critical role in mitochondrial energy metabolism (78, 79). LA can be found from food in cabbage, spinach, liver, meat, whole-wheat and yeast of beer. Moreover, it is also endogenously produced by the liver by the lipoic acid synthase (78).

![Figure 3 The R and S enantiomers of lipoic acid. Modified from (79)](image)

Anti-obesity effects LA

Recently, LA has gained considerable because of its diverse biological actions. It is a unique antioxidant because LA has beneficial effects on fuel metabolism. LA exhibits potential to enhance glucose transport into skeletal muscle of lean and insulin-resistant obese animals (80, 81). The effects of LA contribute potential beneficial effects on anti-obesity properties both in rodents and humans without serious side effects (12, 13, 81-83). Supplementation of a high fat diet induced a lower body weight gain and adipose tissue size in both control and high-fat fed rats (12). Oral administration of LA in diabetic mice fed on a high-fat diet and db/db mice
dramatically reduced the body weight and visceral fat content by activation of SIRT1 (82). In obese individuals, LA also reduce body weight gain (13). The action of LA decreases hypothalamic AMPK activity, which a fuel sensor in the cell and cause profound weight reduction (84).

During the adipocyte differentiation, lipid droplets are gradually accumulated in the cytoplasm for energy storage. The excessive accumulation of adipocytes by adipogenesis generally causes development of obesity (21). LA directly controls adipogenesis through activation of adipogenic transcription factors, the direct modulation of intracellular degradation process and consequently decrease intracellular lipid accumulation of adipocytes (15, 85). LA has a beneficial property to regulate adipogenesis by induction of beige adipocytes in white adipocytes from overweight/obese subjects. The beige adipose features were characterized by increased mitochondrial biogenesis and the acquirement of beige adipocytes features through the SIRT1/PGC-1α pathway (86). Nevertheless, it is not known whether the LA is effective in ADSCs.

Adipose tissue is a target for LA therapeutic properties. Activation of lipolysis has been proposed as a promising therapeutic target for the treatment of obesity. LA exerts lipolysis in white adipocytes by significantly increasing the PKA-mediated phosphorylation of hormone-sensitive lipase (14). Dietary LA supplementation increase plasma free glycerol and the rate of free glycerol release from abdominal adipose tissue (87, 88). Increased free fatty acids levels from adipose tissue has been associated with the obesity-related complications (89). Nevertheless, increased lipolysis in adipose tissue does not necessarily increase serum free fatty acids levels. Free fatty acids are taken by other metabolic tissues toward increased free fatty acids utilization and energy expenditure, and therefore
protects against obesity (14, 90). The effect of LA in lipolysis seem to be independent of its antioxidant capacities, other antioxidants such as vitamin C or N-acetylcysteine are not able to induce lipolysis under similar conditions (14). Additionally, LA has potential to prevent or decrease lipid accumulation not only in adipose tissue, but also in other non-adipose (91-94).

The importance of adipokines in the development of obesity and obesity-related diseases has been widely studied. Indeed, adipocytes secrete multiple bioactive peptides, such as adiponectin, leptin and resistin that play key roles in the regulation of energy metabolism and insulin sensitivity (7). Dysregulated adipokine secretion or production from the adipose tissue of obese individuals contributes to the development of obesity-related disorders. LA has potential to regulate adipokines in adipocytes. Adiponectin is an insulin-sensitizing adipokine that regulates glucose and lipid metabolism, and its levels are inversely correlated with fat accumulation in adipose tissue (66, 67). Dietary supplementation with LA can improve circulating adiponectin levels and adiponectin mRNA expression in adipose tissue (95, 96). However, LA induce a dose-dependent inhibition on adiponectin mRNA expression and protein secretion in 3T3-L1 adipocytes (97). Additionally, LA presents a concentration-dependent (1–500 µM) inhibition of leptin secretion and gene expression (98). Circulating leptin levels are reduced by LA with the reduction of bodyweight and fat mass (93, 98, 99). Resistin is an adipokine that involved in the development of obesity and insulin resistance (100). There is no report the effect of LA in resistin.
3. Nanotechnology

In recent years, there has been widespread interest in the use of nanotechnology as specifically drug delivery system for enhancing therapeutic efficacy. Many substances are under investigation for drug delivery system. Recently, there has been interested in the use of nanoparticles as drug carriers. The reason why nanoparticles are attractive for drug delivery system is based on their important and unique features, such as their surface area is much larger than that of other particles, their quantum properties and their ability to adsorb and carry other compounds. A large (functional) surface of nanoparticles, which can bind, adsorb and carry other compounds such as drugs, probes and proteins (101, 102). Typically, the drug of interest is dissolved, entrapped, adsorbed, attached and/or encapsulated into or onto nanoparticles, which depend on the method of preparation nanoparticles (103). Pharmaceutical nanoparticles can advantageously improve therapeutic efficacy by enhancing the drug bioavailability, serum stability and and/or a reduction in the toxicity of the free drug to non-target organs (101). Various types of nano drug delivery systems, mostly organic and some inorganic ones have been investigated for their ability to serve as carriers in drug delivery. Among various nanoparticles, CaCO$_3$ nanoparticles (CaCO$_3$ NPs) have been introduced and attracted considerable attention as a relatively safe drug vehicle (104, 105)
CaCO$_3$ NPs have been interested as drug carrier because of their innovative property such as availability, biocompatibility and low biodegradability. The drug incorporated into CaCO$_3$ NPs are stable and released very slowly (106). Moreover, CaCO$_3$ NPs appear potential to reduce the cell viability and enhance the apoptosis of the breast cancer cells (107). However, the mechanism of CaCO$_3$ NPs should be considered. The engineered CaCO$_3$ NPs increase the stability and retention time of the drug (108-111). For a positive viewpoint, especially the good sustained-release performance and high stability provide the new approaches for a proper drug delivery carrier.
Research questions
Do LA-CaCO$_3$ NPs affect the lipolysis in adipocytes and differentiation of ADSCs to adipocytes?

Research objectives
To study the effect of LA-CaCO$_3$ NPs in lipolysis and differentiation of ADSCs to adipocytes.

Research hypothesis
The LA-CaCO$_3$ NPs stimulate the lipolysis and differentiation of ADSCs to beige/brown adipocytes.
Conceptual framework
CHAPTER III
MATERIALS AND METHODS

Part I
Preparation of LA-CaCO$_3$ NPs

The synthesis of LA-CaCO$_3$ NPs follow the previous paper (106). Chemical precipitation for the preparation of CaCO$_3$ NPs were used in this study. Briefly, nanoparticles were prepared by the reaction of calcium ions from the aqueous solution from one hand, and carbonate ions from the aqueous solution from another. The aqueous solution of a LA was added into aqueous solutions in the process and then the formation of nanoparticles and entrapment of compounds was performed simultaneously. The LA-CaCO$_3$ NPs were separated by centrifugation.

Characterization of LA-CaCO$_3$ NPs

Thermogravimetric analysis (TGA)

The compositions of the LA-CaCO$_3$ NPs were determined by thermogravimetric analysis (TGA) using a TGA Q50 instrument with heating from 0 to 850 °C at a rate of 10 °C/min.

Zeta potential measurement

For stability analysis of particles, surface charges of NPs were measured by zeta-potential. The zeta potential distribution of nanoparticles was analyzed by a Malvern Zetasizer Nano Series (Malvern Instruments, England).

Transmission electron microscopy

Particle size analysis, LA-CaCO$_3$ NPs were measured by TEM (Hitachi, Japan) at a voltage of 100 kV.
**Adipocytes differentiation**

Adipose-derived stem cells (ADSCs) have capacity to differentiate into adipocytes. ADSCs were differentiated using Adipogenesis differentiation kit (GIBCO, USA). Briefly, ADSCs were harvested in 0.25% trypsin/EDTA for 3-5 minutes at 37°C. The cells were transferred into a centrifuge tube at 2250 rpm for 5 minutes. ADSCs were seeded into 24 well plates at a density $2 \times 10^4$ per well, 96 well plates at a density of $5 \times 10^3$ per well in cell culture medium and incubated for 24 h to allow cell adherence. After that, cells were changed control medium to pre-warmed complete adipogenesis differentiation medium and continue incubation. Cells were changed the complete adipogenesis differentiation medium every 3-4 days. After a specific period of cultivation (14 days), the adipogenic cultures were processed in experiments.

**Cytotoxicity of LA-CaCO$_3$ NPs in ADSCs**

**Cell viability**

We evaluate the influence of LA-CaCO$_3$ NPs on cellular viability with the PrestoBlue™ reagent (Invitrogen, USA). Metabolically active cells can reduce the PrestoBlue reagent, with the colorimetric changes used as an indicator to quantify the viability of cells in culture. Human cell types were used in this experiment including keratinocytes (HaCaT), human dermal fibroblast cells (HDFs), adipose-derived stem cells (ADSCs) and adipocytes. All the cells were seeded in 96 well plates at a density of $5 \times 10^3$ per well in 45 μl of cell culture medium and incubated for 24 h to allow cells adherence. Confluent cells were stimulated with treatment conditions. The plates were incubated for 24, 48 and 72 hours. Untreated cells were used as negative controls. Following incubation, 10 μl PrestoBlue solution were added to each well, and then plates were placed back into the incubator for a further 20 min incubation. Fluorescence
was measured using a microplate reader at 560 nm excitation and 590 nm emission (Thermo, Varioskan Flash, England).

A calculation for estimating the percentage of cell viability is:

\[
\% \text{ Cell viability} = \left( \frac{\text{Fluorescence treatment}}{\text{Fluorescence control}} \right) \times 100
\]

**ROS generation**

To determine the burst of free radicals and reactive oxygen species (ROS), an intracellular ROS-indicator, 2,7-dichlorofluorescin-diacetate (DCFH-DA) was applied in this experiment. HaCaT, HDFs, ADSCs, and adipocytes were seeded into 96-black well plates at a density of 5 x 10^3 cells per well in 100 µL of culture media and incubated at 37°C under 5% CO₂ atmosphere for 24 h. Cells were washed with phosphate-buffered saline and incubated with 0.1 µM of H₂DCF-DA (Invitrogen, USA) at a volume of 100 µL/well for 30 minutes protected from light. After that, cells were washed with phosphate-buffered saline again and stimulated with various conditions. H₂O₂ (0.8 mM) was used as positive control. Fluorescence was immediately measured for 1 hour using a microplate reader at excitation and emission wavelengths of 485 and 528 nm (Thermo, Varioskan Flash, England).

A calculation for estimating the percentage of ROS generation is:

\[
\% \text{ ROS Generation} = \left( \frac{\text{Fluorescence treatment}}{\text{Fluorescence control}} \right) \times 100
\]
Lipid accumulation

Oil Red O staining

To study the effect of LA-CaCO₃ NPs with the correlation between lipolysis and lipid accumulation in adipocytes. ADSCs were differentiated in 96 well plates. The cells were stimulated with all treatment conditions. Untreated cells were used as negative controls. After 24 hours of exposure to treatments, the cells were stained with Oil Red O to identify lipid droplets. Briefly, media was removed, and cells were fixed in 4% formaldehyde at room temperature for 10 minutes. Cells were washed twice with phosphate-buffered saline and then with 60% isopropanol, before staining with 0.3% Oil Red-O (Sigma, USA) for 20 minutes at room temperature. Cells were washed in running tap water. The images were captured using a phase contrast inverted microscope (Nikon, Eclipse TS 100, Japan). After that, cells were eluted dye with 100% isopropanol. The solutions were transferred into 96 well plates and measured at OD 500 nm using a microplate reader (Thermo, Varioskan Flash, England).

Lipolysis measurement

Lipolysis was evaluated by measurement the amount of glycerol released to the media. Lipolysis assay was measured using lipolysis assay kit (Abcam, USA). The amounts of glycerol were detected after 0 and 24 hours of treatment following manufacturer’s instructions. Briefly, the ADSCs were seeded into 96 well plates and differentiated into adipocytes. The cells were stimulated with treatment conditions for 24 h. Untreated cells were used as negative controls. After that, 50 μl reaction mix were added to each well. Plates were incubated at room temperature for 30 minutes protected from light. The amounts of glycerol were measured at OD 570 nm using microplate reader (Thermo, Varioskan Flash, England).
Real-time PCR

To evaluate the effect of LA-CaCO₃ NPs on ADSCs differentiation into adipocytes. ADSCs were differentiated in 24 well plates in cell culture medium and incubated for 24 h to allow cell adherence. The cells were stimulated with all treatment conditions for 14 days. Adipogenesis was determined after 14 days of growth in the presence or absence of treatments in adipogenesis differentiation medium. The total RNA was isolated from cells using TRIzol reagent (Invitrogen). RNA concentrations and quality were measured by Nanodrop Spectrophotometer (Thermo Scientific, USA). One microgram of total RNA was used for cDNA synthesis with a First strand cDNA synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions. qPCR was performed with Express SYBR GreenER qPCR Supermix Universal (Invitrogen) using StepOnePlus Real-Time PCR System (ABI Applied Biosystems). The CT (threshold cycle) values of the target genes were normalized to the endogenous GAPDH level (housekeeping gene) and relative to the normalized calibrator. Primer sequences are listed below.

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<tr>
<td></td>
<td>Reverse</td>
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<td></td>
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<td>Pat2</td>
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<td>Adiponectin</td>
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<td></td>
<td>Reverse</td>
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<tr>
<td>Leptin</td>
<td>Forward</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>TGGAGGAGACTGACTGCCTG</td>
<td></td>
</tr>
<tr>
<td>Resistin</td>
<td>Forward</td>
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<td>(112)</td>
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<tr>
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<td>CATCATCATCATCATCTCCAG</td>
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**Statistical analysis**

The result from triplicates of the sample was expressed as mean ± SD. Statistical significance was analyzed by using one-way ANOVA (Analysis of variance), followed by the Tukey’s multiple comparison tests. Statistical significance was defined as the P-value ≤ 0.05 in comparison to control.

**Ethical consideration**

This study was approved by the Ethic committee of Research affairs, Faculty of medicine, Chulalongkorn University.
Part II
Preparation of LA-CaCO$_3$ NPs

The synthesis of CaCO$_3$ NPs follow the previous paper (106). Chemical precipitation for the preparation of LA-CaCO$_3$ NPs were used in this study. Briefly, NPs were prepared by the reaction of calcium ions from the aqueous solution from one hand, and carbonate ions from the aqueous solution from another. The aqueous solution of a LA was added into aqueous solutions in the process and then the formation of nanoparticles and entrapment of LA were performed simultaneously. The LA-CaCO$_3$ NPs were separated by centrifugation.

Characterization of LA-CaCO$_3$ NPs

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The compositions of the LA-CaCO$_3$ NPs were determined by thermogravimetric analysis (TGA) using a TGA Q50 instrument with heating from 0 to 850 °C at a rate of 10 °C/min.

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**Transmission electron microscopy**

Particle size analysis, LA-CaCO$_3$ NPs were measured by TEM (Hitachi, Japan) at a voltage of 100 kV.
Adipocytes differentiation

Primary human subcutaneous preadipocytes and visceral preadipocytes media were purchased (Lonza, USA), grown and differentiated following the manufacturer’s instructions. Briefly, subcutaneous preadipocytes and visceral preadipocytes were grown in preadipocyte growth media, plated into 96 well plates at a density of $5 \times 10^3$ per well, 24 well plates at a density $1 \times 10^4$ per well and grown until confluent. Preadipocyte Growth Medium-2 BulletKit™ medium was added every two days until day 14. The intracellular lipid vacuoles begin to appear 4 to 5 days after induction and continue to increase in number and size for 7 to 14 days.

Cytotoxicity of LA-CaCO$_3$ NPs in ADSCs

Cell viability

We evaluate the influence of LA-CaCO$_3$ NPs on cellular viability in primary human subcutaneous preadipocytes and visceral preadipocytes using the PrestoBlue™ reagent (Invitrogen, USA) Metabolically active cells can reduce the PrestoBlue reagent, with the colorimetric changes used as an indicator to quantify the viability of cells in culture. The primary human subcutaneous preadipocytes and visceral preadipocytes were seeded in 96 well plates at a density of $5 \times 10^3$ per well in 45 $\mu$l of cell culture medium and incubated for 24 h to allow cells adherence. Confluent cells were stimulated with all treatment conditions. The plates were incubated for 24, 48, and 72 hours. Untreated cells were used as negative controls. Following incubation, 10 $\mu$l PrestoBlue solution were added to each well, and then plates were placed back into the incubator for a further 20 min incubation. Fluorescence was measured using a microplate reader at 560 nm excitation and 590 nm emission (Thermo, Varioskan Flash, England).
A calculation for estimating the percentage of cell viability is:

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To determine the burst of free radicals and reactive oxygen species (ROS), an intracellular ROS-indicator, 2,7-dichlorofluorescin-diacetate (H$_2$DCF-DA) was applied in this experiment. Primary human subcutaneous preadipocytes and visceral preadipocytes were seeded into 96-black well plates at a density of $5 \times 10^3$ cells per well in 100 µL of culture media and incubated at 37°C under 5% CO$_2$ atmosphere for 24 h. Cells were washed with phosphate-buffered saline and incubated with 0.1 µM of H$_2$DCF-DA (Molecular probes, USA) at a volume of 100 µL/well for 30 minutes protected from light. After that, cells were washed with phosphate-buffered saline again and stimulated with all treatment conditions. H$_2$O$_2$ (0.8 mM) was used as positive control. Fluorescence was immediately measured for 1 hour using a microplate reader at excitation and emission wavelengths of 485 and 528 nm (Thermo, Varioskan Flash, England).

A calculation for estimating the percentage of ROS generation is:

% ROS Generation = (Fluorescence treatment / Fluorescence control) × 100
Lipid accumulation

Oil Red O staining

To study the effect of LA-CaCO₃ NPs with the correlation between lipolysis and lipid accumulation in primary human subcutaneous preadipocytes and visceral preadipocytes. The primary subcutaneous and visceral preadipocytes were differentiated in 96 well plates. Cells were stimulated with all treatment conditions. Untreated cells were used as negative controls. After 24 hours of exposure, the cells were stained with Oil Red O to identify lipid droplets. Briefly, media was removed, and cells were fixed in 4% formaldehyde at room temperature for 10 minutes. Cells were washed twice with phosphate-buffered saline and then with 60% isopropanol, before staining with 0.3% Oil Red-O (Sigma, USA) for 20 minutes at room temperature. Cells were washed in running tap water. The images were captured using a phase contrast inverted microscope (Nikon, Eclipse TS 100, Japan). After that, cells were eluted dye with 100% isopropanol. The solutions were transferred into 96 well plates and measured at OD 500 nm using a microplate reader (Thermo, Varioskan Flash, England).
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Real-time PCR

To evaluate the effect of LA-CaCO₃ NPs on primary human subcutaneous and visceral preadipocytes into adipocytes. The primary subcutaneous and visceral preadipocytes were differentiated in 24 well plates in cell culture medium and incubated for 24 h to allow cell adherence. The cells were stimulated with treatment conditions for 14 days. Adipogenesis was determined after 14 days of growth in the presence or absence of treatments in adipogenesis differentiation medium. The total RNA was isolated from cells using TRIzol reagent (Invitrogen). RNA concentrations and quality were measured by Nanodrop Spectrophotometer (Thermo Scientific, USA). One microgram of total RNA was used for cDNA synthesis with a First strand cDNA synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions. qPCR was performed with Express SYBR GreenER qPCR Supermix Universal (Invitrogen) using StepOnePlus Real-Time PCR System (ABI Applied Biosystems). The CT (threshold cycle) values of the target genes were normalized to the endogenous GAPDH level (housekeeping gene) and relative to the normalized calibrator. Primer sequences are listed below.

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Statistical analysis

The result from triplicates of the sample was expressed as mean ± SD. Statistical significance was analyzed by using one-way ANOVA (Analysis of variance), followed by the Tukey’s multiple comparison tests. Statistical significance was defined as the P-value ≤ 0.05 in comparison to control.

Ethical consideration

This study was approved by the Ethic committee of Research affairs, Faculty of medicine, Chulalongkorn University.
Research methodology

Overall experimental layout of this study is presented in below.

Part I

Synthesis of LA-CaCO$_3$ NPs

- Keratinocytes (HaCaT)
- Human dermal fibroblasts (HDFs)
- Adipose-derived stem cells (ADSCs)
- Adipocytes
- PrestoBlue assay
- DCFH-DA assay
- Oil Red O staining
- Lipolysis kit
- TEM
- TGA
- Zeta potential

Lipid reduction

- Lipid accumulation
- Lipolysis
Adipogenesis

• Real-time PCR
  ASC-1, PAT2, P2RX5

Adipokines expression

• Real-time PCR
  ADIPOQ, LEP, RETN
Subcutaneous adipocytes  Visceral adipocytes

- PrestoBlue assay
- DCFH-DA assay

- Oil Red O staining
- Lipolysis kit

Lipid reduction

Adipogenesis

Adipokines expression

- Real-time PCR
  *ASC-1, PAT2, P2RX5*

- Real-time PCR
  *ADIPOQ, LEP, RETN*
CHAPTER IV
RESULTS AND DISCUSSION

Part I
Characterization of LA-CaCO₃ NPs

The chemical structure of LA-CaCO₃ NPs were represented in Figure 4. It is necessary to characterize material because the toxicity of nanomaterials is closely related to its size, shape and structure. The LA-CaCO₃ NPs were obtained by a multi-stage self-assembled strategy. The TEM image of the LA-CaCO₃ NPs was used to determine shape, size and uniformity of the particles. Transmission electron observations showed that the particles are spherical shaped and monodispersed with an approximate size distribution between 10 and 20 nm (Figure 5A). The percentages of LA in CaCO₃ NPs were confirmed by TGA. The concentration of LA in CaCO₃ NPs was approximately 27 percent in CaCO₃ NPs (Figure 5B). The zeta potential analysis showed a negative charge of -30.2 and -30.9 mV for the CaCO₃ NPs and LA-CaCO₃ NPs, respectively (Figure 5C-D).
Figure 4 Schematic diagram representing the synthesis process of LA-CaCO₃ NPs.

Abbreviations: LA, lipoic acids; NaHCO₃, sodium bicarbonate; CO₃⁻², carbonate ion; Ca²⁺, calcium ion; LA-CaCO₃ NPs, lipoic acids-incorporated calcium carbonate nanoparticles.
Figure 5 Characterization of LA-CaCO$_3$ NPs (A) TEM image of LA-CaCO$_3$ NPs. (B) TGA thermograms of CaCO$_3$ NPs and LA-CaCO$_3$ NPs. (C) Zeta potential of CaCO$_3$ NPs and (D) LA-CaCO$_3$ NPs.

Abbreviations: CaCO$_3$ NPs, calcium carbonate nanoparticles; LA-CaCO$_3$ NPs, lipoic acids-incorporated calcium carbonate nanoparticles
The effect of LA-CaCO₃ NPs in the cytotoxicity of human cells

We first examined whether LA-CaCO₃ NPs have an influence on cell viability. After the human cells including HaCaT, HDFs, ADSCs and adipocytes were exposed to NPs for 12, 48 and 72 h, Presto Blue assay was performed to determine the viability of the human cells. (Figure 6A-D) The H₂O₂-treated human cells led to the intensified damage of human cells. When compared with LA, CaCO₃ NPs and LA-CaCO₃ NPs, the treated groups were found to be non-toxic in human cells at any incubation time between the treated and control groups. ROS generation are regarded as the developed paradigms to explain the toxic effects of nanomaterials. ROS is a natural byproduct of the normal metabolism in cells. However, excessive ROS can have potentially damaging biological responses resulting in oxidative stress phenomenon. Consequently, we also studied the effect of LA-CaCO₃ NPs with ROS generation. Assessment of ROS generation following 1 h of exposure to NPs showed that intracellular ROS production is indistinguishable between the treated and control groups. (Figure 6E-H).
Figure 6 Cytotoxicity assessment of LA-CaCO$_3$ NPs in human cells. (A) HaCaT, (B) HDFs, (C) ADSCs, and (D) adipocytes were cultured in the presence of LA-CaCO$_3$ NPs following 24, 48 and 72-hour exposure. Cell viability percentage of human cells were estimated by PrestoBlue® assay. Effect of LA-CaCO$_3$ NPs on the generation of ROS in human cells. (E) HaCaT (F) HDFs (G) ADSCs and (H) adipocytes were monitoring for 1 hour using DCFH-DA. The results were represented by a mean±SD (n=3). Data was expressed as a percentage of the untreated control. *P < 0.05, **P < 0.01, and ***P < 0.001.

Abbreviations: HaCaT, keratinocytes; HDFs, human dermal fibroblasts; ADSCs, adipose hydrogen peroxide-derived stem cells; H$_2$O$_2$; LA, lipoic acids; CaCO$_3$ NPs, calcium carbonate nanoparticles; LA-CaCO$_3$ NPs, lipoic acids-incorporated calcium carbonate nanoparticles
Figure 6 Cytotoxicity assessment of LA-CaCO$_3$ NPs in human cells. (A) HaCaT, (B) HDFs, (C) ADSCs, and (D) adipocytes were cultured in the presence of LA-CaCO$_3$ NPs following 24, 48 and 72-hour exposure. Cell viability percentage of human cells were estimated by PrestoBlue ® assay. Effect of LA-CaCO$_3$ NPs on the generation of ROS in human cells. (E) HaCaT (F) HDFs (G) ADSCs and (H) adipocytes were monitoring for 1 hour using DCFH-DA. The results were represented by a mean±SD (n=3). Data was expressed as a percentage of the untreated control. *P < 0.05, **P < 0.01, and ***P < 0.001.

**Abbreviations:** HaCaT, keratinocytes; HDFs, human dermal fibroblasts; ADSCs, adipose-derived stem cells; H$_2$O$_2$, hydrogen peroxide; LA, lipoic acids; CaCO$_3$ NPs, calcium carbonate nanoparticles; LA-CaCO$_3$ NPs, lipoic acids-incorporated calcium carbonate nanoparticles
LA-CaCO₃ NPs effect lipid accumulation and lipolysis in human adipocytes

The effect of LA-CaCO₃ NPs on lipid accumulation was examined by Oil Red O staining. The LA revealed a significant reduction in the lipid droplets in adipocytes. The lipid accumulation was decreased by 9.8% at 100 μM and 16.4% at 500 μM of LA, respectively (Figure 7A-B). Interestingly, the LA-CaCO₃ NPs were apparently more effective in lipid accumulation than the LA alone at the same concentration. The result showed that the lipid accumulation was decreased by 9.9% at 100 μM and 34.3% at 500 μM of LA-CaCO₃ NPs. Intracellular lipid accumulation is regulated by lipolysis. Therefore, we hypothesized that LA-CaCO₃ NPs directly reduce lipid accumulation by association with lipolysis. To examine this possibility, we next evaluated lipolytic activity of LA-CaCO₃ NP. The amount of glycerol released into the media was observed in adipocytes treated with LA for 24 h (Figure 7C). The LA also exhibited to increase in the amount of glycerol. Nevertheless, the LA-CaCO₃ NPs are more effective in lipolysis than LA-treated group. CaCO₃ NPs were no statistically significant difference in lipid accumulation and lipolysis when compared with the control group. Therefore, these experiments confirmed that LA-CaCO₃ NPs presented anti-adipogenic properties as evidenced by decreasing lipid accumulation and releasing amount of glycerol in adipocytes.
Figure 7 LA-CaCO$_3$ NPs stimulates lipid reduction in adipocytes. Adipocytes were treated with LA-CaCO$_3$ NPs (100–500 $\mu$M) for 24 hours. (A) Lipid accumulation was assessed by the Oil Red O staining and (B) staining quantification. (C) Lipolysis was measured by the amount of glycerol released into media. The results were represented by a mean±SD (n=3). Data was expressed as a percentage of the untreated control. *P < 0.05, **P < 0.01, and ***P < 0.001 between the indicated groups. 

Abbreviations: LA, lipoic acids; CaCO$_3$ NPs, calcium carbonate nanoparticles; LA-CaCO$_3$ NPs, lipoic acids-incorporated calcium carbonate nanoparticles; ns, not significant.
The LA-CaCO₃ NPs inhibit adipocyte differentiation

ADSCs can be differentiated to many types of adipocytes with suitable environments. In this study, we examined whether LA-CaCO₃ NPs influence on adipocytes differentiation. With the identification of the adipocytes marker by USSAR et al. ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes respectively. To study the expression of ASC-1, PAT2, and P2RX5 in differentiated adipocytes using Real-time PCR. After the ADSCs were exposed with LA, CaCO₃ NPs and LA-CaCO₃ NPs for 14 days. The expression of ASC-1, PAT2 and P2RX5 mRNA expression in the differentiated cells were significantly suppressed in both LA and LA-CaCO₃ NPs (Figure 8). Whereas the CaCO₃ NPs did not alter ASC-1, PAT2, and P2RX5 mRNA expression when compared with the control group. All these data suggest that LA and LA-CaCO₃ NPs have a capacity to suppress adipogenesis in ADSCs.
Figure 8 The LA-CaCO$_3$ NPs inhibits adipocytes differentiation in ADSCs. The specific markers were measured by real-time PCR in ADSCs. Both LA- and LA-CaCO$_3$ treated group were suppressed ASC-1, PAT2, and P2RX5 mRNA expression when compared with control groups. GAPDH mRNA expression was used as an internal control. *P < 0.05, **P < 0.01, and ***P < 0.001 between the indicated groups.

**Abbreviations:** ASC-1, neutral amino acid transporter; PAT2, proton assistant amino acid transporter-2; P2RX5, purinergic receptor P2X, ligand-gated ion channel 5; LA, lipoic acids; CaCO$_3$ NPs, calcium carbonate nanoparticles; LA-CaCO$_3$ NPs, lipoic acids-incorporated calcium carbonate nanoparticles, ns; not significant.
The LA-CaCO$_3$ NPs inhibit adiponectin leptin and resistin mRNA expression

Finally, to gain further insights into the function of adipocytes. Adipose tissue functions as a key endocrine organ by releasing numerous protein, known as adipose-derived secreted factors or adipokines, that have pro-inflammatory (such as leptin and resistin) or anti-inflammatory activities (adiponectin). We measured the adipokines expression in differentiated adipocytes using real-time PCR. Adiponectin leptin and resistin mRNA expression were significantly lower in LA compared with the control group (Figure 9). As similarly shown previously, LA-CaCO$_3$ NPs reduced mRNA expression in adiponectin leptin and resistin. In CaCO$_3$ NPs, there was no significant difference in adipokines expression when compared with the control group. Consequently, our results further suggest that LA and LA-CaCO$_3$ NPs inhibit adipokines expression by suppression of adipogenesis.
Figure 9 The LA-CaCO$_3$ NPs inhibits adipokines expression in ADSCs. ADIPOQ, LEP, and RETN mRNA expression were measured by real-time PCR. Adipokines expression was suppressed in both LA- and LA-CaCO$_3$ NPs-treated groups when compared with control groups. GAPDH mRNA expression was used as an internal control. **P < 0.01, and ***P < 0.001 between the indicated groups.

**Abbreviations:** ADIPOQ, adiponectin; LEP, leptin; RETN, resistin; LA, lipoic acids; CaCO$_3$ NPs, calcium carbonate nanoparticles; LA-CaCO$_3$ NPs, lipoic acids-incorporated calcium carbonate nanoparticles; ns, not significant.
Part II

The cytotoxic effects of LA-CaCO$_3$ NPs in subcutaneous and visceral adipocytes

In part II, we examined whether LA-CaCO$_3$ NPs have an influence on cell viability in human subcutaneous and visceral adipocytes. After both cells were exposed to all treatment conditions for 12, 48 and 72 h, Presto Blue assay was performed to determine cell viability. The H$_2$O$_2$-treated adipocytes were significantly decreased cell viability in subcutaneous and visceral adipocytes. When the cells were exposed to LA, CaCO$_3$ NPs, and LA-CaCO$_3$ NPs, we did not observe cytotoxic effects at any incubation time as confirmed by dehydrogenase measurement (Figure 10A-D). ROS generation are regarded as the developed paradigms to explain the toxic effects of nanomaterials. ROS is a natural byproduct of the normal metabolism in cells. However, excessive ROS can have potentially damaging biological responses resulting in oxidative stress phenomenon. Consequently, we also examined the effect of LA-CaCO$_3$ NPs with ROS generation. Assessment of ROS generation following 1 h of exposure to LA-CaCO$_3$ NPs showed that LA-CaCO$_3$ NPs had no effect in intracellular ROS production (Figure 10E-H).
Figure 10  Cytotoxicity assessment of LA-CaCO₃ NPs in human adipocytes. (A) human subcutaneous preadipocytes, (B) human visceral preadipocytes, (C) human subcutaneous adipocytes, and (D) human visceral adipocyte were cultured in the presence of LA-CaCO₃ NPs following 24, 48 and 72-hour exposure. Cell viability percentage of preadipocytes and adipocytes were estimated by PrestoBlue ® assay. Effect of LA-CaCO₃ NPs on the ROS generation. (E) Human subcutaneous preadipocytes, (F) human visceral preadipocytes, (G) human subcutaneous adipocytes, and (H) human visceral adipocyte were monitoring for 1 hour using DCFH-DA. The results were represented by a mean±SD (n=3). Data was expressed as a percentage of the untreated control. *P < 0.05, **P < 0.01, and ***P < 0.001.

Abbreviations: H₂O₂, hydrogen peroxide; LA, lipoic acids; CaCO₃ NPs, calcium carbonate nanoparticles; LA-CaCO₃ NPs, lipoic acids-incorporated calcium carbonate nanoparticles.
Figure 10 Cytotoxicity assessment of LA-CaCO₃ NPs in human adipocytes. (A) human subcutaneous preadipocytes, (B) human visceral preadipocytes, (C) human subcutaneous adipocytes, and (D) human visceral adipocyte were cultured in the presence of LA-CaCO₃ NPs following 24, 48 and 72-hour exposure. Cell viability percentage of preadipocytes and adipocytes were estimated by PrestoBlue® assay. Effect of LA-CaCO₃ NPs on the ROS generation. (E) Human subcutaneous preadipocytes, (F) human visceral preadipocytes, (G) human subcutaneous adipocytes, and (H) human visceral adipocyte were monitoring for 1 hour using DCFH-DA. The results were represented by a mean±SD (n=3). Data was expressed as a percentage of the untreated control. *P < 0.05, **P < 0.01, and ***P < 0.001.

Abbreviations: H₂O₂, hydrogen peroxide; LA, lipoic acids; CaCO₃ NPs, calcium carbonate nanoparticles; LA-CaCO₃ NPs, lipoic acids-incorporated calcium carbonate nanoparticles
Effects of LA-CaCO₃ NPs on lipolysis in subcutaneous and visceral adipocytes

To assess the effects of LA-CaCO₃ NPs on lipid reduction, human subcutaneous and visceral adipocytes were cultured in the presence of LA-CaCO₃ NPs. Lipid accumulation was determined by lipid droplets using oil-red O and total glycerol was measured in culture media in 24 h. LA-treated adipocytes decreased lipid droplets in both subcutaneous and visceral adipocytes. In subcutaneous adipocytes, the lipid accumulation was decreased by 3% at 100 μM and 14.8% at 500 μM. In visceral adipocytes, the lipid accumulation was decreased by 3% at 100 μM and 15.8% at 500 μM. Interestingly, the LA-CaCO₃ NPs were more effective in lipid accumulation than the LA alone at the same concentration in both subcutaneous and visceral adipocytes. The lipid accumulation was decreased by 11.3% at 100 μM and 23.7% at 500 μM in subcutaneous adipocytes (Figure 11A-B). In visceral adipocytes, the lipid accumulation was decreased by 10.6% at 100 μM and 25.8% at 500 μM (Figure 11D-E). While, the CaCO₃ NPs treatment did not affect lipid accumulation in both cells. We next evaluated the effects of LA-CaCO₃ NPs on the lipolysis in subcutaneous and visceral adipocytes. The amount of glycerol released into the media was observed in adipocytes. LA-treated adipocytes increased in the amount of glycerol in culture media. However, the LA-CaCO₃ NPs are more effective in lipolysis than LA alone when compared with control group in both cells (Figure 11C, F). Therefore, LA-CaCO₃ NPs presented anti-adipogenic properties in both subcutaneous and visceral adipocytes as evidenced by decreased lipid accumulation and amount of glycerol in adipocytes.
A

Control

LA

CaCO₃ NPs

LA-CaCO₃ NPs

B

Human subcutaneous adipocytes

C

Glycerol (mM)

Control

LA 500 uM

CaCO₃ NPs

LA-CaCO₃ NPs 500 uM
D. Control vs. LA

E. Human visceral adipocytes

F. Glyceral (%)
Figure 11 LA-CaCO$_3$ NPs decrease lipid accumulation in subcutaneous and visceral adipocytes. Adipocytes were treated with LA-CaCO$_3$ NPs (100–500 $\mu$M) for 24 hours. Lipid accumulation was assessed by the Oil Red O staining in (A) subcutaneous and (D) visceral adipocytes treated for 24 hours and (B, E) staining quantification. Lipolysis in (C) subcutaneous and (F) visceral adipocytes were measured by the amount of glycerol released into media. The results were represented by a mean±SD (n=3). Data was expressed as a percentage of the untreated control. *P < 0.05, **P < 0.01, and ***P < 0.001 between the indicated groups.

Abbreviations: LA, lipoic acids; CaCO$_3$ NPs, calcium carbonate nanoparticles; LA-CaCO$_3$ NPs, lipoic acids-incorporated calcium carbonate nanoparticles; ns, not significant.
The effects of LA-CaCO$_3$ NPs on regulation of differentiation in subcutaneous and visceral adipocytes.

Next, we examined whether LA-CaCO$_3$ NPs influence on adipocytes differentiation. With the identification of the adipocytes marker by Ussar et al. ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes respectively. Human subcutaneous and visceral preadipocytes were treated with differentiating media in the presence or absence of LA, CaCO$_3$ NPs, and LA-CaCO$_3$ NPs for 14 days. After treatment exposure, differentiated adipocytes were measured marker expression using real-time PCR. In subcutaneous adipocytes, the results showed that LA-treated groups were inhibited adipogenesis by suppression of ASC-1 and PAT2. Similarly, LA-CaCO$_3$ NPs treatment significantly reduced the expression of ASC-1 and PAT2. Among the treatment groups, LA-CaCO$_3$ NPs showed the strongest inhibitory effect on the expression of ASC-1. LA, CaCO$_3$ NPs, and LA-CaCO$_3$ did not affect the expression of P2RX5 in subcutaneous preadipocytes (Figure 12A). In visceral adipocytes, LA treatment significantly suppressed the expression of ASC-1, PAT2, and P2RX5. However, LA-CaCO$_3$ NPs-treated groups showed greater ASC1 suppression compared with LA alone. Surprisingly, LA-CaCO$_3$ NPs significantly increased PAT2 expression but did not affect the expression of P2RX5. Moreover, we found that CaCO$_3$ NPs decreased ACS-1 expression in subcutaneous and visceral adipocytes with PAT2 expression in subcutaneous adipocytes.
The LA-CaCO₃ NPs regulate adipocytes differentiation in human subcutaneous and visceral adipocytes. (A) LA-CaCO₃ NP inhibited adipogenesis by suppression of ASC1 and PAT2 expression in subcutaneous adipocytes. (B) LA-CaCO₃ NPs-treated groups in visceral adipocytes were induced PAT2 expression. GAPDH mRNA expression was used as an internal control. *P < 0.05, **P < 0.01, and ***P < 0.001 between the indicated groups.

**Abbreviations:** ASC-1, neutral amino acid transporter; PAT2, proton assistant amino acid transporter-2; P2RX5, purinergic receptor P2X, ligand-gated ion channel 5; LA, lipoic acids; CaCO₃ NPs, calcium carbonate nanoparticles; LA-CaCO₃ NPs, lipoic acids-incorporated calcium carbonate nanoparticles; ns; not significant.
The effects of LA-CaCO$_3$ NPs on adipokines expression in subcutaneous and visceral adipocytes.

Finally, to gain further insights into the functions of differentiated adipocytes. Adipose tissue functions as a key endocrine organ by releasing numerous protein, known as adipose-derived secreted factors or adipokines, that have pro-inflammatory (such as leptin and resistin) or anti-inflammatory activities (adiponectin). We measured the adipokines expression in subcutaneous and visceral adipocytes using real-time PCR. $ADIPOQ$, $LEP$ and $RETN$ mRNA expression were significantly decreased in LA-treated groups compared with the control groups. As similarly shown previously, LA-CaCO$_3$ NPs reduced mRNA expression in $ADIPOQ$, $LEP$ and $RETN$ mRNA in subcutaneous and visceral adipocytes. Moreover, we found that CaCO$_3$ NPs decreased $ADIPOQ$ and $LEP$ in subcutaneous adipocytes. CaCO$_3$ NPs also decreased $ADIPOQ$ expression in visceral adipocytes. Consequently, our results further suggest that LA and LA-CaCO$_3$ NPs inhibit adipokines expression by suppression of adipogenesis.
Figure 13 The LA-CaCO₃ NPs inhibits adipokines expression in adipocytes. ADIPOQ, LEP, and RETN mRNA expression were measured by real-time PCR. Adipokines expression in (A) subcutaneous and (B) visceral adipocytes were suppressed in both LA- and LA-CaCO₃ NPs-treated groups when compared with control groups. GAPDH mRNA expression was used as an internal control. *P < 0.05, **P < 0.01, and ***P < 0.001 between the indicated groups.

Abbreviations: ADIPOQ, adiponectin; LEP, leptin; RETN, resistin; LA, lipoic acids; CaCO₃ NPs, calcium carbonate nanoparticles; LA-CaCO₃ NPs, lipoic acids-incorporated calcium carbonate nanoparticles, ns; not significant.
Discussion

Part I

In the present study, our findings are worth to present that this is the first report on the potential advantages of LA-CaCO$_3$ NPs in ADSCs, their physicochemical characterization and their adipocytes functions by comparing with LA alone. Increasing evidence shows that LA has become increasingly candidate agent with therapeutic potential on several diseases such as diabetes and obesity. In agreement with the limited bioavailability of LA, CaCO$_3$ NPs have been praised for their advantageous drug carrier to improve therapeutic efficacy. Our data demonstrated that LA-CaCO$_3$ NPs presented a promising treatment for the anti-obesity by reducing lipid accumulation, increasing lipolysis, inhibiting adipogenesis as well as adipokine expression. Cytotoxicity of newly synthesized LA-CaCO$_3$ NPs had no obvious toxicity in human cells. Notably, LA-CaCO$_3$ NPs were apparently more effective in lipid accumulation than the LA alone at the same concentration. The significant lipid accumulation of LA-CaCO$_3$ NPs was attributed to the ability of LA-CaCO$_3$ NPs to encourage lipolysis in adipocytes, as confirmed by amounts of glycerol. Previous study suggested that a lipolytic action of LA is mainly mediated by the phosphorylation of hormone-sensitive lipase leading to reduction of lipid accumulation in body.(14, 82) Furthermore, CaCO$_3$ NPs are able to deliver LA, intracellularly at a longer residence time, which results in a sustained therapeutic effect of LA.

Adipogenesis is a differentiation process by stimulation of ADSCs to become terminally differentiated adipocytes. In this study, to our knowledge, this is the first time that the effect of LA was evaluated in differentiation of ADSCs. As reported previously, LA caused inhibition of adipogenesis in 3T3-L1 pre-adipocytes.(15, 85) Here we demonstrate that both LA and LA-CaCO$_3$ NPs inhibited adipocyte differentiation in ADSCs by suppression of ASC1, PAT2 and P2RX5 mRNA expression in ADSCs. Moreover, no significant differences were found between LA- and LA-CaCO$_3$ NPs-treated groups.
The lipid reduction effects have been demonstrated with inhibition of adipogenesis. (113, 114) LA directly modulates the adipocyte differentiation through activation of adipogenic transcription factors, which results in decreased expression of adipocyte-specific genes, consequently contributing to the suppression of adipogenesis. Previous studies reported that LA controls the adipocyte differentiation via the direct modulation of autophagy. (15, 85) However, the precise mechanisms of LA and LA-CaCO₃ NPs in adipogenesis require further investigation, especially in ADSCs.

These results, together with the previous findings, suggest that LA-CaCO₃ NPs significantly reduced in accumulation of lipids in adipocytes, whereas they inhibited adipogenesis in ADSCs.

Adipose tissue has been recognized as an endocrine organ that is capable of producing and secreting a variety of proinflammatory and anti-inflammatory factors known as adipokines. (115-117) Next, we investigated whether LA-CaCO₃ NPs regulates adipokines expression including adiponectin (ADIPQ), leptin (LEP), and resistin (RETN). Adiponectin is mainly secreted by adipocytes. It is an adipokine with insulin-sensitizing property through binding to its receptors AdipoR1 and AdipoR2. (61, 118, 119) The functions of leptin are food intake regulation, glucose and lipid homeostasis, and energy expenditure control via the central nervous system. During food restriction, the leptin levels are decreased while weight loss in mice and humans. (71, 120) Several studies demonstrated that LA treatment up-regulates adiponectin and down-regulates leptin to prevent hyperinsulinemia and insulin resistance. (95, 121) On the contrary, our findings showed a significantly suppressed adiponectin and leptin mRNA expression in both LA- and LA-CaCO₃ NPs-treated groups. These results suggest that LA and LA-CaCO₃ NPs inhibit adipokine expression underlining their inhibitory actions of adipogenesis in differentiated adipocytes. Resistin is an adipocyte hormone that modulates glucose homeostasis. It has been shown to induce pathogenesis of obesity and insulin resistance in humans, both of which appear to contribute to the development of type
2 diabetes. (76, 122, 123) Nevertheless, there have been no reports concerning with the effect of LA in resistin regulation. Our data also showed that LA and LA-CaCO₃ NPs reduced adiponectin leptin as well as resistin mRNA expression. However, we did not find any significant changes between leptin and resistin mRNA expression in LA- and LA-CaCO₃ NPs-treated groups. Adiponectin’s mRNA expression was less inhibited by LA-CaCO₃ NPs when compared with LA alone. Indeed, the adiponectin presents protective actions to have a more pronounced anti-inflammatory property than leptin and resistin. (117) We propose that LA-CaCO₃ NPs act in different pathways, which may possibly be ascribed to suppress adiponectin, leptin and resistin mRNA expression.

The direct effects of LA and LA-CaCO₃ NPs on lipid accumulation, lipolysis, adipokines expression as well as the potential signaling pathways involved in cultured ADSCs and adipocytes still remains to be further investigated.

Considering the cellular uptake mechanisms of LA-CaCO₃ NPs in ADSCs and differentiated adipocytes, it will be also important to further assess its potential effects of LA-CaCO₃ NPs. Although the CaCO₃ NPs did not alter activities in lipid accumulation, lipolysis, adipogenesis as well as adipokines expression. We herein provide evidence that LA-CaCO₃ NPs present the greater effects in reduction of lipid contents, stimulation of lipolysis, inhibition of adipogenesis and adipokine expression. Different uptake mechanisms enable the consequence of diverse responses. The Na⁺/multivitamin transporter is suggested to transport LA into the cells. (124) The incorporation of LA into CaCO₃ NPs possibly become the different uptake mechanisms by structural alteration of LA. LA-CaCO₃ NPs effectively cross cell membranes by ion channel-mediated endocytosis. The pH-sensitive properties of CaCO₃ NPs provide the dissolution of calcium carbonate in the low acidic medium of endosomal compartments. The CaCO₃ NPs are dissolved in the endosome and destabilize it through osmotic imbalance, and finally deliver the LA into the cytosol. (101, 125-127) Our results indicated that these CaCO₃ NPs were used for safe and efficient delivery of
LA or other drugs. The progressive understanding of the drug release kinetics and transport mechanisms in CaCO$_3$ NPs remains deserve further studies for better scientific understanding.
Part II

To the best of our knowledge, this is the first report comparing the efficacy of LA-CaCO₃ NP properties in both human subcutaneous and visceral adipocytes. Human adipose tissue is a diversified tissue organ with different depots secreting a variety of adipokines. Subcutaneous and visceral adipocytes are two major types of adipocytes in adipose tissue. Moreover, subcutaneous and visceral adipocytes are associated with an adverse metabolic effect (128). Here, we developed the new nanoparticles using CaCO₃ NPs as drug carrier to deliver LA into adipocytes. We examined the effect of LA-CaCO₃ NPs in between subcutaneous and visceral adipocytes. Importantly, this study provides the first report that LA-CaCO₃ NPs exhibit anti-obesity properties by a reduction in lipid accumulation and enhanced lipolysis in both human subcutaneous and visceral adipocytes. Additionally, LA-CaCO₃ NPs effects on adipogenic differentiation throughout adipokines expression in both human subcutaneous and visceral adipocytes.

Excessive lipid accumulation due to energy overload causes overweight and obesity. Currently used anti-obesity agents are appetite suppressants (129, 130). However, these drugs often show adverse effects. Due to these undesirable adverse effects of the drugs. Many studies attempt to develop an alternative method for reducing lipid accumulation using natural medicinal agents. Anti-adipogenic activity of LA on adipocytes has been previously well documented. Previous study reveals that the ability of LA to stimulate lipolysis in adipocytes could also contribute to its anti-obesity properties (14). Our results indicate that LA was significantly decreased lipid accumulation and enhanced lipolysis in subcutaneous and visceral adipocytes. Interestingly, the LA-CaCO₃ NPs was also greater than LA in the lipid accumulation. Furthermore, LA-CaCO₃ NPs enhanced lipolytic activity within subcutaneous and visceral adipocytes. Whereas the CaCO₃ NPs treatment did not affect lipid accumulation and lipolytic activity. We postulate that the increased lipid reduction in
subcutaneous and visceral adipocytes is due to CaCO$_3$ NPs properties including slow biodegradation, allowing for LA accumulation at adipocytes.

Adipogenesis, the new key target of anti-obesity drugs, is the process where mesenchymal cells differentiate into preadipocytes followed by second differentiation into lipid accumulating adipocytes. In this study, subcutaneous and visceral preadipocytes were treated with differentiating media in the presence or absence of all treatment conditions for 14 days. ASC-1, PAT2, and P2RX5 are specific marker to identify adipocyte population. Our results indicated that LA treatment significantly decreased ASC-1, PAT2 expression in subcutaneous adipocytes and ASC-1, PAT2, and P2RX5 expression in visceral adipocytes. These findings are in accordance with results of previous studies. They suggest that treatment with 500 $\mu$M LA acts as a PPAR-γ antagonist and inhibits adipogenic differentiation (85). In subcutaneous adipocytes, LA-CaCO$_3$ NPs exhibited same result in LA treatment. The LA-CaCO$_3$ NPs inhibited adipogenic differentiation, which was accompanied with lower expression levels of ASC-1 and PAT2 genes in subcutaneous adipocytes. Whereas the LA-CaCO$_3$ NPs suppressed ASC-1 expression but induced PAT2 expression in visceral adipocytes. The brown and beige adipocytes have similar thermogenic capacities (34). The mitochondria a central role in metabolism of beige, brown adipocytes (34). In addition, specific function performed by brown adipocytes is converting mitochondrial energy into heat in adaptive thermogenesis. The functions of mitochondria in beige, brown adipocytes relate with metabolic homeostasis and weight control. The ability of LA promotes brown-like remodeling in cultured adipocytes. Previous study reveals that the LA not only increased mitochondrial content, but also mitochondrial function via SIRT1-PGC-1α signaling pathway (82, 86, 131-133). Regardless of its natural role, increasing the activity of beige adipocytes by LA-CaCO$_3$ NPs tremendous promise for the treatment of metabolic disease, although the mechanism requires further investigations.
Adipose tissue is essential in energy metabolism. It has endocrine functions and can secrete hundreds of bioactive molecules, many of which are called adipokines. In this study, we explored the effects of LA-CaCO$_3$ NPs in adipokines regulation. We found that LA significantly inhibited expression of ADIPOQ, LEP, and RETN in subcutaneous and visceral adipocytes. Adiponectin has been focused on the potential beneficial effects of obesity. However, treatment of LA inhibits ADIPOQ expression in *in vitro* which contrasts with the stimulation of adiponectin described after *in vivo* supplementation with LA (97). Moreover, many studies report that treatment of adipocytes with LA caused a concentration-dependent inhibition of LEP gene (98). Recently, no data are available concerning LA treatment and resistin expression in adipocytes. Resistin has been identified as an adipokine that links obesity to insulin resistance. Resistin serum levels in our obese study participants were significantly higher than in normal-weight controls (134). Our result indicates that ADIPOQ, LEP, and, RETN also extensively inhibited by LA-CaCO$_3$ NPs, while ASC-1 expression was suppressed by LA and LA-CaCO$_3$ NPs. It is likely that their inhibitory effect of adipogenesis leading to inhibition of adipokines expression, because adiponectin, leptin, and resistin are mainly produced in white adipocytes.

The LA-CaCO$_3$ NPs are synthesized by incorporation between LA and CaCO$_3$ NPs. Our results showed that LA-CaCO$_3$ NPs treatment play different roles with LA in adipogenesis. LA significantly suppressed adipogenic differentiation in subcutaneous adipocytes. On the other hand, LA-CaCO$_3$ NPs induced PAT2 expression in visceral adipocytes. One possibility is that when nanoparticles are combined with LA to create novel properties. Nanomaterials can be designed and synthesized in needed sizes and shapes, and they have unique chemical and physical properties which make them useful as nanocarrier or assistants, excellent signal reporters, transducers, amplifiers or inhibitors (135). Our data indicated the CaCO$_3$ NPs also inhibited adipogenic differentiation and adipokines expression. We hypothesize that CaCO$_3$ NPs may involve
adipogenesis and adipokines expression in adipocytes by calcium-releasing activity. Additionally, a biologically active molecules-incorporating nanoparticles are effective than free form (136, 137). Functional LA-incorporating nanostructures might result in a synergism between the LA and CaCO$_3$ NPs. Subcutaneous and visceral adipocytes exhibit differential gene expression and function (138). The contrast between cell-type preferences for LA-CaCO$_3$ NPs is significant and could contribute to different of functional responses. However, we suggest that this mechanism is difference, improved efficiency of adipogenesis in subcutaneous and visceral adipocytes by CaCO$_3$ NPs will be considered in the future.
CHAPTER V
CONCLUSION

To the best of our knowledge, we successfully synthesized LA-CaCO$_3$ NPs which exhibited lipid reduction properties in ADSCs, subcutaneous, and visceral adipocytes. The LA-CaCO$_3$ NPs has efficient activity in reducing lipid accumulation, increasing lipolysis, decreasing both adipogenesis and adipokines expression. Moreover, LA-CaCO$_3$ NPs regulate adipocytes differentiation to induced \textit{PAT2} expression in visceral adipocytes. Our findings present the new perspective that the delivery of LA to adipose tissue, with the assistance of CaCO$_3$ NPs exerted a potent therapeutic effect in reducing intercellular lipid deposition. The promising potential of CaCO$_3$ NPs and LA-CaCO$_3$ NPs as a stable and effective drug delivery system in targeted adipose tissue for overcoming obesity, metabolic diseases and maintaining a healthy life.
REFERENCES


115. Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Netw. 2006;17(1):4-12.


**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
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<td>ADIPOQ</td>
<td>Adiponectin</td>
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<td>µL</td>
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<td>ADSCs</td>
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<td>ANOVA</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>HDFs</td>
<td>Human dermal fibroblasts</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone-sensitive lipase</td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolt</td>
</tr>
<tr>
<td>LA</td>
<td>Lipoic acid</td>
</tr>
<tr>
<td>LA-CaCO₃ NPs</td>
<td>Lipoic acids-incorporated calcium carbonate nanoparticles</td>
</tr>
<tr>
<td>LEP</td>
<td>Leptin</td>
</tr>
<tr>
<td>MGL</td>
<td>Monoglyceride lipase</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>ns</td>
<td>Not significant</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>P2RX5</td>
<td>Purinergic receptor P2X</td>
</tr>
<tr>
<td>PAT2</td>
<td>Proton assistant amino acid transporter-2</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterases</td>
</tr>
<tr>
<td>RELMs</td>
<td>Resistin-like molecules</td>
</tr>
<tr>
<td>RETN</td>
<td>Resistin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>sWAT</td>
<td>Subcutaneous white adipose tissue</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>-------------------------------------------</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>UCP1</td>
<td>Uncoupling proteins 1</td>
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<tr>
<td>vWAT</td>
<td>Visceral white adipose tissue</td>
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<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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<tr>
<td>WHO</td>
<td>World health organization</td>
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</table>
EQUIPMENTS AND CHEMICALS

Equipment

1. 24-well plate (Corning, USA)
2. 96-well plate (Corning, USA)
3. Autoclave (Hirayama, Japan)
4. Autopipette 10 µL (Biorad, USA)
5. Autopipette 100 µL (Biorad, USA)
6. Autopipette 1000 µL (Biorad, USA)
7. Autopipette 200 µL (Biorad, USA)
8. Biohazard Laminar Flow (Gibco, USA)
9. Cell culture flask (SPL, Korea)
10. Centrifuge tube 1.5 mL (Corning, USA)
11. Centrifuge tube 15 mL (Corning, USA)
12. Centrifuge tube 5 mL (Corning, USA)
13. Centrifuge tube 50 mL (Corning, USA)
14. Class II biohazard safety cabinet (Esco Micro, Singapore)
15. CO2 incubator (Esco, Singapore)
16. CO2 Incubator (Esco Micro, Singapore)
17. Filter Tip (Corning, USA)
18. Laboratory balance (Denver instrument, Germany)
20. Microcentrifuge (Hettich, Germany)
21. Oven (Contherm, New Zealand)
22. Parafilm (Bemis, USA)
23. pH meter (Denver instrument, Germany)
24. Phase contrast inverted microscope (Nikon, Japan)
25. Shaker incubator (Heidolph, Germany)
26. Sonicator (P. Intertrade Equipment Co., Ltd)
27. StepOnePlus Real-Time PCR System (ABI Applied Biosystems, USA)
28. Thermal gravimetric analysis (TGA) (TA Instrument, USA)
29. Transmission Electron Microscope (Hitachi, Japan)
30. Upright Ultra-Low Temperature Freezers (Thermo, England)
31. Varioskan Flash microplate reader (Thermo, England)
32. Veriti 96 Well Thermal Cycler (ABI Applied Biosystems, USA)
33. Vortex mixer (Scientific industries, USA)
34. Water bath (Memmert, Germany)
35. Zetasizer Nano Series (Malvern Instrument, England)
Chemicals

1. Calcium chloride (MERK, Germany)
2. Chloroform (MERK, Germany)
3. Dulbecco's Modified Eagle's Medium (Sigma, USA)
4. Ethanol (MERK, Germany)
5. Fetal Bovine Serum (Gibco, USA)
6. First strand cDNA synthesis kit (Roche®) (Thermoscientific, USA)
7. H2DCFDA (Invitrogen, USA)
8. Hydrochloric acid (MERK, Germany)
9. Isopropanol (MERK, Germany)
10. Lipoic acids (Sigma, USA)
11. Lipolysis kit (Abcam, USA)
12. Oil Red O (Sigma, USA)
13. Paraformaldehyde (Sigma, USA)
14. Penicillin/Streptomycin (Gibco, USA)
15. PrestoBlue™ Cell viability Reagent (Invitrogen, USA)
16. Sodium bicarbonate (MERK, Germany)
17. SYBR GreenER qPCR Supermix Universal (Invitrogen, USA)
18. TRIzol reagent (Invitrogen, USA)
19. Ultrapure distilled water (Invitrogen, USA)
Chemical preparation

1. Phosphate buffer saline

\[
\begin{align*}
KCl & \quad 0.2 \text{ g} \\
KH_2PO_4 & \quad 0.2 \text{ g} \\
NaCl & \quad 8.0 \text{ g} \\
Na_2HPO_4 & \quad 1.15 \text{ g}
\end{align*}
\]

Mix all chemical component and add DI water to 1,000 mL, then adjust pH to 7.4 with HCl.

2. Dulbecco's Modified Eagle's Medium (DMEM)

1) Dissolve 13.4 g of DMEM with 800 mL DI water

2) Add 3.7 g of Na_2HCO_3

3) Adjust pH to 7.2 with HCl

4) Add DI water to 1,000 mL

5) Filtrate by 0.2 µm filter and keep as a stock medium

6) For working medium preparation, add 100 mL of Fetal Bovine Serum, 10 mL of antibiotic (Pen-Strep) into 900 mL of stock medium.
Cell Viability Assay Protocol (PrestoBlue™, Invitrogen, USA, Catalog number A13261)

1. Cell culture

Materials

1. 96-well plate
2. Cell Culture Media
3. Micropipetters
4. CO₂ incubator

Method

1. Cells are seeded in 96-well plates at a density of 1-10 X 10³ cells/well in 45 µL.
2. Incubate at 37°C and 5% CO₂ for 12 h.
2. Cell viability assay

Materials

1. Unknown sample for toxicity test
2. H$_2$O$_2$ (positive control)

Method

1. Add 45 µL of culture medium for negative control.
2. Add 45 µL of H$_2$O$_2$ for positive control.
3. Treat with 45 µL of unknown samples.
4. Incubate at 37°C and 5% CO$_2$ for 24, 48, or 72 h.
5. Add 10 µL PrestoBlue™ reagents and incubate for 30 min.
6. Measure fluorescent product by using a microplate reader at 560 and 590 nm.

Reference

- Product Information Sheet : PrestoBlue™ Cell Viability Reagent Protocol from Invitrogen™
Reactive Oxygen Species (ROS) Generation Protocol (H$_2$DCFDA, Invitrogen, USA, Catalog number D399)

1. Cell culture

Materials

1. 96-black well plate
2. Cell Culture Media
3. Micropipetters
4. CO$_2$ incubator

Method

1. Cells are seeded in 96-black well plate at a density of 1-10 $\times$ $10^3$ cells/well in 100 µL.
2. Incubate at 37°C and 5% CO$_2$ for 12 h.
2. DCFH-DA assay

Materials

1. Unknown sample for ROS generation test
2. H$_2$O$_2$ (positive control)
3. Phosphate Buffer Saline (PBS)

Method

1. Wash the cells 2 times with PBS
2. Add 100 µL of 0.1M H$_2$DCFDA
3. Incubate at 37°C and 5% CO$_2$ for 30 min.
4. Wash 2 times with PBS
5. Add 100 µL of culture medium for control.
6. Add 100 µL of 0.5% H$_2$O$_2$ for positive control.
7. Treat with 100 µL of unknown sample.
8. Measure fluorescence excitation and emission at 485 and 528 respectively by using microplate reader.

Reference

- Product Information Sheet : H$_2$DCFDA from Invitrogen$^\text{TM}$
Miss Sarocha Cherdchom was born in Bangkok, Thailand on April 2, 1991. She was graduated with Bachelor’s Degree in Biology from faculty of Science, Srinakharinwirot University in 2013. She was granted the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship from Graduate School, Chulalongkorn University to enroll in Doctor of Philosophy Program in Medical Sciences in 2014.