A Dissertation for the Degree of Doctor of Philosophy

Anti-obesity effect of 3,3'-diindolylmethane and its mechanisms *in vitro* and *in vivo*

세포주 모델과 동물 모델에서의 3,3'-디인돌릴메탄의 항비만 효과와 작용 기작

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Abstract

Obesity has long been recognized as a major risk factor for a number of metabolic diseases. In order to develop evidence-based reasoning for a novel natural anti-obesity agent for prevention and treatment, I investigated the anti-obesity effect of 3,3'-diindolylmethane (DIM, an active compound in cabbage), in a diet-induced obesity (DIO) mouse model and in 3T3-L1 preadipocytes. I first demonstrated that DIM administration significantly decreases high-fat diet (HFD)-induced weight gain without altering food intake in the DIM mouse model. This effect was due, at least in part, to a decrease in fatty tissue mass, as opposed to tissue such as the kidney, pancreas, or spleen. In addition, DIM significantly decreased hepatic lipid storage and serum triacylglycerol levels. Oil red O staining revealed that DIM inhibited MDI (a mixture of three constituents, methylisobutylxanthine, dexamethasone, and insulin)–induced adipogenesis in 3T3-L1 preadipocytes. DIM was found to inhibit not only MDI-induced adipogenesis, but also various MDI-induced adipogenic and lipogenic expression of genes at the

transcriptional level. These results underline the anti-obesity effects of DIM *in vitro* and *in vivo*.

Next, I investigated the detailed molecular mechanisms of how DIM inhibits adipogenesis in 3T3-L1 preadipocytes. I hypothesized that DIM might target MCE to inhibit adipogenesis. Oil red O staining revealed that the anti-adipogenic activity of DIM was largely confined to the MCE step, rather than the TD step. Since cell proliferation and cell cycle progression occur during MCE, a trypan blue assay and FACS analysis were conducted to estimate cell numbers and analyze cell cycle distribution. The results showed that DIM significantly delays cell cycle progression, and this results in a decrease in cell proliferation. This effect was also due to suppressed cyclin D1 protein expression at the post-transcriptional level. Western blot results showed that DIM decreased cyclin D1 protein expression through the enhancement of proteasome-mediated cyclin D1 degradation, and USP2 was implicated in this process. An *in vitro* deubiquitinase activity assay showed that DIM directly blocked the deubiquitinase activity of USP2. Taken together, these results

indicate that DIM inhibits MDI-induced adipogenesis of 3T3-L1 preadipocytes during the MCE step by targeting USP2 deubiquitinase activity.

Previous results have shown that DIM primarily targets MCE to inhibit adipogenesis of 3T3-L1 preadipocytes, and treatment of DIM during the TD step also decreases intracellular lipid accumulation as evidenced by oil red O staining. Since lipogenesis actively occurs during the TD step, this prompted us to hypothesize that DIM might alter lipid metabolism in mature 3T3-L1 adipocytes. I therefore sought to measure glycerol release. Glycerol assay results revealed that DIM increases glycerol release in mature 3T3-L1 adipocytes. In addition, DIM significantly decreases intracellular lipid accumulation, suggesting that DIM increases triacylglycerol efflux out of adipocytes. Since DIM enhanced lipolysis in mature 3T3-L1 adipocytes, Western blot analysis was conducted to estimate protein expression of lipolytic and lipogenic genes, and the results showed that DIM suppressed lipogenic gene expression significantly while lipolytic gene expression was negligibly affected by DIM treatment, suggesting

that DIM shifts lipid metabolism toward from the lipogenic pathway toward the lipolytic pathway. Unless it is linked to increased thermogenesis, this triacylglycerol efflux could result in increased serum free fatty acid concentrations, thereby causing metabolic syndromes. I then investigated the effects of DIM on thermogenic gene expression in mature 3T3-L1 adipocytes. Real time quantitative PCR results showed that DIM significantly increased thermogenic gene expression in mature 3T3-L1 adipocytes, while Western blot results showed that AMPK phosphorylation was significantly increased by DIM treatment. Taken together, I concluded that DIM exerts lipolytic and thermogenic activity in mature 3T3-L1 adipocytes.

These results reveal important information regardingthe molecular mechanisms of DIM *in vitro* and *in vivo*. Since DIM is a physiological metabolite of indole-3-carbinol (I3C) which is abundant in cabbage, it may explain, at least in part, the anti-obesity effects of cabbage. I have also shown that DIM decreases adipocyte hypertrophy/hyperplasia, and increases lipolysis and

thermogenesis simultaneously, indicating the potential for DIM to be developed as anti-obesity agent without side effects.

Keywords : 3,3'-diindolylmethane (DIM), high-fat diet (HFD), adipogenesis, mitotic clonal expansion (MCE), ubiquitin-specific protease 2 (USP2), lipolysis, thermogenesis

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Contents

Chapter 1.

Strategy for treatment of obesity: A Review

Abstract

Obesity is primarily caused by an imbalance between energy intake and energy expenditure. When energy intake exceeds energy expenditure, the body stores the excess energy in cells called adipocytes, and this causes hypertrophy and hyperplasia of adipocytes, which result in observable obesity. Since obesity has been becoming a serious medical problem across the globe, many researchers have focused efforts on developing novel anti-obesity drugs. To date, several anti-obesity drugs have been developed that target the central nervous system to control appetite or the digestive system to suppress lipid absorption. However, many side effects have been reported, suggesting the need for safer anti-obesity drugs that target new mechanisms. Since natural food phytochemicals have already been proven as safe by common historical consumption, the potential exists for the development of anti-obesity agents using phytochemicals that target adipocytes.

1.1. Introduction

Obesity has remained a serious medical problem in many of the world's developed countries. Obesity occurs when the size and number of adipocytes become excessive, due to an imbalance between energy intake and energy expenditure. Although a major health problem in itself, it also contributes to many other metabolic diseases including Type 2 diabetes, heart diseases, dyslipidemia, cardiovascular diseases, gallstones and respiratory dysfunction [1, 2]. The Second National Health and Examination Survey (NHANES II) showed that the prevalence of hypertension, hypercholesterolemia, diabetes among obese people is 2.9 times, 1.5 times, 2.9 times higher than for non-obese people, respectively [2]. Allison *et al*., also reported that obesity causes between 280,000 and 325,000 deaths per year in the United States alone [3]. The prevalence of obesity has been rapidly increasing in many Asian countries including Korea, and is becoming a serious medical problem [4, 5]. Therefore, renewed attention has been focused on biological research to develop new anti-obesity agents. There are many strategies for controlling obesity, but ultimately, they can be

divided into two types. The first focuses on ‘decreasing energy uptake’, and the second one emphasizes ‘increasing energy expenditure’ [6]. From this perspective, several drugs such as sibutramine, orlistat, rimonabant, lorcaserin and phentermine-topiramate have been approved for the treatment of obesity. However, these drugs target the central nervous system, which controls appetite; or the digestive system, which absorbs lipids, but not the adipocytes which are primarily responsible for obesity. Many adverse effects have also been associated with their activity [7]. This underlines the significant medical need for a better understanding of fat metabolism in adipocytes and studies to find agents that target these pathways. Additionally, for these drugs to have a significant effect on obesity, they must be used steadily for prolonged time periods. Newly synthesized drugs require extensive developmental periods in order to prove their safety in humans. IN contrast, natural food phytochemicals have a long history of human consumption, lowering the risk of severe toxicity. For these reasons, more research is focusing on natural food phytochemicals as novel anti-obesity agents. In this chapter, the pathogenesis of obesity and

metabolic diseases will be discussed, as well as the mechanisms and limits of available anti-obesity drugs, and some natural food phytochemicals that have been reported to have anti-obesity effects.

1.2. Pathogenesis of obesity and its complications

Obesity is ultimately due to an imbalance between energy intake and energy expenditure [8]. When energy intake exceeds energy expenditure, excess energy primarily is stored in the adipocytes. When this happens over a prolonged period of time, adipocyte size (hypertrophy) and number (hyperplasia) increases to meet the increasing demand for lipid storage [9]. Although which process is the major reason for obesity has been a controversial issue, it is now generally accepted that both hypertrophy and hyperplasia are responsible for obesity in rodents and humans [10-12]. Naaz *et al*., reported that p27 and p21 double knock-out (DBKO) mice weighed 100% more than WT mice [10]. They also reported that adipose tissue mass in DBKO mice was increased by 670% compared with WT mice [10]. Harmelen *et al*., reported that an increase in both adipocyte size and number occurs during adipose tissue expansion in obese humans, but the concomitant increase in adipocyte size precedes the increase in adipocyte number [11]. Hirsch *et al*., also reported that mildly obese patients exhibit adipocyte hypertrophy whereas severely obese patients

exhibit adipocyte hyperplasia, with the degree of hyperplasia positively correlated with severity [12]. Collectively, from the results of many previous studies, it can be assumed that both adipocyte hypertrophy and hyperplasia are responsible for adipose tissue expansion, and resultant obesity.

Obesity has become a very serious health issue because it contributes to a number of complications, collectively known as metabolic diseases. There have been many hypotheses created to explain how obesity causes various metabolic syndromes. Some papers report that ectopic lipid accumulation causes metabolic syndromes [13-15]. Adipose tissue stores excess energy primarily as fat during periods of excess nutrition and uses the stored fat during times when nutrition is scarce. This means that fat is not just stored within adipocytes, rather, it is dynamically regulated. To this end, adipocytes have both lipogenic activity and lipolytic activity. Lipogenesis is the process by which fatty acids and triglycerides are synthesized. Fatty acids are synthesized from acetyl-CoA, and three molecules of fatty acid are esterified with one molecule of glycerol to form a single triglyceride. Lipolysis is the process by which

triglycerides are hydrolyzed into three molecules of non-esterified fatty acids and one molecule of glycerol. These non-esterified fatty acids can be oxidized by β-oxidation to produce energy or heat [16]. Adipocytes maintain a balance between lipogenesis and lipolysis in normal states, however, in the obese state, adipocytes expand and lose the ability to control lipid metabolism properly. In this state, fat is ectopically stored, triggering metabolic diseases such as Type 2 diabetes, astherosclerosis, hepatic steatosis and many others [13-15].

Some investigators have reported that adipose tissue inflammation causes metabolic syndrome [17-21]. There are two types of macrophages present in adipose tissue, called M1 and M2 macrophages. In normal states, adipose tissue macrophages are polarized to the M2 type, expressing IL-10 and arginase. In the obese state, when adipocytes grow larger, M1 type macrophages with low IL-10 expression and high iNOS and TNF- production are instead recruited to adipose tissue. This inflammatory response induces insulin resistance and other metabolic diseases [20]. Evidence suggests that endoplasmic reticulum (ER) stress, mitochondrial dysfunction or adipose tissue hypoxia can also

contribute toward obesity-induced metabolic diseases [22-25]. Although various reports regarding obesity-induced metabolic diseases have surfaced, no unifying mechanism as to how obesity induces related metabolic complications has been described.

1.3. Current strategies for the treatment of obesity

Obesity can be prevented or treated by either decreasing energy intake or increasing energy expenditure. To date, the former option has been more preferred than the latter, because energy intake is much easier to control than energy expenditure. Energy intake depends entirely on food intake, whereas energy expenditure depends on various factors including basal metabolism, physical activity and adaptive thermogenesis [8]. In this regard, several anti-obesity drugs that target the central nervous system or digestive system have been developed, but many candidates have been withdrawn or are used only for severely obesity patients due to their side effects.

1.3.1. Anti-obesity agents targeting the central nervous system

The first pharmacological anti-obesity agents were amphetamine derivatives, which were sympathomimetics targeting the central nervous system as appetite suppressants. One such amphetamine congener, desoxyephedrine, has been approved by the FDA to treat obesity since 1947 [26]. It was originally developed as

a drug for narcolepsy. It was an article by Ray HM that let it to be approved for obesity management. That article reported that obese patients receiving 2 mg of desoxyephedrine 3 times daily lost up to 24.5 kg without any blood pressure elevation or addiction [27]. Since then, it has been widely used from the 1950s to 1960s. However, the risk of cardiovascular disease and addiction led to a marked decline in its use by the early 1970s. The next widely used anti-obesity agents were fenfluramine and dexfenfluamine, which are also anorectics. Weintraub *et al*., conducted a clinical trial with fenfluramine plus phentermine [28]. In their experiments, participants receiving the treatment lost approximately 16% of their initial weight, whereas participants receiving placebo lost only about 5% [28]. However, because of some reports about their adverse effect, cardiac valvulopathy [29], However, due to reports of their adverse effects, including cardiac valvulopathy, the FDA asked the manufacturers to withdraw them from the market in 1997 (http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm179871.htm).

Sibutramine (trade names: Reductil, Meridia and Activa)

was developed and approved in 1997 in the USA and 1999 in the European Union [30]. It was originally developed as an anti-depressant, but later was reported to act upon neurons as a serotonin-norepinephrine reuptake inhibitor to enhance satiety [30]. It also is known to have weak thermogenic activity [31]. Although many previous studies have shown the anti-obesity effects of sibutramine [32-34], other studies have uncovered side effects including dry mouth, insomnia, asthenia, anorexia, cardiovascular disease and hypertension [6, 35]. Because of these side effects, sibutramine was withdrawn from the market in 2010 (http://www.fda.gov/safety/medwatch/safetyinformation/safetyalertsforhumanmedicalproducts/ucm228830.htm).

The CB1-receptor has been reported to stimulate appetites in mice and the human brain [36], suggesting that CB1 blockade suppresses appetite. Rimonabant, also known as Acomplia, was first developed as a therapeutic CB1-receptor blocker in the 1990s [37]. Among the many CB1-receptor antagonists that have been reported to suppress appetite [38-40], only rimonabant was approved for use as an anti-obesity drug in Europe in 2006. However, the adverse

effects of rimonabant include nausea, dizziness, insomnia and diarrhea [30] which have been reported since 2008. These reports led to withdrawal (http://www.ema.europa.eu/docs/en_GB/document_library/Press_release/2009/11/WC500014774.pdf).

Recently, lorcaserin hydrochloride (trade name: Belviq) has been approved by the FDA since 2012 [41]. It works as a serotonin receptor 2C activator in the brain to help induce feelings of satiety. Unlike fenfluramine and dexfenfluramine which were withdrawn due to their serious side effects on heart valves, there have been no reported side effects of Belviq on heart valve abnormalities. This may be because it targets a different serotonin receptor isoform. As mentioned earlier, Belviq activates serotonin receptor 2C whereas fenfluramine and dexfenfluramine activate the serotonin 2B receptor. which has been reported to be related to heart valve damage [42] (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm309993.htm).

Single compound therapy has shown good weight loss

effect in initial stages, but readily shows decreased activity during long-term therapy. Due to this problem, many companies have tried to develop combinational therapies. In this context, Qsymia is the first FDA-approved combinational drug [41] to reach the market and is a combination of phentermine and topiramate [43]. Other combinational drugs, such as Tesofensine (a combination of serotonin / dopamine / noradrenaline reuptake inhibitors), Empatic (combination of bupropion and zonisamide), Obinepitide (combination of neuropeptide Y2/Y4 receptor agonist), are also awaiting approval. Since Qsymia has been reported to have serious side effects on the fetuses of pregnant women, the FDA approved this drug with a medication guide, called a Risk Evaluation and Mitigation Strategy (REMS) (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm312468.htm).

1.3.2. Anti-obesity agents targeting the digestive system

Orlistat, also known as Xenical and Alli, was first approved at 1998 [30]. It is a hydrated derivative of Streptomyces toxytrinici

lipstatin, a pancreatic lipase inhibitor [44, 45], that prevents the absorption of fats in the gastrointestinal tract. It inhibits lipase, an enzyme which is responsible for triglyceride disassembly to absorb free fatty acids. Many papers have reported that orlistat not only reduces bodyweight but also improves serum lipid profiles [46-48]. Unlike the other anti-obesity agents mentioned above, orlistat has been reported to have beneficial effects on cardiovascular risk [49, 50]. Side effects of orlistat have also been reported, including oily stools, faecal urgency, oily spotting, diarrhea, flatulence, bloating, abdominal pain and dyspepsia [48, 51]. The absorption of fat-soluble vitamins such as vitamin A and E were also reported to be inhibited, but plasma vitamin concentrations usually remain at normal levels [6]. Orlistat has been steadily used as anti-obesity agent for over 15 years, and is one of the remaining anti-obesity agents approved for long-term treatment.

1.3.3. Disadvantages of current strategies

To date, many anti-obesity drugs have been developed that target the central nervous system to suppress appetite, or target the

digestive system to inhibit fat absorption. Drugs that target the central nervous system such as the sympathetic nerve have been reported to exhibit many side effects which are associated with mental or cardiovascular diseases, as well as risk of addiction. Though the side effects of drugs that target the digestive system seem to be less serious than the former, they have still only been used in strict regulatory environments. Since all drugs which have been developed thus far have targets other than adipocytes, side effects appear inevitable [7, 52]. In addition, researchers have has considerable difficulty to ensure safety. These drawbacks have led come researchers to focus on natural phytochemicals as anti-obesity agents, that target adipocytes.

1.4. New approach for treatment of obesity

Natural phytochemicals have been receiving recent attention from many researchers, as they have relatively simpler safety profiles compared to synthetic drugs. Unlike anti-obesity drugs that exert anti-obesity effects by targeting the central nervous system or digestive system, the anti-obesity effect of phytochemicals has been suggested to primarily target processes involved in adipocyte and lipid metabolism, such as adipogenesis, lipolysis or adipocyte cell death [53, 54].

1.4.1. Anti-adipogenic activity of natural compounds

The adipogenesis of 3T3-L1 preadipocytes has been widely used to study obesity, because they reportedly mimic physiological obesity development [55]. It is generally accepted that natural compounds which have anti-adipogenic activities might exert anti-obesity effects.

Isoflavones in soybeans are the most intensively studied anti-adipogenic agents, perhaps because there have been epidemiologic correlations drawn between soybean consumption

and lower body weight. Genistein is the most well-known anti-adipogenic agent among the isoflavones. Harmon *et al*., reported that genistein inhibits adipogenesis in the 3T3-L1 cell line [56]. Since genistein has been reported as a tyrosine kinase inhibitor, tyrosine phosphorylation of insulin receptor substrate-1 (IRS1) was investigated. They reported that there were no changes in IRS1 tyrosine phosphorylation status between vehicle-treated and genistein-treated groups [56]. Later it was reported that the anti-adipogenic activity of genistein is due to increased C/EBP homologous protein expression [57]. Following this discovery, many other researchers have investigated the molecular mechanisms of the anti-adipogenic activity of genistein. Genistein has been reported to suppress adipogenesis by enhancing the estrogen receptor (ER) and suppressing peroxisome proliferator-activated receptor γ (PPARγ) [58], and represses adipogenesis by activating the transforming growth factor-β1 (TGF-β1) signaling pathway in an ER-dependent manner [59]. Additionally, genistein has been reported to inhibit adipogenesis by activating AMP-activated protein kinase (AMPK) [60], by downregulating

extracellular signal-regulated kinase 1/2 (ERK 1/2) activity [61], and by suppressing p38 and janus-activated kinase 2 (JAK2) [62]. Recently, there have been several reports on combinatorial anti-adipogenic effects of genistein with other natural compounds such as resveratrol [63, 64], quercetin [64], 1,25-dihydroxyvitamin D_3 [65], guggulsterone [66], showing that such compounds synergistically inhibit adipogenesis with genistein. While the anti-adipogenic effects of genistein have been being intensively studied, another isoflavone, daidzein, has received much less focus in terms of anti-adipogenic activity. There have been several papers investigating the role of daidzein on adipogenesis. Kim *et al*., reported that daidzein represses adipogenesis of human adipose tissue-derived mesenchymal stem cells through inhibition of the Wnt/β-catenin signaling or lipolysis [67], while Seo *et al*., reported that the physiological metabolite of daidzein, 6,7,4'-trihydroxyisoflavone (6,7,4'-THIF) inhibits adipogenesis in 3T3-L1 preadipocytes by targeting phosphatidyl inositol 3-kinase (PI3K) [68].

Resveratrol is another well-known anti-adipogenic natural

compound. Resveratrol activates SIRT1 [69] to enable various health promoting effects. As mentioned earlier, resveratrol has been reported to exert synergistic activity with genistein [63, 64], and other compounds [70]. Rayalam *et al*., reported that resveratrol down-regulates various adipogenic genes to inhibit adipogenesis [71]. Kwon *et al*., reported that resveratrol inhibits insulin signaling and mitotic clonal expansion (MCE) [72]. Besides these reports, resveratrol analogs have also been studied. Vingtdeux *et al*., reported that the resveratrol analogs RSVA314 and RSVA405 were reported to activate AMPK to inhibit adipogenesis of 3T3-L1 cells [73], while Kwon *et al*., reported that piceatannol, a structurally similar compound with resveratrol, exhibits anti-adipogenic activity in an insulin receptor (IR)-dependent manner [74]. *In vivo* results have also been reported. Kim *et al*., reported that the anti-obesity effects of resveratrol can be observed in high-fat diet (HFD)-induced obese mice [75]. These *in vitro* and *in vivo* results underline the potential for resveratrol and its analogs (or metabolites) to be used as potent anti-obesity agents.

In addition to these natural compounds, many other

phytochemicals, including epigallocatechin gallate (EGCG) [76-79], conjugated linoleic acid (CLA) [80, 81], ajoene [82], curcumin [83-85], and quercetin [86], have been reported to exhibit anti-adipogenic effects. EGCG, a natural phytochemical found in green tea, has been reported to inhibit adipogenesis by activating the lamine receptor pathway [78], and down-regulates the Wnt/ -catenin signaling pathway [79]. CLA is from a group of linoleic acids that have conjugated double bonds. Among the various forms of CLA, the anti-adipogenic activity of trans-10, cis-12 CLA was reported [80, 81], but isomer-specific anti-adipogenic activity of CLA remains elusive.

1.4.2. Lipolytic and thermogenic activity of natural compounds

Although many anti-adipogenic agents have been studied to date, these agents only inhibit newly synthesized adipocytes. Adipocytes that have been newly-synthesized would remain unaffected by these agents, indicating that the efficacy of this approach would be limited. Also, the inhibition of adipogenesis with excess energy intake may lead to a lack of lipid storage

capacity, causing increased serum fatty acid concentration and various metabolic diseases. It has therefore been postulated that this notion could be combined with another strategy, such as enhancing lipolysis and thermogenesis, to enhance the overall efficacy of treatment and to prevent side effects.

There have been many natural compounds reported to exhibit lipolytic activity [87]. Szkudelska *et al*., reported that genistein inhibits lipogenesis by suppressing fatty acid synthesis from glucose and stimulates lipolysis by targeting upstream adenylyl cyclase activation [87]. Another isoflavone, daidzein, was also reported to exert lipolytic activity [67]. Kim *et al*., reported that daidzein enhances lipolysis by activating PKA-mediated hormone sensitive lipase [67].

Resveratrol has also been reported to have lipolytic activity [88-92]. Baile *et al*., reported that resveratrol increases lipolysis and reduces lipogenesis in mature adipocytes [88]. It was also reported that combinational therapy of resveratrol with vitamin D, quercetin, and genistein synergistically reduces weight gain [88]. Ahn *et al*., reported that resveratrol reverses atherogenic diet-induced up-

regulating lipogenic genes and down-regulating lipolytic genes, indicating that resveratrol activates lipolysis *in vivo* [89]. The thermogenic activity of resveratrol has also been reported [93]. Lagouge *et al*., reported that resveratrol induces oxygen consumption by increasing the capacity for oxidative phosphorylation and mitochondrial biogenesis [93].

In addition, many other natural compounds have been reported to exert lipolytic activity. Quercetin, luteolin, and fisetin were reported to increase lipolysis dose- and time-dependently in rat adipocytes [94]. CLA has been reported to influence lipolysis *in vitro* [95, 96], in mice [97] and in humans [98]. Evans *et al*., reported that trans-10, cis-12 CLA enhances lipolysis and fatty acid oxidation [99], highlighting the TG-lowering effects of CLA. Sun *et al*., determined the role of docosahexanoic acid (DHA) on lipid metabolism-related genes in various tissues [100], however, the effect of DHA on lipid metabolism-related gene in different tissues remains controversial.

1.5. Conclusion

Since obesity has become a serious global health problem, various studies have been conducted to develop novel anti-obesity agents. As a result of these studies, several anti-obesity drugs have been developed that target the central nervous system or digestive system, instead of focusing on targeting adipocytes and lipid metabolism, which are directly related to the pathogenesis of obesity. Due to this discrepancy, many side effects have been reported. In recent years, adipocyte and lipid metabolism has therefore been receiving renewed interest as a target of natural phytochemicals for the prevention or treatment of obesity. However, this approach has also been reported to entail difficulties in terms of weak physiological effects. To solve this problem, researchers have investigated combinational therapies using several compounds that target different pathways. Though this approach has many challenges to overcome, it is thought that the development of safe anti-obesity agents without serious side effects may be possible in the near future.

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Chapter 2.

3,3'-diindolylmethane (DIM) suppresses high fat-diet (HFD)-induced obesity *in vivo* and MDI-induced adipogenesis *in vitro*

Abstract

Obesity has been become a major global health problem and contributes to many other metabolic syndromes. Obesity occurs when excess energy is stored in adipocytes, resulting in hyperplasia and hypertrophy of the cells. To date, cabbage has been regarded as a common home remedy for obesity, but the molecular mechanisms for this notion remain unknown. In the present study, *in vivo* experiments rshowed that DIM significantly reduces high fat-diet (HFD)-induced weight gain in a diet-induced obesity (DIO) mouse model. DIM reduced HFD-induced epididymal fat mass and adipose cell size. In addition, DIM significantly reduced serum triglyceride levels and hepatic lipid storage. Adipogenesis of 3T3-L1 preadipocytes was induced to confirm these *in vivo* results and to investigate the anti-adipogenic activity of 3,3'-diindolylmethane (DIM), and the metabolite indole-3-carbinol (I3C), which is a natural compound derived from cruciferous vegetables. Results showed that DIM, but not I3C, suppressed adipogenesis in 3T3-L1 preadipocytes. Additionally, DIM suppressed the expression of various adipogenic and lipogenic genes including peroxisome

proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα), adipocyte protein 2 (aP2), fatty acid synthase (FAS), and adipocyte determination- and differentiation-dependent factor1/sterol response element-binding protein 1c (ADD1/SREBP1c). Taken together, these results highlight the anti-obesity effects of DIM *in vitro* and *in vivo*, although the specific molecular mechanisms responsible remain elusive.

2.1. Introduction

The obesity rate in eastern countries has been gradually increasing, as approaching that of western countries. It is well-documented that obesity contributes to several chronic diseases, including diabetes, arthritis, hypertension, and heart diseases [1]. Therefore, the prevention of obesity has become a critical unmet medical need. Obesity is primarily characterized by hypertrophy (increase in size) and hyperplasia (increase in number) of adipocytes [2]. Both events contribute to increases in adipose tissue mass.

3T3-L1 preadipocytes are well-established cell lines that can mimic the differentiation process of preadipocytes to adipocytes under appropriate conditions. This adipocyte differentiation step is known as adipogenesis. Upon hormonal stimulation, 3T3-L1 preadipocyte cell lines differentiate into adipocytes. A hormone cocktail can induce this process, and is composed of a mixture of three materials, isobutylmethylxanthine (IBMX), dexamethasone and insulin, together referre to as MDI [3]. IBMX has been known to induce CCAAT-enhancer-binding protein β (C/EBPβ), and

dexamethasone has been known to induce C/EBPδ, which are both important regulators of adipogenesis [4]. In addition, IBMX has been reported to induce peroxisome proliferator-activated receptor γ (PPARγ) transcriptional activity by increasing cAMP levels [5]. Insulin activates various signaling pathways when it binds to the insulin receptor. The insulin signaling pathway has been reported to induce glucose uptake and intracellular lipid accumulation in adipocytes [6].

Adipogenesis consists of two primary stages, mitotic clonal expansion (MCE) and terminal differentiation (TD) [7]. The MCE step is a type of cell proliferation. During the MCE stage, growth arrested cells synchronously re-enter the cell cycle and cell numbers increase. In many previous studies, the MCE stage has been shown to be a prerequisite step for TD and consequently the entire adipogenesis process [8-11]. In accordance with these previous studies, Kwon *et al*., reported that resveratrol and piceatannol can block MCE to suppress adipogenesis [12, 13], suggesting that of the arrest of MCE is sufficient to inhibit the entire adipogenesis process. The MCE stage is followed closely by the TD stage. It is the TD

stage during which 3T3-L1 preadipocytes acquire an adipocyte-like phenotype. Their morphology changes from fibroblast-like shapes into a more rounded shape, and the cells acquire the ability to store intracellular lipid droplets. During the TD stage, the adipogenic master regulator, PPARγ, and other lipogenic genes, including the C/EBP family, adipocyte protein 2 (aP2), fatty acid synthase (FAS) and adipocyte determination- and differentiation-dependent factor1/sterol response element-binding protein 1c (ADD1/SREBP1c) are highly expressed. These genes have been known to confer adipocyte-like phenotypes in 3T3-L1 preadipocytes [14-18].

3,3'-Diindolylmethane (DIM) is a physiological metabolite of indole-3-carbinol (I3C) which is converted from glucobrassicin [19, 20], a glucosinolate compound that is abundantly detected in various brassica species including cabbage, chinese cabbage, broccoli, and others [21-23]. Glucobrassicin and sinigrin are major glucosinolates in cabbage, and they have been reported to be metabolized by myrosinase to I3C and allylisothiocyanate (AITC), respectively, while I3C has been reported to be further oligomerized

to DIM in the human stomach [19, 20] (structures of these compounds are depicted at Fig. 1). Choi, Y. *et al.*, reported that I3C inhibits adipocyte differentiation *in vitro* by targeting SIRT1 (silent mating type information regulation 2 homolog 1) [24] and prevents diet-induced obesity *in vivo* [25]. It was also reported that I3C protects against diet-induced hepatic steatosis in mice through the activation of the SIRT1-AMPK (AMP-activated protein kinase) signaling cascade [26]. Although the anti-obesity effect of an I3C, active compound of cruciferous vegetables, was revealed, together with its mechanism of action *in vitro* and *in vivo*, it is still unclear whether I3C really exerts anti-obesity effects in the humanssince previous studies have shown that the metabolism an breakdown of I3C takes place in the human body [19, 20]. Collectively, it appears more likely that DIM, rather than I3C, exerts anti-obesity effects in physiological conditions. To clarify this hypothesis, I investigated the anti-adipogenic activity of DIM in 3T3-L1 preadipocytes and the anti-obesity effect of DIM in a diet-induced obesity (DIO) mouse model.

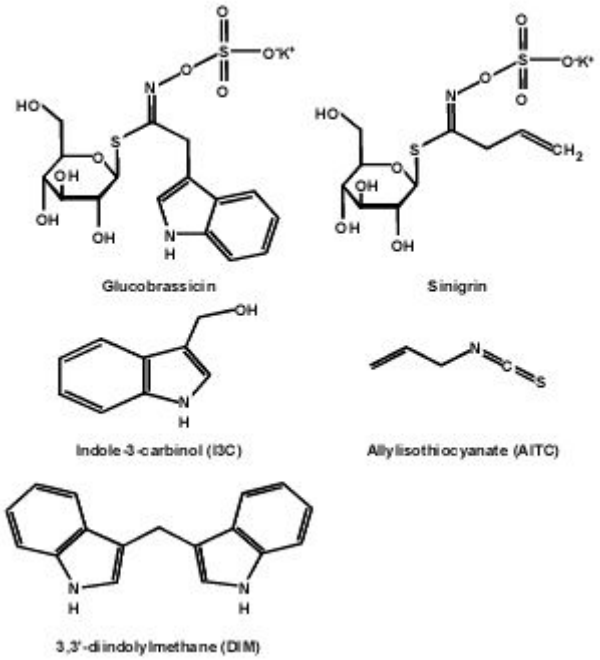


Figure 1. Chemical Structure of various glucosinolates and their metabolites.

2.2. Materials and Methods

2.2.1. Chemicals and reagents

3,3'-diindolylmethane (DIM), indole-3-carbinol (I3C), and antibody against β-actin were obtained from Sigma (St. Louis, MO). Antibody against PPARγ was obtained from Santa Cruz (Santa Cruz, CA). Antibodies against C/EBPα, FAS, aP2, and SREBP-1c were obtained from Cell Signaling Biotechnology (Beverly, MA). Dexamethasone, isobutylmethylxanthine (IBMX) and insulin were purchased from Sigma (St. Louis, MO).

2.2.2. Animals and diets

Animal experiment was performed as described previously [27]. Male C57BL/6J mice (6-week-old) were purchased from Central Lab. Animal Inc. (Seoul, Korea). After 1 week acclimation, mice were housed in climate-controlled quarters (23 ± 3 ^{o}C, 50 ± 10% humidity) with a 12-hr light-dark cycle. Mice were divided into five different dietary groups (n=7 each group): a low-fat diet (LFD), a high-fat diet (HFD), a HFD with 10 mg kg^{-1} body weight

(BW) of DIM administration, a HFD with 50 mg kg^{-1} BW of DIM administration and a HFD with 250 mg kg^{-1} BW of DIM administration. The LFD and HFD were purchased from Research diets Inc. (New Brunswick, NJ), and provided in the form of pellets for 12 weeks. Animals were treated with either DIM, which was dissolved in phosphate buffered saline (PBS), or vehicle by oral gavage evergyday. BW and food intake were monitored on a weekly basis.

2.2.3. Serum triacylglycerol content

Serum triglycerides content was measured as described previously [27]. Blood was collected in serum separator tubes (Becton Dickinson; Franklin Lakes, NJ) and centrifuged at 3000 rpm for 20 mins. Serum triglyceride contents were measured by enzymatic assay using kits purchased from Sigma (St. Louis, MO).

2.2.4. Cell culture

All cell culture materials were obtained from GIBCO BRL (Grand Island, NY). 3T3-L1 mouse preadipocytes were maintained

in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS), at 5% CO2 and 37°C. Cells were subcultured every two or three days at a density of 2×10^5 cells per dish.

2.2.5. MTS assay

3T3-L1 preadipocytes were seeded in 24-well plate at a density of 2.5×10^4 cells per well. Cells were incubated until confluence. Two days after confluence, cells were treated with DMEM supplemented with 10% fetal bovine serum (FBS) in the presence or absence of DIM or I3C at indicated concentrations. After three days, MTS solution was added to each well according to the manufacturer's instructions. After 30 minutes incubation, absorbance of formazan product was read at 490nm.

2.2.6. Trypan blue assay

3T3-L1 preadipocytes were seeded in 24-well plates at a density of 2.5×10^4 cells per well, and incubated in DMEM supplemented with 10% FBS and MDI cocktail in the presence or

absence of DIM or I3C at indicated concentrations. Cells were trypsinized and stained with 0.4% trypan blue. The stained cells were loaded onto a hematocytometer, and both the viable cells and non-viable cells were counted.

2.2.7. Adipogenesis

3T3-L1 preadipocytes were seeded and cultured until confluence. Two days after confluence, cells were incubated for two days in DMEM supplemented with 10% FBS and mixture of isobutylmethylxanthine, dexamethasone, and insulin (MDI) cocktail [dexamethasone (1 μM), IBMX (0.5 mM), and insulin (5 μg/mL)]. After two days, medium was replaced with DMEM containing 10% FBS and 5 μg/mL of insulin, and cells were incubated for two days. After two days, medium was replaced with DMEM containing 10% FBS.

2.2.8. Oil red O staining

3T3-L1 preadipocytes were seeded in 24-well plates at a density of 2.5×10^4 cells per well and incubated until confluence.

Cells were differentiated to adipocytes as mentioned above in the presence or absence of DIM and I3C. After induction of cell differentiation for 6 days, the media were removed, and the mature 3T3-L1 adipocytes were fixed in 10% formaline. The fixed cells were stained with Oil red O solution as previously described [27]. Stained Oil red O solution was eluted with isopropyl alcohol (Sigma), and the concentration was measured using a spectrophotometer set at 515 nm.

2.2.9. Western blot analysis

3T3-L1 preadipocytes were seeded in 6-cm dishes at a density of 1.5×10^5 cells per dish and treated as mentioned in the figure. The cells were lysed and centrifuged (10 min, 14,000 rpm, 4^oC), and supernatants were collected. The supernatants were loaded onto SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare; Piscataway, NJ). The membranes were blocked with 5% skim milk and incubated with specific primary antibodies followed by HRP-conjugated secondary antibodies. Protein bands were visualized using a

chemiluminescence detection kit (Amersham Pharmacia Biotech; Piscataway, NJ).

2.2.10. Quantitative real-time PCR

3T3-L1 preadipocytes were seeded in 6-cm dishes at a density of 1.5×10^5 cells per dish and treated as mentioned in the figure. Messenger RNA (mRNA) was extracted using RNA-BEE (TEL-TEST; Pearland, TX) and reverse transcribed into complementary DNA (cDNA) using Promega (Fitchburg, WI) product. Quantitative real-time PCR was performed as described previously [28] using SYBR Green RT-PCR kit from Fermentas (Glen Burnie, MD). Results are expressed relative to β-actin.

2.2.11. Statistical analysis

Data are expressed as means ± SD, and Student's *t*-test was used for single statistical comparisons. A probability value of $p < 0.05$ was used as the criterion for statistical significance.

2.3. Results

2.3.1. 3,3'-diindolylmethane (DIM) suppresses high fat-diet (HFD)-induced weight gain in diet-induced obesity (DIO) mouse model

I evaluated the anti-obesity effects of DIM in a diet-induced obesity (DIO) mouse model. The body weight of mice fed on an HFD increased significantly compared with mice fed on a low fat-diet (LFD), while DIM decreased HFD-induced body weight gain in a dose-dependent manner (Fig. 2A, 2B). To investigate whether DIM acts as an appetite suppressant, I monitored food intake of the mice in each group. Food intake quantification showed that DIM did not affect intake significantly, with all four groups of mice showing similar values (Fig. 2C). The food efficiency ratio (FER) is an indicator of how efficiently ingested food increases body weight. Figure 2D shows that DIM significantly decreased FER (Fig. 2D). Next, I analysed the weights of several organs. The results showed that DIM decreased white adipose tissue (WAT) mass, but not that of the pancreas, spleen or kidney (Fig. 3). These results together suggest that DIM reduces HFD-induced weight gain by decreasing

adipose tissue mass, but not via appetite suppression.

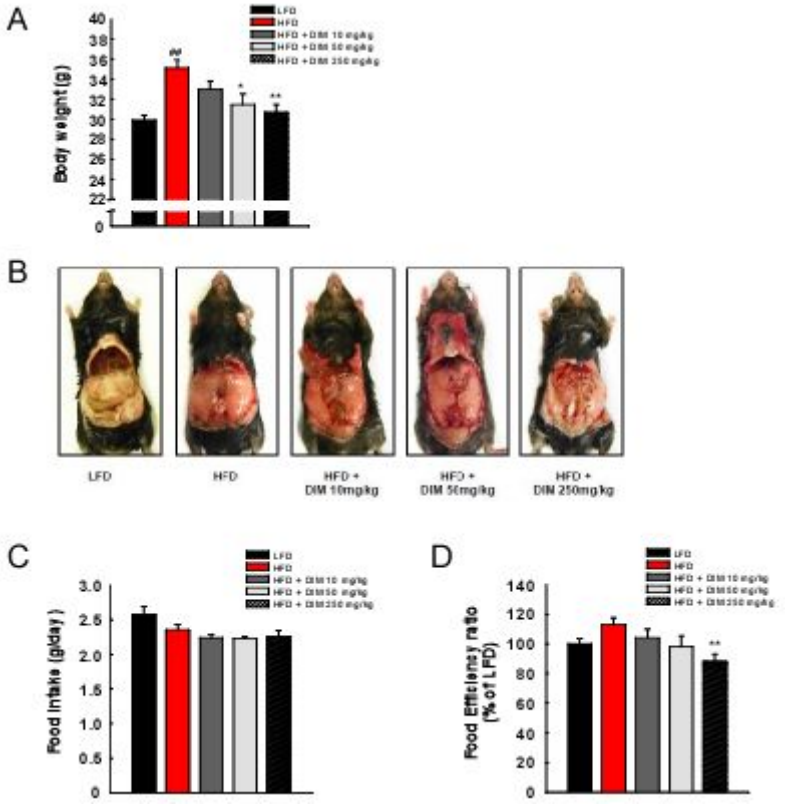


Figure 2. (A-B) Effects of DIM on HFD-induced weight gain in DIO mouse model. (A) Animal experiment was conducted as described at Materials and Methods section. Mice were administrated as indicated at the figure. Mice body weights were measured every week. The presented result was body weight of 12th week. Mice body weights were presented as means ± SE (n = 7). * : compared with HFD ($p < 0.05$). ** : compared with HFD ($p <$

0.01). ## : compared with LFD ($p < 0.01$). (B) Animal experiment was conducted as described at Materials and Methods section. 12 week after HFD with or without DIM administration, mice were sacrificed and visualized by photograph. The presented photographs are of representative mouse at each group. (C) Effect of HFD and DIM on food intake in DIO mouse model. Animal experiment was conducted as described at Materials and Methods section. Mice were administrated as indicated at the figure. Food intakes were measured three times per week. Mice food intakes were presented as means ± SE (n = 7). (D) Effect of HFD and DIM on food efficiency ratio in DIO mouse model. Animal experiment was conducted as described at Materials and Methods section. Mice were administrated as indicated at the figure. Food efficiency ratio is body weight gain normalized with food intake. Food efficiency ratio was calculated according to this formula, “weight gain / food intake”. Mice food efficiency ratios, expressed as a percentage of LFD values, were presented as means ± SE (n = 7). ** : compared with HFD ($p < 0.01$).

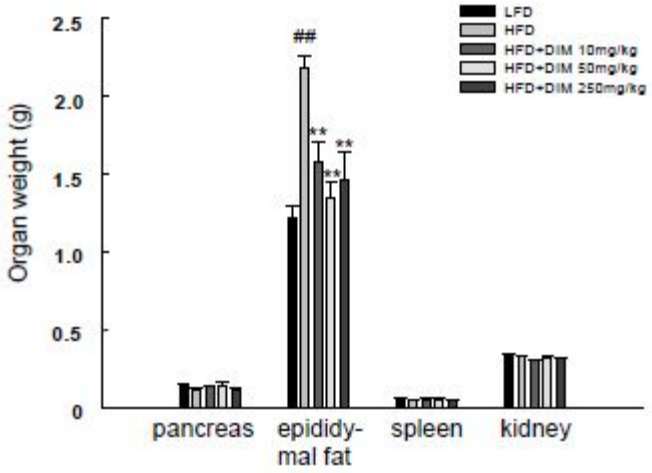


Figure 3. Effects of DIM on various tissue mass in DIO mouse model. Animal experiment was conducted as described at Materials and Methods section. Mice were administrated as indicated at the figure. 12 weeks after HFD with or without DIM administration, mice were sacrificed. After that, pancreas, epididymal fat, spleen, and kidney mass were measured. Each tissue mass were presented as means ± SE (n = 7). ** : compared with HFD ($p < 0.01$). ## : compared with LFD ($p < 0.01$).

2.3.2. 3,3'-diindolylmethane (DIM) decreases high fat-diet (HFD)-induced serum and hepatic lipid storage in a diet-induced obesity (DIO) mouse model

Since DIM reduced adipose tissue mass, I next investigated whether DIM increases ectopic lipid storage. First, photographs were taken of adipose tissue under the microscope to confirm the mass-reducing effect of DIM in the DIO mouse model. As evidenced by photography, DIM reduced adipocyte size significantly (Fig. 4A). Next, I measured serum triglyceride levels, and the results showed that DIM dose-dependently decreased serum triglyceride concentrations, which were significantly elevated by the HFD (Fig. 4B). In addition, hepatic lipid storage, which is closely related to hepatic insulin resistance, was significantly decreased by DIM administration (Fig. 4C). These results suggest that DIM decreases serum-circulating lipid levels and ectopic lipid storage.

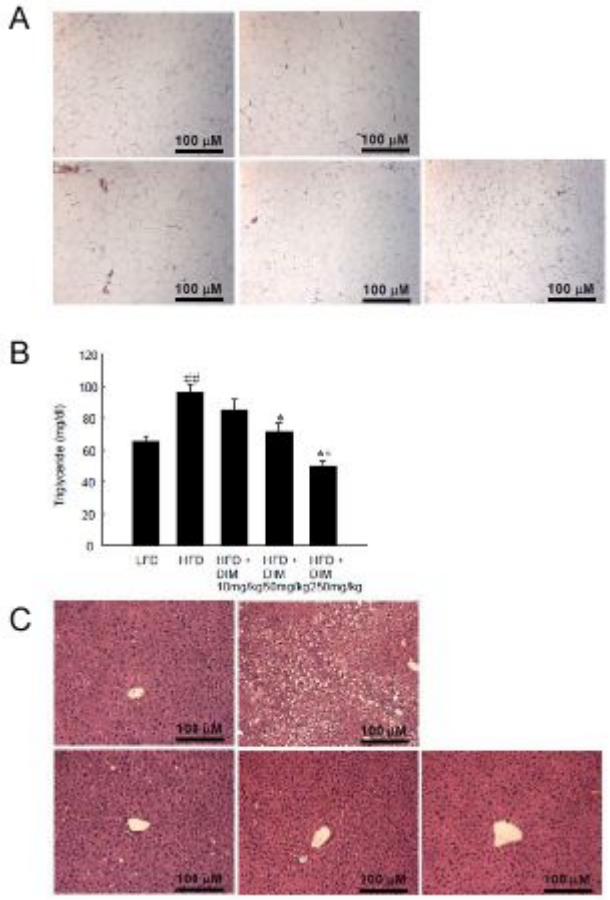


Figure 4. (A) Effect of DIM on adipocyte size in DIO mouse model. Animal experiment was conducted as described at Materials and Methods section. Mice were administrated as indicated at the figure. 12 weeks after HFD with or without DIM administration, mice were sacrificed. After that, epididymal fat tissue was paraffin-sectioned and stained with hematoxylin & eosin. Tissues were visualized

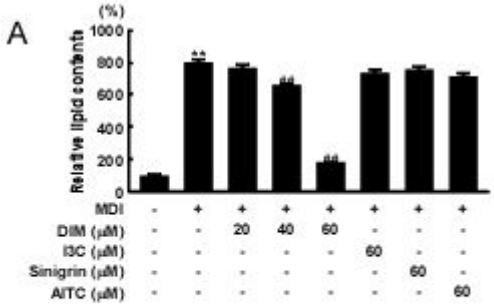
under the microscope. The presented photographs are of representative tissue at each group (upper left : LFD, upper right : HFD, lower left : HFD + DIM 10 mg/kg, lower middle : HFD + DIM 50 mg/kg, lower right : HFD + DIM 250 mg/kg). (B) Effect of DIM on serum triglyceride concentration in DIO mouse model. Animal experiment was conducted as described at Materials and Methods section. Mice were administrated as indicated at the figure. 12 weeks after HFD with or without DIM administration, mice serum was collected and triglyceride concentration was measured as described at Materials and Methods section. Serum triglyceride concentrations were presented as means ± SE (n = 7). * : compared with HFD ($p < 0.05$). ** : compared with HFD ($p < 0.01$). ## : compared with LFD ($p < 0.01$). (C) Effect of DIM on hepatic lipid accumulation in DIO mouse model. Animal experiment was conducted as described at Materials and Methods section. Mice were administrated as indicated at the figure. 12 weeks after HFD with or without DIM administration, mice were sacrificed. After that, liver tissue was paraffin-sectioned and stained with hematoxylin & eosin. Tissues were visualized under the microscope.

The presented photographs are of representative tissue at each group (upper left : LFD, upper right : HFD, lower left : HFD + DIM 10 mg/kg, lower middle : HFD + DIM 50 mg/kg, lower right : HFD + DIM 250 mg/kg).

2.3.3. 3,3'-diindolylmethane (DIM) is the most potent anti-adipogenic agent among the four glucosinolates in MDI-induced adipogenesis of 3T3-L1 preadipocytes

To confirm that DIM is the most potent anti-obesity agent among the cabbage glucosinolates, I compared the anti-adipogenic activity of four different glucosinolates, indole-3-carbinol (I3C) and its physiological metabolite, 3,3'-diindolylmethane (DIM), sinigrin and its aglycone, allylisothiocyanate (AITC) (Fig. 1). Oil red O staining showed that DIM completely blocked MDI-induced adipogenesis in 3T3-L1 preadipocytes at 60 M whereas the other three glucosinolates did not (Fig. 5A). Since DIM exhibited the best anti-adipogenic activity in 3T3-L1 preadipocytes, I compared the anti-adipogenic activity of DIM and its parent compound, I3C, dose-dependently. Oil red O staining results showed that DIM suppressed MDI-induced adipogenesis of 3T3-L1 preadipocytes in a dose-dependent manner, but indole-3-carbinol did not significantly suppress adipogenesis of 3T3-L1 preadipocytes (Fig. 5B, 5C). The anti-adipogenic activity of DIM in 3T3-L1 preadipocytes was confirmed by CARS imaging analysis.

Consistent with the Oil red O staining result, DIM significantly decreased lipid accumulation at 60 M. However, 60 M of I3C did not exhibit any anti-adipogenic activity (Fig. 5D). To clarify that this anti-adipogenic effect of DIM was not due to cytotoxicity, I conducted an MTS assay and a trypan blue assay. The MTS assay result showed that both DIM and I3C did not exert significant cytotoxicity (Fig. 5E). In addition, the trypan blue assay result showed that neither DIM nor I3C decrease viable cell number even if treated with MDI (Fig. 5F). Taken together, these results suggest that DIM specifically suppress MDI-induced adipogenesis in 3T3-L1 preadipocytes.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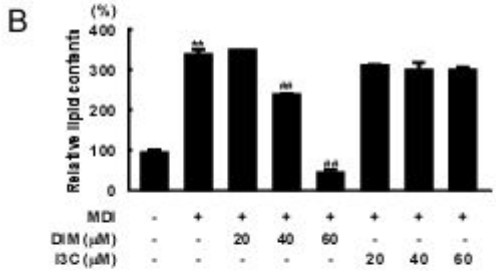
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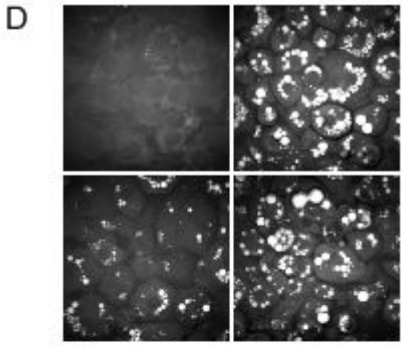

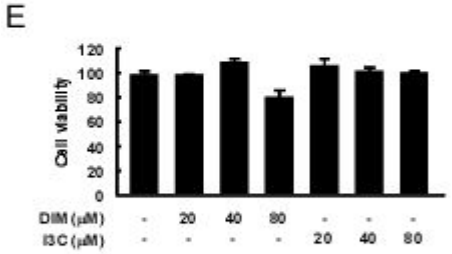

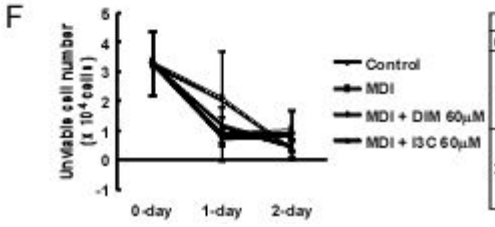

% of dead cell (%)		Average	STDEV
0-day	Control	4.99	1.89
1-day	Control	3.27	2.28
	MDI	1.06	1.05
	MDI + DIM 60μM	1.23	0.17
	MDI + I3C 60μM	1.58	0.35
2-day	Control	0.65	0.56
	MDI	0.88	0.67
	MDI + DIM 60μM	1.44	1.41
	MDI + I3C 60μM	0.47	0.15

Figure 5. (A-D) Effects of various glucosinolates on MDI-induced adipogenesis of 3T3-L1 preadipocytes. (A) 3T3-L1 preadipocytes were induced to differentiate to adipocyte in the presence of various glucosinolates at indicated concentrations. Intracellular lipid accumulations were stained by oil red O staining. Stained oil red O

was eluted with isopropyl alcohol and absorbance was measured at 515 nm. The intracellular lipid accumulations, expressed as a percentage of control values, were presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$). ## : compared with MDI ($p < 0.01$). (B) 3T3-L1 preadipocytes were induced to differentiate to adipocyte in the presence of DIM or I3C at indicated concentrations. Intracellular lipid accumulations were stained by oil red O staining. Stained oil red O was eluted with isopropyl alcohol and absorbance was measured at 515 nm. The intracellular lipid accumulations, expressed as a percentage of control values, were presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$). ## : compared with MDI ($p < 0.01$). (C) 3T3-L1 preadipocytes were induced to differentiate to adipocyte in the presence of DIM or I3C at indicated concentrations. Intracellular lipid accumulations were stained by oil red O staining. Stained oil red O was visualized by photograph. (D) 3T3-L1 preadipocytes were induced to differentiate to adipocyte in the presence of DIM or I3C at 60 μM. Intracellular lipid droplets were visualized by CARS imaging (upper left : control, upper right : MDI, lower left : MDI +

DIM 60μM, lower right : MDI + I3C 60μM). (E-F) Cytotoxicity of DIM and I3C on 3T3-L1 preadipocytes. (E) 3T3-L1 preadipocytes were cultured until confluence, then they were treated with DIM or I3C at indicated concentrations for three days. Relative cell viabilities were measure by MTT assay. The relative cell viabilities, expressed as a percentage of control values, were presented as means ± SE (n = 3). (F) 3T3-L1 preadipocytes were cultured until confluence, then they were treated with MDI in presence or absence of DIM and I3C at 60 μM. At indicated time points, cells were trypsinized and stained with trypan blue, then they were loaded on hematocytometer to be counted. The number of unviable cells were presented as means ± SE (n = 3).

2.3.4. 3,3'-diindolylmethane (DIM) suppresses both mRNA and protein expression of adipogenic and lipogenic gene

Since DIM inhibited MDI-induced adipogenesis completely, I next evaluated adipogenic and lipogenic gene expression. Protein expression of two adipogenic genes, PPARγ and C/EBPα, were decreased by DIM treatment (Fig. 6A). Additionally, protein expression of aP2, a PPARγ target gene, was decreased by DIM treatment (Fig. 6A). Furthermore, DIM abolished protein expression of various lipogenic genes, including FAS and SREBP1. To verify that DIM inhibited expression of these genes at the transcriptional level, I performed quantitative real time PCR (qRT-PCR). The qRT-PCR results showed that mRNA expression of various adipogenic and lipogenic genes are down-regulated by DIM treatment (Fig. 6B). Taking these results together, I concluded that DIM suppresses MDI-induced adipogenesis of 3T3-1 preadipocytes and decreases various adipogenic and lipogenic gene expression at the transcriptional level.

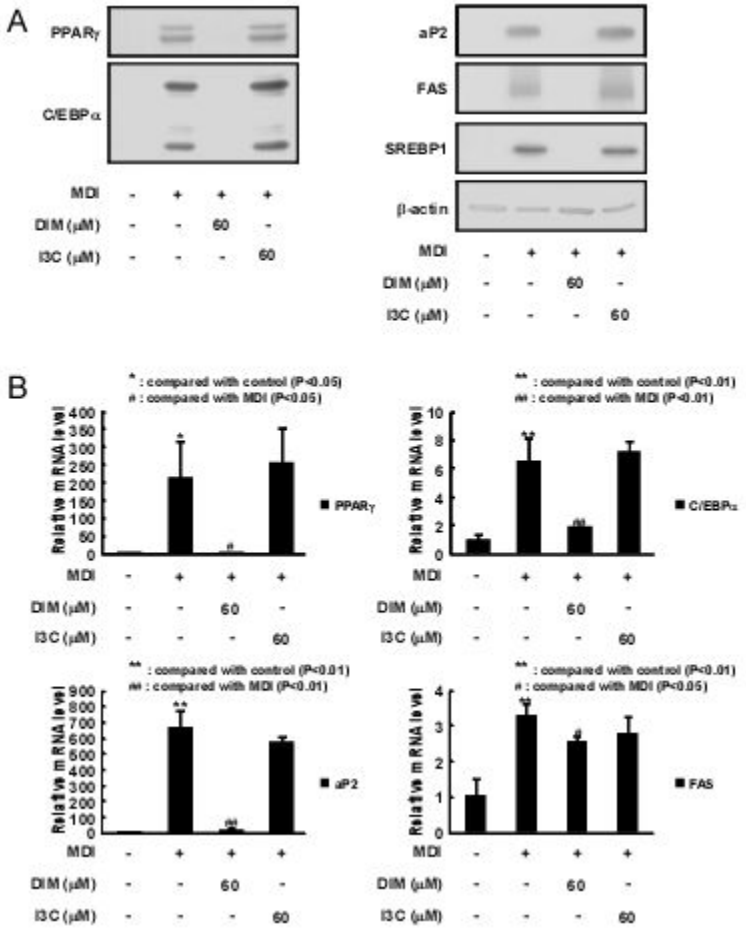

Figure 6. (A) Effects of DIM and I3C on MDI-induced protein expression of various adipogenic and lipogenic genes in 3T3-L1 peadipocytes. 3T3-L1 preadipocytes were induced to differentiate to adipocyte in the presence of DIM or I3C at 60 μM. Then they were lysed as described in Materials and Methods section. The amount of

PPARγ, C/EBPα, aP2, FAS, and SREBP1 protein expressions were determined by Western blotting as described in Materials and Methods section. β-actin was measured as loading control. The data are representative of three independent experiments. (B) Effects of DIM and I3C on MDI-induced mRNA expression of various adipogenic and lipogenic genes in 3T3-L1 peadipocytes. 3T3-L1 preadipocytes were induced to differentiate to adipocyte in the presence of DIM or I3C at 60 μM. RNA was extracted as described in Materials and Methods section. The amount of PPARγ, C/EBPα, aP2, and FAS mRNA expressions were determined by qRT-PCR as described in Materials and Methods section. β-actin was used as reference gene. The relative mRNA expression level, expressed as a percentage of control values, was presented as means ± SE (n = 3). * : compared with control ($p < 0.05$). ** : compared with control ($p < 0.01$). # : compared with MDI ($p < 0.05$). ## : compared with MDI ($p < 0.01$).

2.4. Discussion

This study showed that DIM exerts anti-obesity effects in a DIO mouse model without significantly altering food intake (Fig. 2A-2D). Further analysis revealed that reduced adipose tissue mass was responsible for the decreased body weight (Fig. 3A-3D). . In addition, serum lipid concentrations and ectopic lipid storage were reduced by DIM administration (Fig. 4A-4C). In previous studies, adipogenesis of 3T3-L1 preadipocytes has been generally recognized as an *in vitro* model for obesity [25, 27, 29-31]. In the present study, I demonstrated the anti-adipogenic activity of DIM *in vitro* and the anti-obesity effect of DIM *in vivo*. DIM exhibited superior anti-adipogenic activity among the four different glucosinolates (Fig. 5A). DIM, but not I3C, inhibited MDI-induced adipogenesis of 3T3-L1 preadipocytes (Fig. 5B-5D), and adipogenic/lipogenic gene expression levels (Fig. 6A, 6B).

Generally, hypertrophy or hyperplasia of adipose tissue has been reported to be responsible for obesity-related metabolic syndromes, such as Type 2 diabetes. However, Reitman *et al*., reported a contrasting situation involving lipoatrophic diabetes [32].

They reported that mice with too little or too much adipose tissue, developed metabolic syndromes. In accordance with this result, Gavriolva *et al*., reported that the provision of lipid storage by surgical implantation of adipose tissue ameliorates diabetes in lipoatrophic mice [33], underlining the importance of adipose tissue as lipid storage.

Considering these previous studies, I hypothesized that the anti-adipogenic effects of DIM may cause increased serum triglyceride concentrations and ectopic lipid storage, because DIM decreased adipose tissue mass without significantly altering food intake. This prompted us to monitor serum triglyceride level and hepatic lipid accumulation. The results revealed that DIM increased neither serum triglyceride concentrations nor hepatic lipid accumulation, but that DIM decreased HFD-induced serum triglyceride concentrations and hepatic lipid accumulation significantly. This suggests that DIM may have unknown effects such as lipolytic activity or thermogenic activity. These notions should be further elucidated in future studies.

Additionally, the exact molecular mechanism of the anti-

adipogenic activity of DIM in 3T3-L1 preadipocytes should be determined. Though I have investigated the anti-adipogenic activity of DIM *in vitro*, the precise mechanism responsible for this activity remains elusive. Since adipogenesis is composed of two steps, mitotic clonal expansion (MCE) and terminal differentiation (TD) [7], either MCE or TD, or both could be the target of anti-adipogenic activity induced by DIM.

In summary, I have demonstrated the anti-obesity effects of DIM in a DIO mouse model and its anti-adipogenic activity in 3T3-L1 preadipocytes. Since DIM is a physiological metabolite of I3C and glucobrassicin, these outcomes could help to explain, at least in part, the anti-obesity effects of cruciferous vegetables, although the exact molecular mechanism of how DIM exerts anti-obesity remains elusive.

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Chapter 3.

3,3'-diindolylmethane (DIM) inhibits MDI-induced adipogenesis of 3T3-L1 preadipocytes by regulating USP2 activity

Abstract

In vitro adipogenesis is regarded as a widely-used, well-established cell culture model for obesity research, since it mimics physiological obesity development. Adipogenesis is a process composed of two distinct steps, mitotic clonal expansion (MCE) and terminal differentiation (TD), with MCE reported as prerequisite step for the entire adipogenesis process. Here, I investigated the molecular mechanisms of the reported anti-adipogenic activity of 3,3'-diindolylmethane (DIM), which is a physiological metabolite of indole-3-carbinol (I3C), an active compound in various cruciferous vegetables. I observed that the anti-adipogenic activity of DIM was primarily confined at the MCE step, although relatively weak anti-adipogenic activity also occurred during the TD step. I have also shown that DIM suppresses MDI-induced cell number increase by delaying cell cycle progression. This effect was due to the induction of an enhanced cyclin D1 degradation rate. Since ubiquitin-specific protease 2 (USP2) has been reported to regulate cyclin D1 degradation, I measured USP2 deubiquitinase activity *in vitro*. Deuqibuitinase assay results showed

that DIM directly inhibits the deubiquitinase activity of USP2 *in vitro*. However, USP2 protein expression levels remained unchanged by DIM treatment. Taken together, I have concluded that DIM directly targets USP2 to enhance the cyclin D1 degradation rate, resulting in suppressed MCE and adipogenesis in 3T3-L1 preadipocytes.

3.1. Introduction

Some decades ago, obesity was regarded as just a problem related to aesthetic appearance. However, in recent years, there have been an increasing number of reports on the health problem of obesity in terms of metabolic syndromes [1, 2]. The World Health Organization (WHO) defined obesity as a disease in 1996. In this regard, the development of anti-obesitic agents has been receiving great interest by many research group. Adipocyte hyperplasia (an increase in cell number) and hypertrophy (increases in cell size) are both responsible for obesity [3, 4], and these can be mimicked *in vitro* during adipogenesis of 3T3-L1 preadipocytes [5-7]. Upon hormonal stimulation, 3T3-L1 preadipocytes differentiate into mature adipocytes [8].

Growth-arrested 3T3-L1 preadipocytes were induced to proliferate using a hormonal cocktail called MDI (a mixture of isobutylmethylxanthin, dexamethasone and insulin). This step is referred to as mitotic clonal expansion (MCE). Since MCE is a type of cell proliferation, the increased expression or activation of various cell cycle-related proteins such as cyclins and cyclin-

dependent kinases (cdks) have been reported during the MCE step [9]. The MCE step is followed by the terminal differentiation (TD) step [10]. 3T3-L1 preadipocytes acquire adipocyte-like phenotypes at this step. In many previous studies, MCE has been suggested as a prerequisite step for TD and consequently the whole adipogenesis process [11-14]. In a similar vein to these previous studies, some studies have reported that resveratrol, piceatannol or cocoa polyphenols can block MCE to suppress adipogenesis [15-17], suggesting that the blockade of MCE is sufficient to inhibit the whole adipogenesis process. These results have provided evidence that suppression of MCE represents a promising strategy for the prevention of adipogenesis during the development of obesity.

Ubiquitin specific protease (peptidase) 2 (USP2) is a deubiquitinating enzyme that removes ubiquitin from its substrates [18, 19]. USP2 has been reported to regulate various biological processes, including cell cycle progression [20, 21], fatty acid synthesis [22, 23], circadian rhythm [24-26], and hepatic gluconeogenesis [26]. Many previous studies have intensively investigated the relationship between USP2 and carcinogenesis [20-

23] or USP2 and circadian rhythms [24-26]. However, there have been no studies conducted on the relationship between USP2 and adipogenesis or USP2 and obesity.

3,3'-diindolylmethane (DIM) is a physiological metabolite of indole-3-carbinol (I3C) in cruciferous vegetables [27, 28]. DIM has been reported to inhibit cell cycle progression in various cancer cells [29-31], suggesting the possibility that DIM might regulate MDI-induced cell cycle progression of 3T3-L1 preadipocytes. Hence I investigated whether DIM inhibits MDI-induced adipogenesis through the blockade of cell cycle progression in 3T3-L1 preadipocytes by targeting USP2.

3.2. Materials and Methods

3.2.1. Chemicals and reagents

3,3'-diindolylmethane (DIM) and indole-3-carbinol (I3C) were obtained from Sigma (St. Louis, MO). Antibody against PPARγ was obtained from Santa Cruz (Santa Cruz, CA). Antibody against C/EBPα was obtained from Cell Signaling Biotechnology (Beverly, MA). Dexamethasone, isobutylmethylxanthine (IBMX) and insulin were purchased from Sigma (St. Louis, MO).

3.2.2. Cell culture

All cell culture materials were obtained from GIBCO BRL (Grand Island, NY). 3T3-L1 mouse preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS), at 5% CO2 and 37^{o}C. Cells were subcultured every two or three days at a density of 20×10^4 cells per dish.

3.2.3. Adipogenesis

3T3-L1 preadipocytes were seeded and cultured until confluence. Two days after confluence, cells were incubated for two days in DMEM supplemented with 10% FBS and mixture of isobutylmethylxanthine, dexamethasone, and insulin (MDI) cocktail [dexamethasone (1 μM), IBMX (0.5 mM), and insulin (5 μg/mL)]. After two days, medium was replaced with DMEM containing 10% FBS and 5 μg/mL of insulin, and cells were incubated for two days. After two days, medium was replaced with DMEM containing 10% FBS.

3.2.4. Oil red O staining

3T3-L1 preadipocytes were seeded in 24-well plates at a density of 2.5×10^4 cells per well and incubated until confluence. Cells were differentiated to adipocytes as mentioned above in the presence or absence of DIM and I3C. After induction of cell differentiation for 6 days, the media were removed, and the mature 3T3-L1 adipocytes were fixed in 10% formaline. The fixed cells were stained with Oil red O solution as previously described [17]. Stained Oil red O solution was eluted with isopropyl alcohol

(Sigma), and the concentration was measured using a spectrophotometer set at 515 nm.

3.2.5. Trypan blue assay

3T3-L1 preadipocytes were seeded in 24-well plates at a density of 2.5×10^4 cells per well, and incubated in DMEM supplemented with 10% FBS and MDI cocktail in the presence or absence of DIM or I3C at indicated concentrations. Cells were trypsinized and stained with 0.4% trypan blue. The stained cells were loaded onto a hematocytometer, and both the viable cells and non-viable cells were counted.

3.2.6. Fluorescence-activated cell sorter (FACS) analysis

3T3-L1 preadipocytes were seeded in 6-cm dishes at a density of 1.5×10^5 cells per dish, and incubated until confluence. Confluent cells were cultured with MDI cocktail in the presence or absence of DIM or I3C at indicated concentrations. After indicated time periods, cells were trypsinized and centrifuged at 2,000 rpm for 2 min. Supernatants were removed and pellets were resuspended

in PBS. Then, cells were centrifuged at 2,000 rpm for 2 min again. After centrifugation, supernatants were removed and pellets were resuspended in ice-cold 70 % (*v*/*v*) ethanol and fixed at 4°C until they were stained with propidium iodide (PI). For PI staining, cells were centrifuged at 2,000 rpm for 2 min and resuspended in 1 ml of PBS. Cells were centrifuged at 2,000 rpm for 2 min again, then resuspended in 500 μl of PBS containing PI (20 μg/ml; Sigma) and RNase (0.2 mg/ml; Amresco; Solon, OH). The mixture was incubated at 37°C for 10 min in the dark. Fluorescence was measured using a FACS Calibur flow cytometer (Becton-ickinson; San Jose, CA). Ten thousand cells in each sample were analyzed.

3.2.7. Western blot analysis

3T3-L1 preadipocytes were seeded in 6-cm dishes at a density of 1.5×10^5 cells per dish and treated as mentioned in each figure. The cells were lysed and centrifuged (10 min, 14,000 rpm, 4°C), and supernatants were collected. The supernatants were loaded onto SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare; Piscataway, NJ). The

membranes were blocked with 5% skim milk and incubated with specific primary antibodies followed by HRP-conjugated secondary antibodies. Protein bands were visualized using a chemiluminescence detection kit (Amersham Pharmacia Biotech; Piscataway, NJ).

3.2.8. Quantitative real-time PCR

3T3-L1 preadipocytes were seeded in 6-cm dishes at a density of 1.5×10^5 cells per dish and treated as mentioned in each figure. Messenger RNA (mRNA) was extracted using RNA-BEE (TEL-TEST; Pearland, TX) and reverse transcribed into complementary DNA (cDNA) using Promega (Fitchburg, WI) product. Quantitative real-time PCR was performed as described previously [32] using SYBR Green RT-PCR kit from Fermentas (Glen Burnie, MD). Reasults are expressed relative to β-actin.

3.2.9. USP2 activity assay

Materials which are required to conduct USP2 deubiquitinase activity assay, including USP2 core protein, were all

purchased at Life Sensors (Malvern, PA). USP2 deubiquitinase activity assay was conducted according to the manufacturer's instructions.

3.2.10. Statistical analysis

Data are expressed as means ± SD, and Student's *t*-test was used for single statistical comparisons. A probability value of $p < 0.05$ was used as the criterion for statistical significance.

3.3. Results

3.3.1. 3,3'-diindolylmethane (DIM) inhibits MDI-induced adipogenesis at the mitotic clonal expansion (MCE) stage

Adipogenesis of 3T3-L1 preadipocytes consists of two steps, mitotic clonal expansion (MCE; Day 0-2) and terminal differentiation (TD; After Day 2) [33]. To verify our hypothesis that DIM could be targeting MCE to inhibit adipogenesis, I treated cells with DIM at different timepoint (Fig. 1A). Oil red O staining results showed that the anti-adipogenic effect of DIM was largely confined to Day 0-2, that is, MCE (Fig. 1B, 1C). Intracellular lipid storage of cells that were treated with DIM during the two days (Day 0-2) was similar with that of cells treated with DIM over six days (Day 0-6) duration, suggesting that treatment with DIM during MCE is sufficient to inhibit the entire adipogenesis process. Although cells that were treated with DIM for four days (Day 2-6) exhibited decreased lipid storage, it was higher than the lipid storage of cells that were treated with DIM for only two days (Day 0-2). These results suggested that DIM primarily targets MCE to inhibit

adipogenesis in 3T3-L1 preadipocytes.

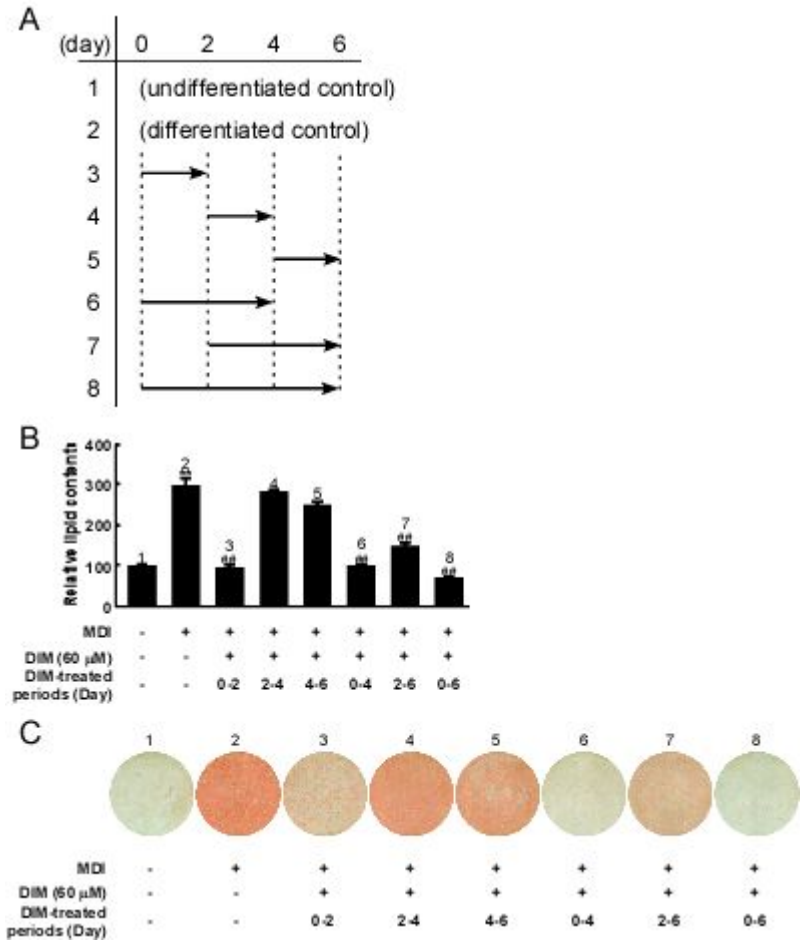


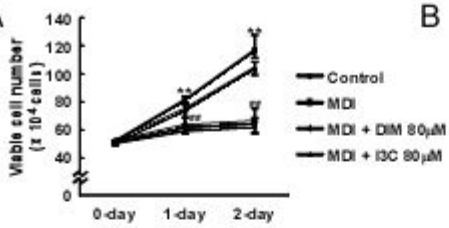
Figure 1. (A) Schematic experimental schedule. (B-C) Time-dependent effect of DIM on MDI-induced adipogenesis of 3T3-L1 preadipocytes. (B) 3T3-L1 preadipocytes were induced to differentiate to adipocyte in the presence of DIM at 60 μM at indicated time periods. Intracellular lipid accumulations were

stained by oil red O staining. Stained oil red O was eluted with isopropyl alcohol and absorbance was measured at 515 nm. The intracellular lipid accumulations, expressed as a percentage of control values, were presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$). ## : compared with MDI ($p < 0.01$). (C) 3T3-L1 preadipocytes were induced to differentiate to adipocyte in the presence of DIM at 60 μM at indicated time periods. Intracellular lipid accumulations were stained by oil red O staining. Stained oil red O was visualized by photograph.

3.3.2. 3,3'-diindolylmethane (DIM) suppresses MDI-induced cell cycle progression and subsequent cell number increase

It has been previously demonstrated that cell cycle progression occurs and cells proliferate during MCE [13, 33]. I therefore investigated whether DIM affects cell proliferation and cell cycle progression during the MCE step. First, I conducted a trypan blue assay to assess viable cell numbers during MCE in either the presence or absence of DIM and I3C. During the two days of MCE, cell number increased by approximately 2-fold in response to MDI. However, DIM significantly reduced MDI-increased cell number, whereas I3C had no effect (Fig. 2A, 2B). Next, I conducted FACS analysis to investigate cell cycle profiles. As depicted in Figure 2C, MDI induced cell cycle progression to S phase at 16 hr and G2/M phase between 20 to 24 hrs (Fig. 2C, 2E). However, DIM significantly delayed cell cycle progression in 3T3-L1 preadipocytes. A large portion of cells (approximately 30%) still remained at G2/M phase even at 48 hr (Fig. 2D). These results together suggest that DIM inhibits cell proliferation and delays cell cycle progression during MCE in 3T3-L1 preadipocytes.

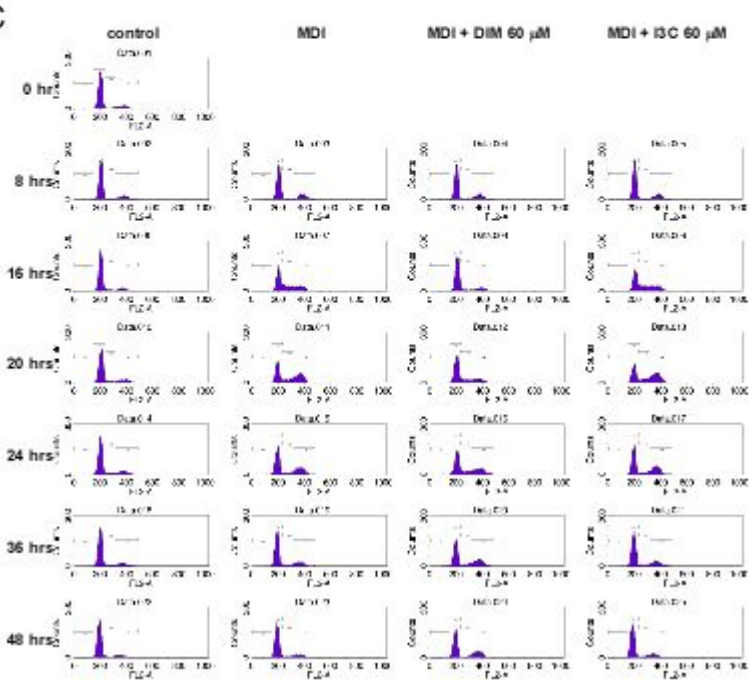
A



B

% of viable cell (%)		Average	STDEV
0-day	Control	51.6	1.59
1-day	Control	60.3	2.16
	MDI	81.5	2.91
	MDI + DIM 60μM	63.6	5.46
	MDI + I3C 60μM	74.7	5.40
2-day	Control	63.7	5.53
	MDI	117.3	11.40
	MDI + DIM 60μM	66.7	9.03
	MDI + I3C 60μM	104.5	4.78

C



D

	Control			MDI			MDI + DIM 60μM			MDI + I3C 60μM		
	G1	S	G2/M	G1	S	G2/M	G1	S	G2/M	G1	S	G2/M
0 hr	82.4	4.0	13.6									
8 hr	82.0	3.4	14.8	76.9	3.5	19.7	75.4	3.8	20.8	78.3	3.1	18.7
16 hr	82.3	5.6	12.3	53.2	21.8	25.4	77.7	9.2	13.8	48.9	23.8	28.0
20 hr	78.3	7.2	15.1	45.9	14.9	39.6	67.3	15.2	17.9	39.8	15.2	45.3
24 hr	82.0	3.9	14.4	64.0	6.8	29.5	55.2	14.1	31.1	60.5	7.0	32.7
36 hr	84.8	3.5	11.8	79.2	5.1	15.8	62.4	7.2	30.3	75.2	5.4	19.6
48 hr	85.0	5.0	9.9	80.6	5.5	13.9	65.8	5.3	28.5	80.6	6.1	13.0

E

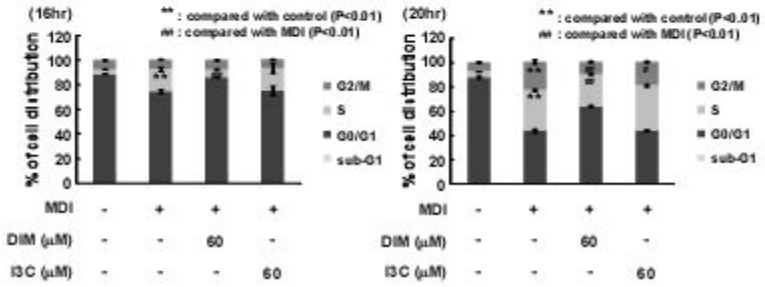


Figure 2. (A-E) Effects of DIM on MDI-induced cell proliferation and cell cycle progression in 3T3-L1 preadipocytes. (A) 3T3-L1 preadipocytes were cultured until confluence, then they were treated with MDI in presence or absence of DIM and I3C at 60 μM. At indicated time points, cells were trypsinized and stained with trypan blue, then they were loaded on hematocytometer to be counted. The number of viable cells were presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$). ## : compared with MDI ($p <$

0.01). (B) Number of viable cells at 2-day was presented. (C) 3T3-L1 preadipocytes were cultured until confluence, then they were treated with MDI in presence or absence of DIM and I3C at 60 μM. At indicated time points, cells were trypsinized and fixed with ice-cold 70% ethanol. Cells were stained with DAPI and loaded on FACS machine. The data are representative of three independent experiments. (D) Percentage of cells at each cell cycle phase was presented in table. The data are representative of three independent experiments. (E) 3T3-L1 preadipocytes were cultured until confluence, then they were treated with MDI in presence or absence of DIM and I3C at 60 μM. At indicated time points, cells were trypsinized and fixed with ice-cold 70% ethanol. Cells were stained with DAPI and loaded on FACS machine. The percentage of cells in each cell cycle phase was presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$). ## : compared with MDI ($p < 0.01$).

3.3.3. 3,3'-diindolylmethane (DIM) reduces cyclin D1 protein expression at the post-transcriptional level

I next checked cell cycle-related gene expression levelsby confirming cyclin D1, cyclin A1 protein expression and Rb phosphorylation in 3T3-L1 preadipocytes. Cyclin D1 has been reported to be expressed at the G1 phase and be responsible for G1-S transition [34]. Cyclin D1 has been reported to phosphorylate the Rb protein, allowing it to dissociate from E2F, a transcription factor for genes that are responsible for S-G2/M transition, such as cyclin A1 [35]. Since Rb protein suppresses E2F activity, E2F is activated when it dissociates from the Rb protein.

Western results showed that MDI-induced cyclin D1 protein expression and Rb phosphorylation were significantly decreased by DIM treatment, suggesting that E2F might be associated with Rb (Fig. 3A). In addition, MDI-induced cyclin A1 protein expression was decreased by DIM treatment (Fig. 3A). Since DIM reduced cyclin D1 and cyclin A1 protein expression levels, I next confirmed mRNA expression levels of cyclin D1 and cyclin A1 to investigate whether these suppressive effects occur at

the post-transcriptional level. qRT-PCR results showed that DIM significantly reduces mRNA levels of cyclin A1, but not cyclin D1 (Fig. 3B). Taking these results together, I concluded that DIM suppresses MDI-induced cyclin D1 expression at the post-transcriptional level.

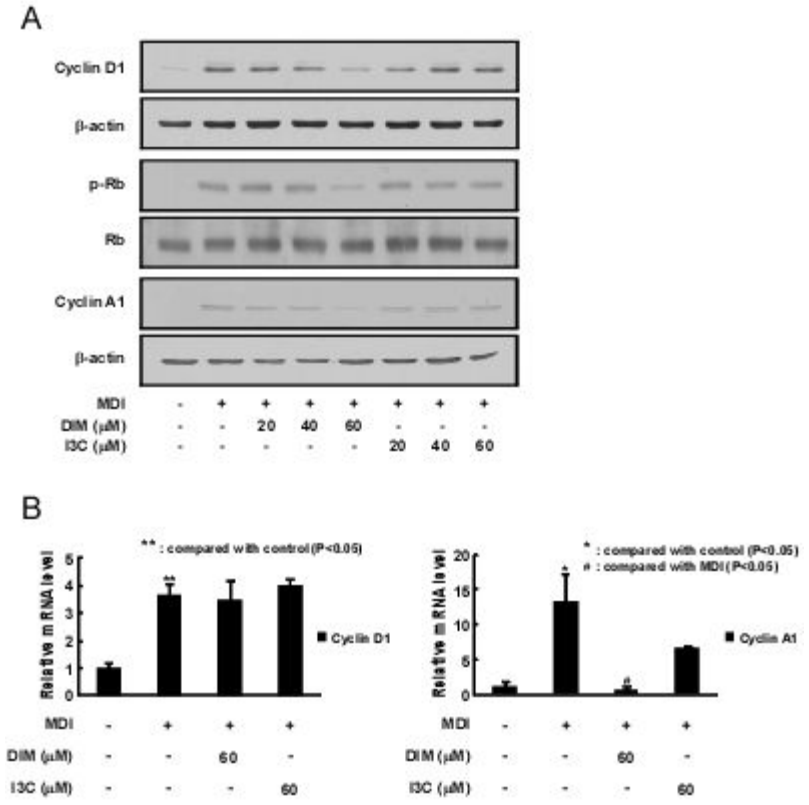


Figure 3. (A) Effects of DIM and I3C on MDI-induced cell cycle-related protein expression in 3T3-L1 peadipocytes. 3T3-L1 preadipocytes were cultured until confluence. They were treated with MDI in presence or absence of DIM and I3C at indicated concentrations. Then they were lysed as described in Materials and Methods section. The amount of cyclin D1 and cyclin A1 protein expressions and Rb phosphorylation were determined by Western

blotting as described in Materials and Methods section. β-actin and Rb were measured as loading control. The data are representative of three independent experiments. (B) Effects of DIM and I3C on MDI-induced cell cycle-related mRNA expression in 3T3-L1 peadipocytes. 3T3-L1 preadipocytes were cultured until confluence. They were treated with MDI in presence or absence of DIM and I3C at indicated concentrations. RNA was extracted as described in Materials and Methods section. The amount of cyclin D1 and cyclin A1 mRNA expressions were determined by qRT-PCR as described in Materials and Methods section. β-actin was used as reference gene. The relative mRNA expression level, expressed as a percentage of control values, was presented as means ± SE (n = 3). * : compared with control ($p < 0.05$). ** : compared with control ($p < 0.01$). # : compared with MDI ($p < 0.05$).

3.3.4. 3,3'-diindolylmethane (DIM) enhances cyclin D1 ubiquitination in 3T3-L1 preadipocytes

Since I observed that DIM inhibited cyclin D1 protein expression but not mRNA expression, I hypothesized that DIM might be reducing cyclin D1 stability. First, I measured the cyclin D1 turnover rate using cycloheximide (a protein synthesis inhibitor). Reductions in cyclin D1 protein expression levels began within 15 minutes after cycloheximide treatment, and was almost completely abrogated within 1 hour (Fig. 4A).

Previous studies have reported that cyclin D1 expression is regulated by ubiquitin-mediated degradation [36, 37]. I therefore investigated whether DIM enhanced the degradation rate of cyclin D1, by using MG132 (a proteasome inhibitor). Consistent with previous data, I confirmed that MDI-induced increases in cyclin D1 protein expression level was significantly reduced by DIM treatment (Fig. 4B). However, MG132 recovered the suppression of DIM-mediated scyclin D1 protein expression (Fig. 4B). These results suggested that DIM increases the cyclin D1 degradation rate by enhancing proteasome-mediated cyclin D1 proteolysis.

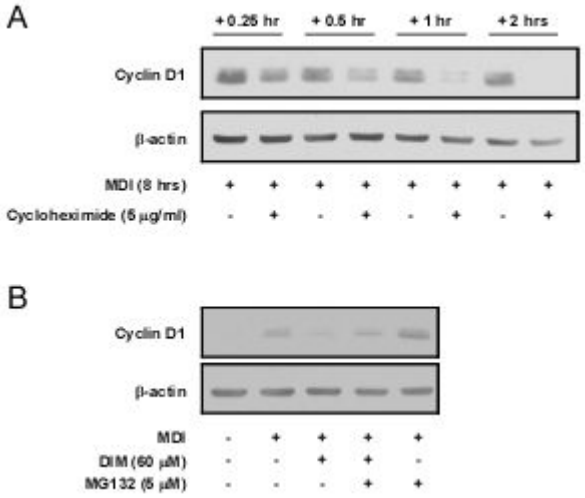


Figure 4. (A) Effect of cycloheximide on MDI-induced cyclin D1 protein expression in 3T3-L1 peadipocytes. 3T3-L1 preadipocytes were cultured until confluence. They were treated with MDI for 6 hours. Then they were treated with cycloheximide at 5 μg/ml. After indicated time periods, they were lysed as described in Materials and Methods section. The amount of cyclin D1 protein expression was determined by Western blotting as described in Materials and Methods section. β-actin was measured as loading control. The data are representative of three independent experiments. (B) Effect of MG132 on DIM-mediated cyclin D1 protein suppression in 3T3-L1 peadipocytes. 3T3-L1 preadipocytes were cultured until confluence.

They were treated with MDI for 6 hours. Then they were treated with DIM (60 μM) and/or MG132 (5 μM) for 2 hours. They were lysed as described in Materials and Methods section. The amount of cyclin D1 protein expression was determined by Western blotting as described in Materials and Methods section. β-actin was measured as loading control. The data are representative of three independent experiments.

3.3.5. 3,3'-diindolylmethane (DIM) directly inhibits USP2 deubiquitinase activity *in vitro*

Previous studies have reported that cyclin D1 ubiquitination is regulated specifically by ubiquitin-specific protease (USP2) [21]. USP2 has been reported to remove ubiquitin from its substrates such as cyclin D1 [21]. I hypothesized that DIM might target USP2 to enhance the cyclin D1 degradation rate. I first confirmed the effect of MDI or DIM treatment on USP2 protein expression level in 3T3-L1 preadipocytes, but USP2 protein expression remained unchanged by MDI or DIM treatment (Fig. 5A). Next, I investigated whether DIM directly targets USP2 deubiquitinase activity *in vitro*. USP2 activity assay results showed that DIM inhibited USP2 deubiquitinase activity to control levels (Fig. 5B, 5C). This inhibitory activity was quite comparable to the peptide inhibitor, Ub-aldehyde (Fig. 5B, 5C). Taken together, I conclude that DIM directly targets USP2 deubiquitinase activity to decrease cyclin D1 protein expression levels.

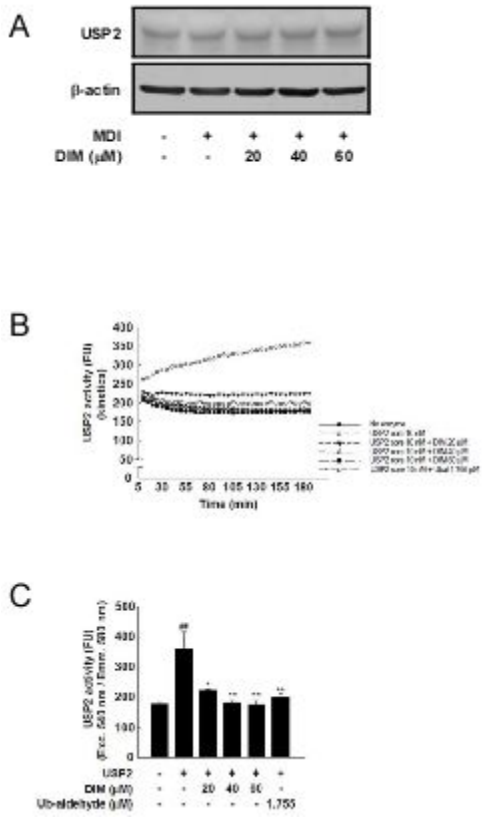


Figure 5. (A) Effect of DIM on USP2 protein expression in 3T3-L1 peadipocytes. 3T3-L1 preadipocytes were cultured until confluence. They were treated with MDI in presence or absence of DIM at indicated concentrations for 8 hours. After that, they were lysed as described in Materials and Methods section. The amount of USP2 protein expression was determined by Western blotting as described

in Materials and Methods section. β-actin was measured as loading control. The data are representative of three independent experiments. (B-C) Effect of DIM on USP2 deubiquitinase activity *in vitro*. (B) USP2 core protein was incubated with DIM *in vitro*. Deubiquitinase activity assay was conducted as described in Materials and Methods section. Kinetic of USP2 deubiquitinase activity was presented. (C) USP2 deubiquitinase activity at 3-hour time point was presented. Fluorescence unit (FU) was presented as means ± SE (n = 3). ## : compared with control ($p < 0.05$). * : compared with USP2 ($p < 0.01$). ** : compared with USP2 ($p < 0.05$).

3.4. Discussion

The process of adipogenesis is composed of two major steps, mitotic clonal expansion (MCE) and terminal differentiation (TD). A number of previous studies have shown that MCE is an essential step for the entire adipogenesis process [11-14]. In the present study, I have demonstrated that the anti-adipogenic activity of 3,3'-diindolylmethane (DIM) was primarily confined to MCE. Treatment with DIM during Day 0-2 is sufficient to block MDI-induced adipogenesis of 3T3-L1 preadipocytes (Fig. 1B, 1C). Since cell proliferation and cell cycle progression is a characteristic of MCE, I quantified viable cell numbers and analyzed cell cycle profiles. The results showed that DIM delays cell cycle progression to significantly decrease cell proliferation (Fig. 2A-2D). DIM reduced the expression of cell cycle-related genes, such as cyclin D1 and cyclin A1. I then determined that DIM suppresses cyclin D1 expression level at post-transcriptional level (Fig. 3A, 3B). Next, I investigated whether DIM can enhance the degradation rate of cyclin D1. I measured the turnover rate of cyclin D1 (Fig. 4A), and showed that MG132 recovers cyclin D1 expression, which was

suppressed by DIM treatment, suggesting that DIM increases the cyclin D1 degradation rate through the enhancement of proteasome-mediated proteolysis (Fig. 4B). Since previous studies have demonstrated that cyclin D1 expression is regulated by USP2 [21], I investigated whether DIM directly inhibits USP2 activity. Deubiquitinase activity assay results showed that DIM directly suppresses USP2 deubiquitinase activity *in vitro*, whereas DIM did not affect USP2 protein expression levels (Fig. 5A-5C).

I have shown that the anti-adipogenic activity of DIM was confined to MCE, but cells that were treated with DIM during TD (Day 2-6) exhibited significantly decreased intracellular lipid storage (Fig. 1A-C), suggesting DIM might exert other actions other than inhibiting MCE alone. Since lipogenesis and lipid accumulation occur during the TD step, it is possible that DIM might regulate lipid metabolism during TD. This notion should be addressed in further studies. However, it was the MCE step that DIM primarily affected, because adipogenesis was most dramatically inhibited when DIM was treated between Day 0-2 (Fig. 1A-1C).

I have shown that cyclin D1 protein expression was down-regulated by DIM treatment (Fig. 3A). Since cell cycle-related protein expression is precisely regulated according to cell cycle status, it is difficult to distinguish whether decreased cyclin D1 protein expression leads to an altered cell cycle profile or altered cell cycle status leads to decreased cyclin D1 protein expression. To clarify this problem, I confirmed the cell cycle profiles of 3T3-L1 preadipocytes at various time points in the presence of MDI. FACS analysis results showed that cell cycle distribution was similar during the first 8 hours after MDI treatment (Fig. 2B, 2C). At the 16-hour time point a difference was detectable in the cell cycle profiles as a result of DIM treatment (Fig. 2B, 2C). I also confirmed cyclin D1 protein expression at various time points after treatment with MDI, and observed that cyclin D1 protein expression was induced from 8 hours after MDI treatment (data not shown). I then evalulated the effects of DIM on MDI-induced cyclin D1 protein expression at the 8-hour time point, and confirmed that cyclin D1 protein expression was diminished by DIM treatment (Fig. 3A). Taking these results together, I have concluded that decreased cyclin

D1 protein expression leads to altered cell cycle distribution.

In the present study, I have shown that DIM directly inhibits USP2 deubiquitinase activity in vitro, whereas neither DIM nor MDI affected USP2 protein expression (Fig. 5A-5C). Through these results, I have concluded that DIM directly targets USP2 deubiquitinase activity to suppress cyclin D1 protein expression, without affecting USP2 protein expression levels. However, it is still unclear how MDI specifically affects USP2 activity in 3T3-L1 preadipocytes. In addition, direct evidence for the binding of USP2 with DIM and the exact binding site should be further investigated.

In summary, I have shown that the anti-adipogenic activity of DIM was more effective during MCE than TD. This anti-adipogenic activity occurred through the inhibition of cyclin D1 protein expression at the post-transcriptional level by directly targeting USP2 deubiquitinase activity. These shed further light on the molecular mechanism of the anti-adipogenic effect of DIM in 3T3-L1 preadipocytes. The precise nature of the binding between USP2 and DIM should be further studied.

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Chapter 4.

3,3'-Diindolylmethane (DIM) induces lipolysis and thermogenesis in 3T3-L1 adipocytes

Abstract

Increases in lipolysis have been traditionally thought to result in elevation of serum free fatty acid concentrations. However, recent reports have demonstrated that when lipolysis is simultaneously activated with thermogenesis, it does not cause an increase in serum free fatty acid levels and thus can be a novel strategy to treat obesity. Based on previous *in vivo* results, I hypothesized that 3,3'-diindolylmethane (DIM) might increase lipolysis and thermogenesis in 3T3-L1 adipocytes. In the present study, I showed that DIM exerts the strongest lipolytic activity among four different glucosinolates from cruciferous vegetables. DIM treatment increased glycerol release in 3T3-L1 adipocytes. To understand the molecular mechanism of the lipolytic activity of DIM, Western blot was performed to check several lipid metabolism-regulating gene expression such as hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), and adipocyte protein 2 (aP2). Results showed that DIM inhibited lipogenic gene expression with a

concomitant reduction in lipolytic gene expression in 3T3-L1 adipocytes, suggesting that DIM alters lipid metabolism from lipogenesis to lipolysis. Also, qRT-PCR results demonstrated that DIM enhances expression of thermogenic genes, including uncoupling protein 1 (UCP1) and PPAR gamma coactivator 1α (PGC-1α). In addition, treatment of DIM significantly increased AMPK phosphorylation which has been reported to regulate thermogenesis. Therefore, I concluded that DIM possesses lipolytic and thermogenic activities against 3T3-L1 adipocytes.

4.1. Introduction

Obesity is thought to be one of the main causes for metabolic syndromes [1], and hence, obesity is now recognized as a major global health problem. Due to the prevalence of obesity, developing anti-obesity agents has become a crucial issue. The majority of the current anti-obesity drugs target the central nervous system to suppress appetite [2-7] or target the digestive system to inhibit lipid absorption [8, 9], however, most of these have been reported to show various side effects [4, 10-15]. Due to these side effects, new approaches for treating obesity are required. Several papers have reported natural phytochemicals with anti-obesity effects by targeting adipogenesis of 3T3-L1 preadipocytes [16-20]. However, blocking adipogenesis while maintaining the same energy intake will only lead to prevention of fat cell hyperplasia and hypertrophy, and eventually lead to an increase in serum fatty acid / triacylglycerol concentration or ectopic lipid storage which could result in metabolic diseases. Therefore, to prevent and treat metabolic diseases, merely suppressing adipogenesis is not sufficient, and energy expenditure-enhancing activity is required.

Whereas energy intake solely depends on food consumption, energy expenditure is affected by various factors such as resting metabolic rate, physical activity, thermogenesis, and lifestyle patterns [21]. Energy expenditure is composed of three steps which can result in the production of adenosine triphosphate (ATP) or heat. The first step is β-oxidation. β-oxidation is the process by which fatty acids are broken down into acetyl-CoA. Since fatty acids are located in the cytosol and β-oxidation occurs in the mitochondria, fatty acids should be transferred to the mitochondria. In this process, carnitine palmitoyltransferase 1 (CPT-1) has been reported as a key regulator of β-oxidation [22, 23]. During the second step, acetyl-CoA enters the TCA cycle to produce NADH and FADH2 which are further used by the electron transport chain (ETC) to pump out H+ through the mitochondrial inner membrane to generate an H+ gradient. During the last step, the H+ gradient is used to generate chemical energy or heat. If H+ (which is pumped out during ETC) flows back through the membrane, ATP is generated by ATP synthase. Generation of heat, or thermogenesis has been reported to be regulated by uncoupling protein 1 (UCP1) [24]. UCP1 has been

reported to dissipate H+ gradient as heat rather than producing ATP [25]. Activating thermogenesis through UCP1-mediated mechanisms could be another way of preventing obesity that can overcome the side effects of previously developed anti-obesity agents.

Here, I show that 3,3'-diindolylmethane (DIM) shifts lipid metabolism toward lipolysis to provide fuel for energy or heat generation. In addition, I show that DIM enhances thermogenic gene expression, underlining the possibility of DIM's thermogenic activity.

4.2. Materials and Methods

4.2.1. Chemicals and reagents

3,3'-Diindolylmethane (DIM) and indole-3-carbinol (I3C) were obtained from Sigma (St. Louis, MO). Antibody against p-HSL, HSL, and ATGL were obtained from Cell Signaling Biotechnology (Beverly, MA). Antibody against PPARγ was obtained from Santa Cruz (Santa Cruz, CA). Antibodies against C/EBPα, FAS, aP2, and SREBP-1c were obtained from Cell Signaling Biotechnology (Beverly, MA). Dexamethasone, isobutylmethylxanthine (IBMX) and insulin were purchased from Sigma (St. Louis, MO).

4.2.2. Cell culture

All cell culture materials were obtained from GIBCO BRL (Grand Island, NY). 3T3-L1 mouse preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS), at 5% CO2 and 37^{o}C. Cells were subcultured every two or three days at a density of 20 $\times$ 10^4

cells per dish.

4.2.3. Adipogenesis

3T3-L1 preadipocytes were seeded and cultured until confluence. Two days after confluence, cells were incubated for two days in DMEM supplemented with 10% FBS and mixture of isobutylmethylxanthine, dexamethasone, and insulin (MDI) cocktail [dexamethasone (1 μM), IBMX (0.5 mM), and insulin (5 μg/mL)]. After two days, medium was replaced with DMEM containing 10% FBS and 5 μg/mL of insulin, and cells were incubated for two days. After two days, medium was replaced with DMEM containing 10% FBS.

4.2.4. Glycerol release

3T3-L1 preadipocytes were seeded in 24-well plates at a density of 2.5×10^4 cells per well and incubated until confluence. Cells were differentiated to adipocytes as mentioned above. Mature 3T3-L1 adipocytes were treated with various compounds at indicated concentrations. At indicated time points, 30 μl of media in

each well were collected and stored at -20°C. Glycerol concentration in each media was measured using free glycerol reagent (Sigma; St. Louis, MO), according to the manufacturer's instructions.

4.2.5. Non-esterified fatty acid (NEFA) release

3T3-L1 preadipocytes were seeded in 24-well plates at a density of 2.5×10^4 cells per well and incubated until confluence. Cells were differentiated to adipocytes as mentioned above. Mature 3T3-L1 adipocytes were treated with various compounds at indicated concentrations. At indicated time points, 30 μl of media in each well were collected and stored at -20°C. NEFA concentration in each media was measured using LabAssay (TM) NEFA (Wako pure chemical industries, Ltd; Osaka, Japan), according to the manufacturer's instructions.

4.2.6. Oil red O staining

3T3-L1 preadipocytes were seeded in 24-well plates at a density of 2.5×10^4 cells per well and incubated until confluence.

Cells were differentiated to adipocytes as mentioned above. After induction of cell differentiation for 6 days, the media were removed, and the mature 3T3-L1 adipocytes were treated with various compounds at indicated concentrations. At indicated time periods, the media were removed, and the mature 3T3-L1 adipocytes were fixed in 10% formaline. The fixed cells were stained with Oil red O solution as previously described [18]. Stained Oil red O solution was eluted with isopropyl alcohol (Sigma), and the concentration was measured using a spectrophotometer set at 515 nm.

4.2.7. Western blot analysis

3T3-L1 preadipocytes were seeded in 6-cm dishes at a density of 1.5×10^5 cells per dish and treated as mentioned in each figure. The cells were lysed and centrifuged (10 min, 14,000 rpm, 4^oC), and supernatants were collected. The supernatants were loaded onto SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare; Piscataway, NJ). The membranes were blocked with 5% skim milk and incubated with specific primary antibodies followed by HRP-conjugated secondary

antibodies. Protein bands were visualized using a chemiluminescence detection kit (Amersham Pharmacia Biotech; Piscataway, NJ).

4.2.8. Quantitative real-time PCR

3T3-L1 preadipocytes were seeded in 6-cm dishes at a density of 1.5×10^5 cells per dish and treated as mentioned in each figure. Messenger RNA (mRNA) was extracted using RNA-BEE (TEL-TEST; Pearland, TX) and reverse transcribed into complementary DNA (cDNA) using Promega (Fitchburg, WI) product. Quantitative real-time PCR was performed as described previously [26] using SYBR Green RT-PCR kit from Fermentas (Glen Burnie, MD). Results are expressed relative to β-actin.

4.2.9. Statistical analysis

Data are expressed as means ± SD, and Student's *t*-test was used for single statistical comparisons. A probability value of $p < 0.05$ was used as the criterion for statistical significance.

4.3. Results

4.3.1. 3,3'-Diindolylmethane (DIM) enhances lipolysis in 3T3-L1 adipocytes

I first compared the lipolytic activity of four different glucosinolates, indole-3-carbinol (I3C) and its physiological metabolite, DIM, sinigrin and its aglycone, allylisothiocyanate (AITC). Since lipolysis is the process by which triglyceride breaks down into one molecule of glycerol and three molecules of non-esterified fatty acid (NEFA), glycerol release and NEFA release were used as markers of lipolysis.

To compare the lipolytic activity, I treated four glucosinolates to 3T3-L1 adipocytes, and collected media to measure glycerol or NEFA concentration. Results showed that DIM increased both glycerol and NEFA release more significantly than three other glucosinolates, suggesting that DIM possesses the strongest lipolytic activity in 3T3-L1 adipocytes (Fig. 1A, 1B). Additionally, DIM decreased intracellular lipid accumulation in 3T3-L1 adipocytes whereas other glucosinolates did not (Fig. 1C).

This lipolytic activity was dose-dependent and time-dependent, and was effective only in 3T3-L1 adipocytes, and not in 3T3-L1 preadipocytes (Fig. 2A, 2B). These results suggested that DIM exhibits the best lipolytic activity in 3T3-L1 adipocytes among four different glucosinolates, in a dose-dependent and time-dependent manner.

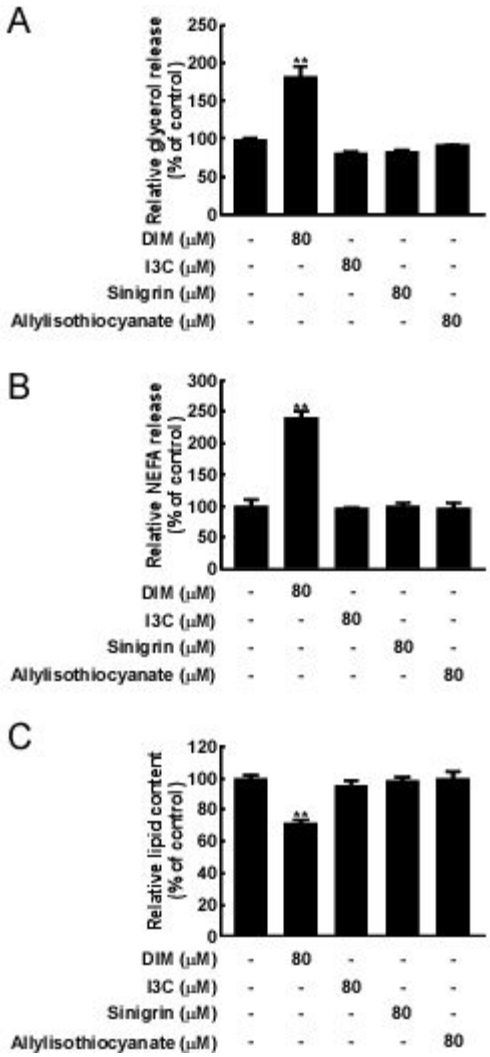

Figure 1. (A) Effects of various glucosinolates on glycerol release in mature 3T3-L1 adipocytes. 3T3-L1 preadipocytes were induced to differentiate to adipocyte. Mature 3T3-L1 adipocytes were treated with various glucosinolates at indicated concentrations for three

days. Glycerol release was measured as described in Materials and Methods section. The relative glycerol release, expressed as a percentage of control values, was presented as means ± SE (n = 3). * : compared with control ($p < 0.05$). ** : compared with control ($p < 0.01$). (B) Effects of various glucosinolates on NEFA release in mature 3T3-L1 adipocytes. 3T3-L1 preadipocytes were induced to differentiate to adipocyte. Mature 3T3-L1 adipocytes were treated with various glucosinolates at indicated concentrations for three days. NEFA release was measured as described in Materials and Methods section. The relative NEFA release, expressed as a percentage of control values, was presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$). (C) Effects of various glucosinolates on intracellular lipid accumulation in mature 3T3-L1 adipocytes. 3T3-L1 preadipocytes were induced to differentiate to adipocyte. Mature 3T3-L1 adipocytes were treated with various glucosinolates at indicated concentrations for four days. Intracellular lipid accumulations were stained by oil red O staining. Stained oil red O was eluted with isopropyl alcohol and absorbance was measured at 515 nm. The intracellular lipid accumulations,

expressed as a percentage of control values, were presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$).

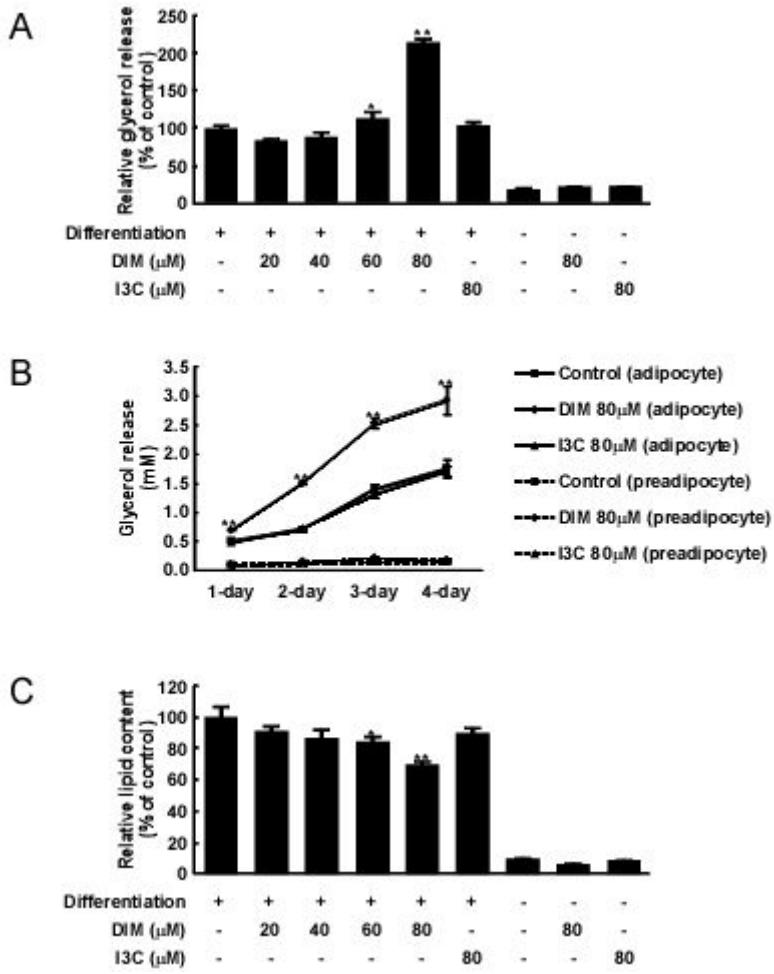

Figure 2. (A) Dose-dependent effects of DIM and I3C on glycerol release in mature 3T3-L1 adipocytes. 3T3-L1 preadipocytes were either induced to differentiate to adipocyte or not. 3T3-L1 preadipocytes and mature 3T3-L1 adipocytes were treated with

DIM or I3C at indicated concentrations for three days. Glycerol release was measured as described in Materials and Methods section. The relative glycerol release, expressed as a percentage of control values, was presented as means ± SE (n = 3). * : compared with control ($p < 0.05$). ** : compared with control ($p < 0.01$). (B) Time-dependent effects of DIM and I3C on glycerol release in mature 3T3-L1 adipocytes. 3T3-L1 preadipocytes were either induced to differentiate to adipocyte or not. 3T3-L1 preadipocytes and mature 3T3-L1 adipocytes were treated with DIM or I3C at 80 μM for indicated time periods. Glycerol release was measured as described in Materials and Methods section. The relative glycerol release, expressed as a percentage of control values, was presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$). (C) Dose-dependent effects of DIM and I3C on intracellular lipid accumulation in mature 3T3-L1 adipocytes. 3T3-L1 preadipocytes were either induced to differentiate to adipocyte or not. 3T3-L1 preadipocytes and mature 3T3-L1 adipocytes were treated with DIM or I3C at indicated concentrations for four days. Intracellular lipid accumulations were stained by oil red O staining. Stained oil

red O was eluted with isopropyl alcohol and absorbance was measured at 515 nm. The intracellular lipid accumulations, expressed as a percentage of control values, were presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$).

4.3.2. 3,3'-Diindolylmethane (DIM) shifts lipid metabolism from lipogenesis to lipolysis in 3T3-L1 adipocytes

Among the many lipolytic genes such as hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), and acyl-CoA synthetase long chain (ACSL), HSL (also previously known as cholesteryl ester hydrolase [27]) has been reported to be a key enzyme in lipolysis. Since previous studies have reported that phosphorylation of HSL on Ser563 enhances lipolysis [28], I confirmed whether DIM enhances phosphorylation of HSL at Ser563 in 3T3-L1 adipocytes. DIM increased HSL phosphorylation in 3T3-L1 adipocytes (Fig. 3A). DIM treatment significantly reduced peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) protein expression in 3T3-L1 adipocytes (Fig. 3B). Since DIM enhanced HSL phosphorylation on S563 and decreased lipogenic protein expression, these results together suggested that DIM shifts lipid metabolism toward lipolysis rather than lipogenesis.

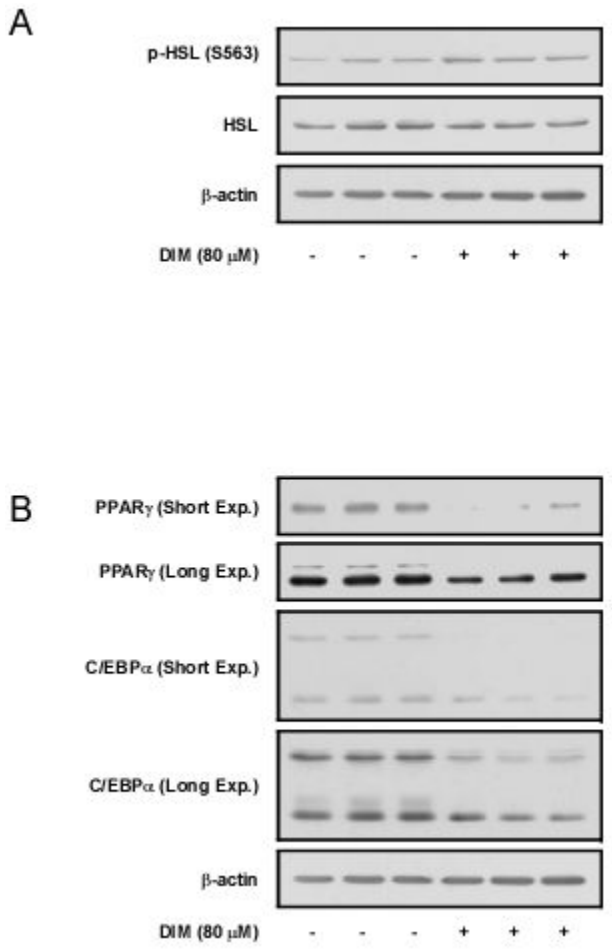

Figure 3. (A) Effects of DIM on phosphorylation of HSL in mature 3T3-L1 adipocytes. 3T3-L1 preadipocytes were induced to differentiate to adipocyte. Mature 3T3-L1 adipocytes were treated with DIM at 80 μM for one day. Then they were lysed as described in Materials and Methods section. HSL phosphorylation and

expression were determined by western blotting as described in Materials and Methods section. β-actin was measured as loading control. The relative protein expression level, expressed as a fold of control values, was presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$). (B) Effects of DIM on expression of PPARγ and C/EBPα in mature 3T3-L1 adipocytes. 3T3-L1 preadipocytes were induced to differentiate to adipocyte. Mature 3T3-L1 adipocytes were treated with DIM at 80 μM for one day. Then they were lysed as described in Materials and Methods section. PPARγ and C/EBPα expression were determined by western blotting as described in Materials and Methods section. β-actin was measured as loading control. The relative protein expression level, expressed as a fold of control values, was presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$).

4.3.3. 3,3'-Diindolylmethane (DIM) elevates thermogenic gene expression in 3T3-L1 adipocytes

Although increased lipolysis can decrease intracellular lipid accumulation in adipose tissue, serum free fatty acid concentration would be increased, causing various metabolic syndromes. In regard to this problem, Ahmadian M. *et al*., reported that increased lipolysis in adipose tissue causes a shift within adipocytes towards increased fatty acid utilization and energy expenditure [29, 30]. Considering these previous studies, I hypothesized that DIM enhanced thermogenesis in 3T3-L1 adipocytes. To verify this hypothesis, I confirmed expression of several thermogenic genes including uncoupling protein 1 (UCP1), carnitine palmitoyltransferase 1 (CPT-1), and PPAR gamma coactivator 1α (PGC-1α) by quantitative real time PCR (qRT-PCR). qRT-PCR results showed that DIM increased UCP1 mRNA expression 8-fold and PGC-1α mRNA 2.5-fold compared to the control group in 3T3-L1 adipocytes (Fig. 4A, 4B). However, DIM did not increase CPT-1b mRNA expression (data not shown).

AMP-activated protein kinase (AMPK) has been reported

to regulate energy homeostasis. AMPK is activated by cellular demand for ATP to enhance cellular energy level through stimulating catabolic pathways such as fatty acid oxidation and thermogenesis. I performed Western blot to examine the effect of DIM on AMPK phosphorylation in 3T3-L1 adipocytes and the results demonstrate that DIM significantly increases AMPK phosphorylation (Fig. 4C). Taken together, I concluded that DIM enhances thermogenic gene expression and stimulates AMPK phosphorylation in 3T3-L1 adipocytes, resulting in increased thermogenesis.

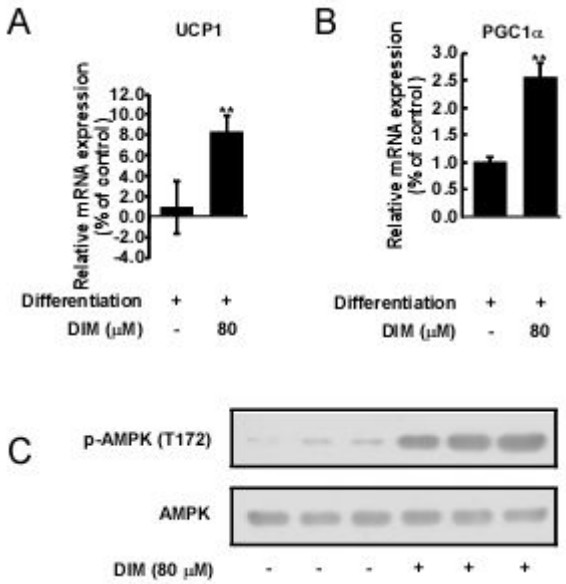

Figure 4. (A-B) Effects of DIM on mRNA expression of thermogenic gene in mature 3T3-L1 adipocytes. (A) 3T3-L1 preadipocytes were induced to differentiate to adipocyte. Mature 3T3-L1 adipocytes were treated with DIM at 60 μM for three days. RNA was extracted as described in Materials and Methods section. The amount of UCP1 mRNA expressions was determined by qRT-PCR as described in Materials and Methods section. β-actin was used as reference gene. The relative mRNA expression level, expressed as a percentage of control values, was presented as means

± SE (n = 3). ** : compared with control ($p < 0.01$). (B) 3T3-L1 preadipocytes were induced to differentiate to adipocyte. Mature 3T3-L1 adipocytes were treated with DIM at 60 μM for three days. RNA was extracted as described in Materials and Methods section. The amount of PGC-1α mRNA expressions was determined by qRT-PCR as described in Materials and Methods section. β-actin was used as reference gene. The relative mRNA expression level, expressed as a percentage of control values, was presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$). (C) Effects of DIM on phosphorylation of AMPK in mature 3T3-L1 adipocytes. 3T3-L1 preadipocytes were induced to differentiate to adipocyte. Mature 3T3-L1 adipocytes were treated with DIM at 80 μM for one day. Then they were lysed as described in Materials and Methods section. AMPK phosphorylation and expression were determined by western blotting as described in Materials and Methods section. AMPK was measured as loading control. The relative protein expression level, expressed as a fold of control values, was presented as means ± SE (n = 3). ** : compared with control ($p < 0.01$).

4.4. Discussion

Previously I showed that DIM blocks MDI-induced adipogenesis of 3T3-L1 preadipocytes by targeting USP2 deubiquitinase activity *in vitro* and HFD-induced obesity *in vivo*. In previous results, DIM decreased fat mass significantly without affecting energy intake (calorie intake), suggesting the possibility that it might increase serum fatty acid/triaclyglyerol concentration or ectopic lipid storage. However, surprisingly, DIM decreased HFD-induced serum triacylglycerol concentration and ectopic lipid storage. This prompted us to hypothesize that DIM might elevate energy expenditure through lipolysis and thermogenesis. To verify this hypothesis, I measured glycerol and NEFA release in 3T3-L1 adipocytes. I compared the lipolytic activity of four different glucosinolates derived from cruciferous vegetables, DIM exhibited the best lipolytic activity in 3T3-L1 adipocytes among the four glucosinolates (Fig. 1A-1C). DIM significantly increased glycerol release (Fig. 1A) and NEFA release (Fig. 1B), and it decreased intracellular lipid accumulation (Fig. 1C) in 3T3-L1 adipocytes. This lipolytic activity of DIM was confined to adipocytes in a dose-

and time-dependent manner (Fig. 2A-2C). Next, I confirmed the effect of DIM on lipid metabolism-regulating protein phosphorylation and expression. Western blot results showed that DIM significantly decreased lipogenic protein expression whereas phosphorylation of HSL, lipolytic protein, was slightly increased by DIM treatment (Fig. 3A, 3B), suggesting that DIM shifts lipid metabolism toward the lipolytic pathway. Further analysis showed that DIM induced thermogenic gene expression such as UCP1 and PGC-1α and enhanced AMPK phosphorylation (Fig. 4A-4C). These results indicated that DIM might enhance thermogenesis in 3T3-L1 adipocytes.

In the present study, I analyzed the mRNA levels of thermogenic genes. It is still unclear whether DIM induces thermogenesis in 3T3-L1 adipocytes. However, I could find several lines of evidence on the thermogenic activity of DIM. The first one is the increase in thermogenic gene expression. Since UCP1, PGC-1α, and AMPK have been used as biomarkers of thermogenesis [31-34], enhanced UCP1 and PGC-1α expression by DIM treatment might suggest increased thermogenesis in 3T3-L1 adipocytes. The

second line of evidence is the decrease in ectopic lipid storage in a diet-induced obesity (DIO) mouse model. Previously I determined the anti-obesity effect of DIM in a DIO mouse model. I observed that DIM decreased fat tissue mass without altering calorie intake (Chapter 2; Fig. 2C, Fig. 3). This means that lipid storage was decreased but calorie intake remained the same. In this case, if energy expenditure is not increased, serum triglyceride and/or free fatty acid concentration, as well as ectopic lipid storage should be increased. But, there was no change in serum free fatty acids (data not shown) and serum triglyceride concentration and moreover, hepatic lipid storage levels were decreased (Chapter 2; Fig. 4B, 4C). Taking these results together, I suggested that DIM might induce thermogenesis. It should be further investigated whether DIM directly induces thermogenesis in physiological conditions.

Lipolysis is the process by which triglyceride breaks down into one molecule of glycerol and three molecules of NEFA. I found that glycerol and NEFA release was significantly increased by DIM treatment. Stoichiometrically, released glycerol and NEFA concentrations should be 1:3, however, released NEFA

concentrations were lower than released glycerol concentrations. There may be several possible reasons regarding this problem. Hashimoto T. el al., reported fatty acid re-esterification into micro-lipid droplets [35]. According to them, the partner of fatty acid re-esterification is not glycerol, but glycerol-3-phosphate. However, since adipocytes exhibit poor activity of glycerol kinase, the released glycerol by lipolysis is not reused into fatty acid re-esterification. They observed significantly increased fatty acid release when they used a fatty acid re-esterification inhibitor, such as 2-bromooctanoate or triacsin C [35]. In addition to re-esterification, thermogenesis could be another reason for unexpected stoichiometry. Since thermogenesis includes fatty acid β-oxidation, this could be a possible reason why released fatty acid is lower than expected. Further studies with re-esterification inhibitor or β-oxidation inhibitor are required to verify these hypotheses.

In summary, DIM exerted lipolytic activity in mature 3T3-L1 adipocytes by shifting lipid metabolism toward lipolysis. Also, DIM enhanced expression of thermogenic genes, UCP1 and PGC-

1α, and stimulated phosphorylation of AMPK. Therefore, I concluded that DIM induces lipolysis and thermogenesis in mature 3T3-L1 adipocytes.

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Chapter 5.

Conclusion

5.1. Conclusion

Obesity is one of the primary causes of various metabolic syndromes [1, 2], and prevention and treatment of obesity has emerged as a major public health issue [3], Though there have been many attempts to develop anti-obesity drugs, the majority have been unsuccessful due to side effects [4, 5]. Thus, the need for a novel approach to treat obesity is greater than ever.

In previous studies, Choi, Y. *et al*., reported the anti-obesity effects of I3C, which is a metabolite of glucobrassicin in cruciferous vegetables [6-8]. Since I3C has been reported to be further metabolized to DIM in our body [9, 10], I hypothesized that DIM might possess anti-obesity effects.

I investigated the anti-obesity effect of DIM *in vitro* and *in vivo*. First, an *in vivo* experiment was conducted to evaluate anti-obesity effects of DIM in a diet-induced obesity (DIO) mouse model. Results showed that high fat-diet (HFD) increased body weight of mice and DIM suppressed HFD-induced weight gain significantly. Decreased body weight in the DIM administered group was due to a reduction in fat mass, but not other tissue mass.

In addition, DIM reduced hepatic lipid accumulation and serum triacylglycerol levels which are critical factors in obesity-induced metabolic syndromes. Since, adipogenesis of 3T3-L1 preadipocytes mimic physiological obesity development, this *in vitro* model was utilized to evaluate the anti-adipogenic activity of DIM. Oil red O staining results showed that DIM, but not I3C, blocked adipogenesis of 3T3-L1 preaipocytes. Also DIM inhibited the expression of adipogenic and lipogenic genes, PPARγ, C/EBPα, aP2, FAS and SREBP1, at the transcriptional level. Collectively, I concluded that DIM has anti-adipogenic activity and anti-obesity effects.

Next, I investigated the molecular mechanisms of how DIM suppresses adipogenesis of 3T3-L1 preadipocytes. Adipogenesis is composed of two steps, mitotic clonal expansion (MCE) and terminal differentiation (TD) [11]. MCE have been reported to be a prerequisite step for adipogenesis [12]. Many previous studies showed that inhibition of MCE is sufficient to block the adipogenesis process [13-15].

Hence, I have examined whether DIM can block MCE to

inhibit adipogenesis of 3T3-L1 preadipocytes. Oil red O staining result showed that anti-adipogenic activity of DIM was dominant in the MCE step. Fluorescence-activated cell sorter (FACS) analysis results showed that DIM delayed MDI-induced cell cycle progression significantly. This delayed cell cycle was mediated by reduced cyclin D1 expression level. Western blot and quantitative real-time PCR (qRT-PCR) results showed that DIM regulated cyclin D1 expression at the post-transcriptional level. Further studies with MG132 revealed that DIM increased degradation rate of cyclin D1. A previous study has reported that USP2 can regulate cyclin D1 ubiquitination, thus I analyzed the inhibitory effect of DIM on USP2 activity in silico. Result showed that DIM directly inhibited USP2 deubiquitinase activity in silico. Taken together, I concluded that DIM suppresses adipogenesis of 3T3-L1 preadipocytes by targeting USP2 deubiquitinase activity.

Finally, I investigated the effect of DIM on lipolytic and thermogenic activity in mature 3T3-L1 adipocytes. *In vivo* results showed that administration of DIM reduced HFD-induced weight gain while DIM treatment did not affect calorie intake. When the

total body weight decreases with no change in calorie intake, the lipid synthesis level would be the same but lipid storage capacity should be decreased. This would subsequently cause the extra lipids which are not stored in adipose tissue to circulate in the blood and eventually to be stored in other organs such as the liver or the muscle. However, *in vivo* results showed that DIM completely decreased serum triacylglyceride level and hepatic lipid accumulation.

These inconsistencies led us to hypothesize that DIM might enhance lipolysis and thermogenesis to reduce the blood lipid levels. To verify this hypothesis, I first measured glycerol and non-esterified NEFA release. DIM treatment in adipocytes increased glycerol release and NEFA release. Analysis of lipid metabolism-regulating protein expression and phosphorylation showed that DIM inhibited lipogenic protein expression significantly and slightly increased HSL phosphorylation, indicating that DIM shifted lipid metabolism of mature 3T3-L1 adipocytes toward lipolysis. Further, western blot results showed that DIM enhanced thermogenic gene expression and phosphorylation of AMPK. Taking these results

together, I concluded that DIM shifts lipid metabolism toward lipolysis and activated thermogenesis in 3T3-L1 adipocytes.

Collectively, DIM can act as a potent anti-obesity agent through suppressing adipogenesis and inducing lipolysis and thermogenesis. However, a more detailed mechanism of action of DIM in the molecular level should be investigated to better understand how DIM exerts anti-obesity effects *in vitro* and *in vivo*. For example, X-ray crystallography can help reveal the binding structure of USP2 with DIM and *in vivo* experiments with metabolic cages will elucidate the physiological thermogenic activity of DIM. Further studies of DIM using detailed molecular and structural biology approaches and advanced animal study will help understand the mechanism and effect of DIM as a potent natural anti-obesity agent.

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국문초록

대사성질환 등 여러 만성질환의 원인이 되는 비만을 예방하고 치료하기 위한 새로운 천연물 소재를 개발하기 위하여 십자화과 식물의 식물성분인 3,3'-디인돌릴메탄(DIM)의 비만 예방 및 억제 효과와 그 기작을 3T3-L1 지방전구세포주 모델과 식이로 유도되는 비만 동물 모델에서 연구하였다. 먼저, 고지방식이로 유도되는 비만 동물모델에서 DIM 이 식이섭취량의 변화 없이 고지방식이로 유도되는 체중증가를 억제함을 확인하였다. 이러한 체중 조절 효능은 지방조직 무게의 감소에 기인함을 확인하였으며, 신장, 췌장, 비장 등 다른 장기의 무게에는 큰 변화가 없음을 알 수 있었다. 또한 DIM 이 간 내의 지방 저장과 혈중 중성 지방 농도를 감소시킴을 확인하였다. 다음으로, 이와 같은 DIM 의 항비만 효과를 세포주 모델에서 확인하였다. DIM 이 3T3-L1 지방전구세포의 지방세포분화를 억제함을 확인하였고, 지방세포의 지표 유전자와 지방합성에 관련된 유전자의

발현을 억제함을 확인하였다. 이 결과들을 종합하여 DIM 이 세포주 모델과 동물 모델에서 항비만 효능을 가진다는 결론을 내릴 수 있었다.

다음으로 DIM 이 유사분열성세포증식 단계를 타겟으로 하여 지방세포분화를 억제할 수 있다는 가설을 세워 실험을 진행하였다. 실험 결과, DIM 의 지방세포분화 억제 효능은 유사분열성세포증식 단계에서 가장 큼을 확인하였다. 유사분열성세포증식 단계에서는 세포 증식과 세포주기진행이 일어나기 때문에 이에 DIM 이 미치는 영향을 확인 해 보았는데, DIM 이 세포주기진행을 지연시키고, 세포 증식을 억제하였다. 이러한 효과는 싸이클린 D1 단백질의 발현을 감소시키는 효능을 통해서 일어남을 알 수 있었다. 또한 DIM 이 싸이클린 D1 단백질의 분해속도를 증가시켰고, 이러한 효능은 유에스피 2 단백질의 유비퀴틴 분리 활성 억제에 의한 것이었다. 이 결과들을 토대로 DIM 이 유에스피 2 단백질의 유비퀴틴 분리 활성을 억제하는 기작을 통해

유사분열성세포증식 단계에서 지방세포분화를 잘 억제하는 효능을 지닌다는 결론을 내릴 수 있었다.

이상의 결과에서 DIM 의 지방세포분화 억제 효과가 유사분열성세포증식 단계에서 가장 크게 나타남을 확인하였지만, 최종분화 단계에 DIM 을 처리하였을 때도 세포 내 지방 저장량이 감소함을 확인할 수 있었다. 최종분화 단계에서는 지방합성이 주로 일어나기 때문에, 이러한 결과를 토대로 DIM 이 분화가 완료된 3T3-L1 지방세포에서 지방대사의 균형에 영향을 줄 수 있을 것이라는 가설을 세우게 되었다. 먼저 글리세롤과 자유지방산의 방출량을 측정 해 보았는데, DIM 이 분화가 완료된 3T3-L1 지방세포에서 글리세롤과 자유지방산의 방출을 크게 증가시켰다. 또한 분화가 완료된 3T3-L1 지방세포에 DIM 을 처리하였을 때 세포 내 지방 저장량이 유의적으로 감소함을 확인하였다. 이는 DIM 이 분화가 완료된 3T3-L1 지방세포에서 중성지방의 유출을 가속화시킨다는 것을 의미한다. 이에 관련된 단백질의

발현을 살펴보았는데, DIM 은 지방합성에 관련된 단백질의 발현을 억제하고, 지방분해에 관련된 단백질인 호르몬-민감성 지방분해 효소인 HSL 의 인산화를 증가시켰다. 이를 통해 DIM 이 지방대사의 균형을 지방합성 쪽에서 지방분해 쪽으로 이동시킨다는 결론을 내렸다. 지방분해가 증가되는 것이 지방산분해 및 열생성으로 이어지지 않을 경우 대사성질환을 유발하는 요인인 혈중 자유지방산의 농도가 높아질 가능성이 있기 때문에 DIM 이 열생성에 관련된 유전자의 발현에 미치는 영향과 에이엠피케이의 인산화에 미치는 영향에 대한 실험을 진행하였다. 실험 결과, DIM 이 열생성에 관련된 유전자의 발현을 증가시키고, 에이엠피케이의 인산화를 촉진함을 확인할 수 있었고, 이러한 결과들을 통해 분화가 완료된 3T3-L1 지방세포에서 DIM 이 지방분해와 열생성을 증가시킴을 알 수 있었다.

이 결과들을 모두 종합하여 DIM 이 세포주 모델과 동물 모델에서 항비만 효능을 지님을 확인하였고, 그

작용기작을 밝혀내었다. DIM 은 양배추의 주요 성분인 인돌-3-카비놀의 체내 대사체이기 때문에 이러한 연구 결과는 부분적으로 양배추의 항비만 효과에 관한 작용 기작을 제시하고 있으며, DIM 이 지방세포의 비대화를 막고, 동시에 지방분해 및 열생성을 촉진시켰기 때문에 현재까지 개발된 약과는 다른 작용기작을 가지는, 그러면서도 부작용은 없는 새로운 항비만제재의 개발 가능성을 시사하고 있다.

검색어 : 3,3'-디인돌릴메탄 (DIM), 고지방식이, 지방세포분화, 유사분열성세포증식, 유에스피 2 (USP2), 지방분해, 열생성

감사의 글

박사과정에 입학한지 4 년이 지났습니다. 내가 무사히 박사과정을 졸업할 수 있을지 걱정이 많았었는데, 여러 교수님의 지도 덕분에 무사히 졸업을 할 수 있게 된 것 같습니다. 부족한 저를 졸업할 수 있게 잘 지도해 주신 이형주 교수님, 이기원 교수님, 그리고 다른 모든 교수님들께 감사드립니다. 그리고 지난 4 년간 가족보다 더 많은 시간을 함께 보낸 기능성식품학실 식구들에게도 감사의 인사를 올립니다. 또한 미국에서 연구와 생활에 많은 도움을 주신 이경상 박사님과 실험실원 분들께도 감사의 인사를 드립니다. 마지막으로 제가 박사과정에 진학하여 공부에 전념할 수 있도록 뒤에서 물심양면으로 도와주신 부모님과 제가 박사과정에 재학하는 동안 나 대신 집안 경제에 보탬을 주고 있는 동생에게도 감사하는 마음을 전합니다. 이 모든 분들 중 한 분이라도 계시지 않았다면 지금의 저도 없었을 것입니다. 제가 졸업할 수

있도록 도와주신 모든 분들께 감사의 마음을 전하면서 감사의 글을 마치겠습니다. 감사합니다.