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Beneficial effects of naringenin and indomethacin on white and brown adipocytes

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Beneficial effects of naringenin and indomethacin on white and brown adipocytes

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DEDICATION

To my late mother, Sheila Simpson Kearns, who instilled in me the importance of education. To my sister and brother-in-law, Jessica and Ben Puffer who have encouraged and supported me through every step.

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ABSTRACT

As obesity continues to grow and medical costs in the United States are estimated at $147 billion annually, novel ways to prevent and treat obesity are needed. One approach is to promote thermogenesis to improve energy balance by increasing the activities of thermogenic brown and beige adipocytes.

Naringenin, a citrus flavanone, has been shown to act as anti-inflammatory and lipid lowering agent as well as activate PPARgamma. However, it is unclear whether it can activate thermogenic activity in white adipocytes, i.e., promote formation of beige adipocytes. Indomethacin (INDO) is an FDA approved drug used to treat pain related to inflammation by inhibiting cyclooxygenase (COX). It has been demonstrated that INDO is a PPARgamma agonist and is protective against weight gain in mice fed a high fat and high sucrose diet. Whether INDO independently induces brown adipocyte differentiation has not been studied.

In this thesis, I investigated the effect of naringenin combined with isoproterenol, a beta-adrenergic receptor agonist on thermogenic activation of a common white adipocyte cell line, 3T3-L1. In addition, I investigated whether INDO induces brown adipocyte differentiation. 3T3-L1 cells were differentiated into mature adipocytes with a standard differentiation cocktail in the presence of naringenin and then stimulated with isoproterenol. While naringenin had little effect at the basal level, it significantly increased mRNA and protein expression of UCP-1 and PGC-1alpha, browning marker genes. Moreover, naringenin increased mitochondrial DNA, which is indicative of increased mitochondrial biogenesis. The results suggest that in addition to increased UCP-1 expression, naringenin can promote up regulation of PGC-1alpha, leading to increased mitochondrial biogenesis in thermogenic activation of 3T3-L1.

To study the effects of INDO on brown adipocyte differentiation I differentiated brown preadipocytes in the presence of increasing doses of INDO using a modified differentiation protocol. INDO dose-dependently increased lipid accumulation and mRNA expression of brown specific marker genes PGC-1alpha, UCP-1 and PRDM16. Protein expression of PGC-1alpha and UCP-1 was confirmed by western analysis. Consistently, INDO dose-dependently increased mitochondrial biogenesis. Mechanistically, INDO increased PPAR responsive promoter activities. These results suggest that INDO may promote brown adipogenesis through activation of PPARgamma.

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CHAPTER I
INTRODUCTION

A major health issue in the United States (US) is obesity. One-third of US adults, totaling 78.6 million, are obese.[1] Children are also experiencing this disease with childhood obesity affecting 17% of individuals 2-19 years of age.[2] The estimated cost of adult obesity is 147 billion dollars annually. An obese individual costs an additional $1,429 in healthcare each year. This is due to the complications of obesity that include; type 2 diabetes, cardiovascular disease, hypertension, dyslipidemia and certain cancers. Many factors contribute to obesity such as environment, genetics, diet, behaviors, socioeconomic status and particular illnesses. All of these factors contribute to positive energy balance, that creates excess adiposity.[1]

Adipose tissue includes white, brown and beige phenotypes. White adipose tissue (WAT), the primary site of energy storage within the body, is composed of mostly triglycerides, up to 85%,[3]and has been an evolving topic of interest for research due to its endocrine contributions and its role in systemic inflammation.

The second type of adipose tissue is brown adipose tissue (BAT), whose main function is non-shivering thermogenesis in addition to lipid storage. The non-shivering thermogenic function of BAT is due primarily to the expression of the unique protein uncoupling protein 1 (UCP-1).[3] It was originally assumed that BAT was exclusively present in neonates. The recent discovery of BAT in human adults along with its ability to increase non-shivering thermogenesis has made BAT a novel target for obesity treatment and prevention.[4-6]

A third adipose cell phenotype is beige, which is thought to be derived from WAT adipose depots and can be induced to express UCP-1 for increased thermogenic activity. Much

interest has been sparked in this area of research as it may lead to novel strategies to combat obesity and its related diseases.[7]

Peroxisome proliferator activated receptor gamma coactivator- 1 alpha (PGC-1α) is a transcription factor coactivator that is known for its function in regulating mitochondria biogenesis and oxidative metabolism.[8] PGC-1α acts to coactivate peroxisome proliferator activated receptor gamma (PPARγ) that binds at the PPRE site of various adipogenic genes to promote transcription, including those related to differentiation and that of the UCP-1 promoter. PGC-1α is important in cellular metabolism and is regulated by other important signals such as increased cAMP,[9] p38MAPK phosphorylation,[10] and SIRT1 deacetylation.[11] Due to its role in metabolism, it may be beneficial target to increase thermogenesis in adipocytes.

Several compounds including nutrients and pharmaceuticals are being investigated for their potential to influence cellular targets for obesity treatment and prevention. Polyphenols have become more popular in research as they have been reported to exert a variety of beneficial effects on metabolism and health.[12] Naringenin a member of the flavanone class of polyphenols has been shown to improve lipid metabolism and adiposity in animal models.[13] Furthermore, it has been shown to be a PPARγ agonist in hepatic cells.[14] However, it is not clear whether naringenin can induce thermogenic activation in white adipocytes or what role PGC-1α

In addition to using nutrient based strategies to target thermogenesis for obesity, pharmaceutical agents are also studied. Indomethacin is a non-selective cyclooxygenase (COX) inhibitor used in the treatment of pain and inflammation.[15] It has been shown to promote adipogenesis and is protective against high fat/high sucrose diet induced obesity in mice.[16] However, the mechanism that exerts its effects on brown adipocytes is not clear. Additionally it

is unknown whether INDO can induce differentiation of brown adipocytes independent of other inducers in a typical differentiation cocktail[17] (i.e. dexamethasone and IBMX).

Both naringenin and INDO have been shown to be PPARγ agonists. Rosiglitazone, a known PPARγ agonist and browning agent, has been shown to promote thermogenic activation in 3T3-L1 cells. PPARγ increases differentiation and this process in brown adipocytes may be applied to increased BAT mass in vivo. We hypothesize that naringenin will promote thermogenic activity with a phenotype similar to that of rosiglitazone and that INDO will promote brown adipocyte differentiation through PPARγ activation. To fill the knowledge gap, the objective of this thesis is to study the effects of both naringenin and INDO on browning and brown adipocyte differentiation respectively. Specifically we aim to study the beneficial effects of naringenin on PGC-1α in thermogenic activation of 3T3-L1 cells. For INDO, we aim to determine if INDO can promote brown adipocyte differentiation of BAT without an induction period that includes a corticosteroid using a modified differentiation protocol. The results presented add to our understanding of nutrient and pharmacological regulation of adipose tissue and suggest great potential of naringenin and INDO in obesity treatments and prevention.

CHAPTER II
LITERATURE REVIEW

2.1 Brown Adipose Tissue and Browning: Novel Targets for Intervention

2.1.1 Function, anatomic location and discovery in adult humans

Brown adipose tissue (BAT) is a distinct tissue type in the body. It was originally accepted that BAT was only present in neonates and there was a rapid regress afterwards.[18] Evidence of BAT in adult humans was found utilizing fluoro-deoxyglucose (FDG) positron emission tomography (PET) and X-ray computed tomography (CT). This showed increased 2-deoxyglucose uptakes during cold stimulation in the supraclavicular and paraspinal regions. The presence of BAT was correlated with younger age and lower BMI. [5] A study using the same scanning technique and larger sample size found similar results and a higher mass of BAT in women.[4] These studies confirm the presence of active BAT in humans, and suggest that BAT could be a novel target for obesity treatment and prevention.

BAT is estimated to compose 0.05%- 0.1% of total body weight in an adult human compared to 5-10% in mice and 2-5% in human infants.[19] The tissue is highly innervated with sympathetic nerves, which is the major activator for brown cell thermogenic activity.[20] BAT is highly vascularized, allowing for access to circulating glucose and lipids as well as distributing heat produced by thermogenesis.[3]

Recruitment and activation of BAT is due to stimulation of the sympathetic nervous system and the release of norepinephrine that activates β-adrenergic receptors expressed on the cell surface. This leads to the mobilization of triglycerides from all adipose tissues to increase energy availability that can be utilized for thermogenesis.[3] Norepinephrine activation of β-adrenergic receptors has been shown to stimulate thermogenesis in BAT as well as increase cell growth and differentiation.[3, 21, 22]

2.1.2 Cellular origins of brown adipose tissue

Like white adipocytes, brown adipocytes are derived from mesenchymal stem cells. Conventional brown cells are from a Myf5+ precursor cell whereas white cells are from Myf5- precursor cells. Brown adipose cells arising from the Myf5+ precursor have a closer relation to myocytes rather than a white adipocytes.[23] It was found in brown preadipocytes, on day 1 of differentiation that myogenin, a protein found in skeletal muscle, was transiently expressed then declined during the course of adipocyte differentiation; this was not found in white adipocytes.[24]

However, both types of adipocytes utilize many common transcriptional factors for complete differentiation. Since both types of adipose accumulate lipids during the differentiation process it has been shown that 72% of the genes up regulated and 52% that are down regulated are similar between the two types.[24] In contrast, brown adipocytes up regulated about 100 genes that are related to mitochondria.[24] Brown cells contain multilocular lipid droplets and more mitochondria compared to WAT, that contain unilocular lipids with fewer mitochondria.[21]

Another related cell type is called "beige" adipocytes. There are various hypotheses about the origin of these cells. It is disputed whether they have unique precursors or if they are from transdifferentiation from white adipocytes. Transdifferentiation has not been proven false but studies have isolated pre beige cells that have different marker expression than those of white preadipocytes.[25] Beige cells are localized inside WAT depots but they can be induced to appear and function as brown like cells, i.e. with increased mitochondrial content and UCP1 expression. This phenomenon is called "browning".[21, 26]

2.1.3 Uncoupling protein 1 (UCP-1) and other key markers of brown adipocytes

A major identifying protein for brown adipocytes is uncoupling protein 1 (UCP-1). Respiration is uncoupled from ATP synthesis by the UCP-1 protein, which makes the inner

mitochondrial membrane more permeable to protons, thus lowering the proton gradient created by the electron transport chain, preventing the synthesis of chemical energy (in the form of ATP). Five different uncoupling proteins have been identified; however, UCP-1 is specific to adipose tissue and its function is the best understood.[22]

Wild type and UCP-1 knockout mice were given a β_3-andrenergic receptor agonist (CL-316, 243) to determine the effect of β-adrenergic receptor activation on UCP-1. The wild type mice had an increase in oxygen consumption with decreased lipid levels after administration of the β-adrenergic agonist. The UCP-1 knockout mice did not experience these changes.[27] These results point to the importance of UCP-1 in the increased thermogenesis from the stimulation of β_3-adrenergic receptors in BAT from the SNS.

The UCP-1 gene shares a common structure between mice, rats and humans near the transcriptional start site as well as a conserved upstream enhancer. UCP-1 promoter contains multiple binding sites that allow for the action of PPARs, retinoids, and thyroid hormones. UCP-1 transcription is mainly activated by norepinephrine released from the nervous system to activate β_3-andrenergic receptors and its downstream pathways.[28, 29]

A transcription factor highly specific to adipocytes is peroxisome proliferator-activated receptor gamma (PPARγ). This transcription factor is the key regulator in the differentiation and maintenance of both white and brown adipocytes. Low levels of PPARγ are present in preadipocytes and are increased to peak at the beginning of differentiation.[30] The essential role of PPARγ is evidenced by an in vitro study where cells derived from embryonic stem cells were used to make a PPARγ knockout. It was found that knock out cells did not differentiate while the wild type did.[31]

Rosiglitazone is a known anti-diabetic drug that has been shown to decrease circulating lipid levels and act as PPARγ agonists.[32] When administered in mice a marked increase in UCP-1 expression in both lean and obese mice was seen. These results point towards the link between the activation of PPARγ and UCP-1 expression. [33] This is due to PPARγ activity binding to the PPRE site of the UCP-1 promoter to increase transcription.[28] At this site is where PGC-1α (peroxisome proliferator activated receptor γ coactivator- 1α) coactivates PPARγ to help drive UCP-1 expression.[34]

A protein specific to brown adipocytes important for the differentiation of the Myf5+ precursor is PR domain-containing protein-16 (PRDM16).[35] An experiment with knockdown of PRDM16 in primary brown fat cells led to myogenesis and decreased expression of UCP-1, PCG-1α and other brown fat identifiers.[35, 36] In addition increased PRDM16 expression in myoblasts promoted adipogenesis leading to mature brown adipocytes, which may result from an increase in PPARγ and UCP-1 expression. PRDM16 has been shown to bind to PCG-1α, PPARα[37], PPARγ and members of the C/EBP family to enhance their action as DNA-binding transcription factors and coactivators.[38] Seale and colleagues proposed that a major action of PRDM16 was through its interaction with PPARγ[35] and it was also shown that PPARγ agonist (rosiglitazone) stabilized PRDM16 in white adipocytes to promote browning.[39]

2.2 Browning and Beige Adipocytes

2.2.1 Function, origin, and markers

Beige adipocytes are of interest as they can be found in WAT depots but are active in thermogenic uncoupling, contributing to energy expenditure. While beige adipocytes have been studied and found to have a variety of origins, they are a therapeutic target for obesity and other

metabolic diseases.[40] In animal studies, the presence of beige cells in classical WAT depots has shown to be effective in protecting against diet-induced obesity.

Some markers have been tested for the identification of beige adipocytes. However, the literature varies in what parameters define a beige adipocyte. Many publications rely on the increased expression of brown fat cell markers such as UCP-1, and increased energy expenditure along with a white fat origin to define a beige adipocyte. However, beige cells still express UCP-1 at a lower level than classical brown adipocytes. Studies seeking to determine markers that are exclusive to beige cells have identified CD137, Tmem26 and HoxC9.[41] A study comparing expression of markers in different adipose tissue depots and cell culture of primary adipocytes found that Tmem26 and CD137 were reliable markers in tissue but were less specific when primary cells were isolated from the tissue and cultured. Cells cultured with rosiglitazone and norepinephrine expressed decreased Tmem26 whereas CD137 was increased. This study also measured HoxC9 and found that it did not distinguish between beige and white adipocytes; however, it did distinguish white and beige cells from brown indicating HoxC9 is able to differentiate the depot of origin.[42] Another study comparing inguinal WAT as well as primary cells from the stromal vascular (SVF) and the adipocyte fraction showed that Tmem26 decreased when differentiated into beige cells. In contrast, CD137 increased with differentiation to a beige adipocyte in vitro.[43] Together these studies indicate that these beige markers may be more specific to the precursor beige cells in the tissue rather than fully differentiated cells.

Zic1 and Meox-2 are brown adipose markers.[24, 36] In beige cells; Petrovic and coauthors found that these two markers were not detectable even when PGC-1α and UCP-1 expression was increased.[44] There is variation in expression of white, brown and beige marker genes between

cell lines, animal studies, and human derived cells. Therefore, it is unclear which are the best markers to be used that are consistent among cell culture, animals, and humans.[41]

2.2.2 Common cell models of browning

3T3-L1 cells are the most widely used cell line for studying adipose and were utilized in 392 publications in the last five years comparing to other cell lines that have been used less than 15 each.[45] The 3T3-L1 cell line is derived from mouse embryos of Swiss 3T3 mice at 17 to 19 days of development. They are preadipocytes that appear like fibroblasts and can be differentiated into mature adipocytes.[46] They are frequently used due to their low cost and ease of use compared to primary cells. However, they can be difficult to transfect and lose the ability to differentiate after several passages.[47]

3T3-L1 cells have been shown to express higher levels of UCP-1 when treated with Rosiglitazone, IBMX, and T3.[48] Isoproterenol is a potent, but nonspecific, β-adrenergic receptor activator that can induce expression of beige and brown related genes in 3T3-L1 cells. Treatments after 6 or 48 hours increased expression of UCP-1, PRDM16 and CD137 and increased uncoupling.[49]

In contrast, to the cell line, primary cultures from the stromal vascular region (SVF) are also used as a model in studies related to adipose tissue cell differentiation process. Unlike other cell types, these preadipocytes are more committed and can only be differentiated into adipocytes. Primary adipocytes may also have more characteristics related to the depot of origin compared to cell lines, providing more information about the differences in cell origin.[50] Human derived primary cells and cell lines are becoming more available and can be beneficial in confirming results found in cell models of animal origin. In addition, human derived primary

cells can be used to confirm what is known about cell lineages and differences between cell origin related to the fat depot and genetic differences between donors.[51, 52]

2.2.3 Stimuli and known browning agents

Several agents to promote "browning" process have been identified. Similar to BAT, prolonged cold exposure can promote browning in WAT depots that has been shown in vivo. Additionally, many pharmacological inducers of UCP-1 expression in beige cells are β-adrenergic agonists such as isoproterenol, CGP-12177, or CL-316243.[49, 53 54] PPARγ ligands such as rosiglitazone, is another well-known inducer of browning. PPARγ agonist activity has been shown to increase mitochondrial biogenesis, mitochondrial gene expression, and increase UCP-1 expression.[44, 55, 56]

Beyond pharmaceuticals, dietary components, such as polyphenols and lipids have been studied in relation to browning. Capsaicin has been shown to promote browning in vitro, and increased browning alongside resistance to HFD-induced obesity in rats.[57, 58] Resveratrol has been shown to increase UCP-1 expression and mitochondrial content in mouse embryonic fibroblasts (MEF) but failed to increase UCP-1 in 3T3-L1 adipocytes.[59] Additionally, omega-3 fatty acids have been shown to increase the expression of PGC-1α, Nrf (nuclear respiratory factor), and CPT1 (Carnitine palmitoyltransferase 1) with increased beta-oxidation but did not increase UCP-1 in WAT in mice.[60] In rats fed a high fat diet, thermogenesis increased with the addition of omega-3 fatty acids to the lipid composition of the diet.[61]

2.3 Peroxisome Proliferator-Activated Receptor γ Co-activator-1a (PGC-1α)

Peroxisome proliferator-activated receptor γ co-activator-1a (PGC-1α) was identified as a critical part of adaptive thermogenesis in brown adipose tissue as it was found to be increased upon cold exposure. It was identified for its role in co-activating PPARγ by forming a heterodimer with RXR on the UCP-1 promoter region.[34] This led to the interest in studying the interaction with PPARγ in promoting UCP-1 expression and thermogenesis. Further studies reveal many more capacities of PGC-1α, including mitochondria biogenesis and energy adaptation.[62] PGC-1α is expressed at the highest levels in tissues that have high rates of oxidative metabolism including liver, kidney, and muscle tissues. In relation to thermogenesis in adipose tissue, in addition to its role in UCP-1 expression, PGC-1α induces expression of respiratory chain components that are encoded by mitochondrial DNA such as cytochrome oxidase subunits and ATP synthase subunits.[8] A study that generated a PGC-1α- null brown fat cell line demonstrated that PGC-1α was required for mitochondrial biogenesis and increased thermogenesis in response to cAMP increases. However, it was not required for the phenotypic differentiation of BAT.[63]

2.3.1 PGC-1α activation and modification

PGC-1α is activated through β-adrenergic activation via the cAMP dependent pathway. This pathway increases the transcriptional activity of PGC-1α by increasing the binding of CREB to the PGC-1α promoter. It was found in hepatic cells that when CREB was virally knocked down the induction of PGC-1α by a cAMP agonist was lost, leading to the conclusion that PGC-1α is a direct target of CREB.[64] However, in brown adipocytes p38 MAPK can

increase transcription of PGC-1α and assists in regulation of the protein through phosphorylation.[9, 10]

PGC-1α is activated by exercise in skeletal muscle where it contributes to energy adaptations.[65, 66] It is also possible that with exercise an up regulation of PGC-1α in adipose is part of the associated metabolic improvements.[67] This activation in adipose and muscle cells leads to increased respiration, which is considered positive for prevention of obesity and a potential therapeutic approach. However, p38MAPK phosphorylation of PGC-1α has been shown to be activated by cytokines and this activation is thought to be a driver of cachexia and muscle wasting that is clinically harmful in disease states such as cancer and trauma.[68]

Posttranslational modifications are important for the regulation of PGC-1α as it has a half-life of 2.28 hours. Phosphorylation by p38MAPK can triple the half-life allowing for higher PGC-1α protein expression and increased activity.[68] Deacetylation by SIRT1 is another modification that can increase PGC-1α activity to help modulate energy status of the cell and inhibition.[11] The variety of modifications lends itself to the differential regulation and activity of PGC-1α in various tissue types. For example, in the liver deacetylation of PGC-1α increases the transcription of genes involved in gluconeogenesis but does not increase genes related to the electron transport chain as in adipose tissue. In adipose tissue PGC-1α increases UCP-1 but not aP2 which are both regulated by PPARγ, indicating greater specificity than coactivating PPARγ.[69]

Several different pathways can also down regulate PGC-1α activity. RIP140 is a nuclear receptor corepressor that directly binds to PGC-1α and prevents its binding its downstream targets.[70] When a chemical screen of transient receptor potential vanilloid 4 (TRPV4) agonists is done, there is an increase in PGC-1α expression, indicating the receptor can regulate expression.

In TRPV4 null mice, there was increased thermogenesis and protection from diet-induced obesity.[71]

2.3.2 PGC-1α promotes mitochondrial biogenesis

Mitochondrial biogenesis is the growth and division of mitochondrial that result in variation in number and size of cellular mitochondria.[72] For its role in mitochondrial biogenesis, PGC-1α increases the gene expression of Nrf1 and Nrf2. These transcription factors work to promote a portion of mitochondria related genes including cytochrome c subunits by up regulating expression of Tfam. Tfam plays a critical role in its ability to translocate to the mitochondria in order to activate and coordinate mitochondrial DNA replication and transcription. Unlike increases in Nrf or Tfam alone, PGC-1α also plays a role in oxidative metabolism in skeletal muscle and cardiac muscle, indicating its importance in overall energy regulation.[62]

This pathway is important for regulating the health of mitochondria. Dysfunction of mitochondria has been linked to aging, cancer, Alzheimer's, and diabetes.[72] Several studies have looked at muscle biopsies in humans to understand mitochondria content and insulin resistance. *Morino etal* looked at young lean individuals with insulin resistance that had parents with Type 2 Diabetes. Insulin-mediated glucose update was 60% lower and mitochondrial density was 38% less than controls that had no insulin resistance or history of diabetes.[73] In addition, two other studies found decreases in mitochondrial associated genes including PGC-1α, Nrf-1 and other downstream oxidative phosphorylation genes in diabetic subjects.[74, 75] Further evidence of PGC-1α in relation to diabetes is shown by a study that treated mice with the common anti-diabetic drug Metformin and found increases in muscle content of PGC-1α.[76] All of this points to the

importance of PGC-1α and how its role in mitochondrial biogenesis and cellular metabolism can be beneficial in fighting against metabolic diseases.

2.4 Overview of Naringenin

2.4.1 Structure, dietary sources, and metabolism

Naringenin has been found to function as an anti-inflammatory and antioxidant agent. It has been shown to be beneficial for hyperlipidemia, hypertension, hyperglycemia, diabetes, atherosclerosis, steatosis, inflammation as well as to be cardiac, and hepatic protective properties.[77-82]

Plant based compounds such as polyphenols are actively researched to understand their beneficial properties in treating various diseases. Naringenin is a flavanone found in citrus fruits grapefruit, oranges and in much smaller amounts in tomatoes and mint.[83] Naringin is the flavanone responsible for the bitter taste and when this compound is ingested it is converted by gut microbiota into the aglycone form; naringenin.[84] When ingested in the aglycone form, naringenin is readily absorbed in the small intestine although the transporter has not been identified. Once absorbed, naringenin is then metabolized in the liver through methylation, sulfation and glucuronidation. The aglycone form in the blood is typically low after ingested at doses found in foods that contain naringenin. Even though the aglycone form is more efficiently absorbed in the small intestine, the glucuronides are found at the highest concentration in the blood. Polyphenols in general can be transported in circulation bound to albumin. However, it remains unclear where polyphenols accumulate within the body. Their excretion of polyphenols, especially flavanones, is through urine output but can also be excreted via the biliary route.

Additionally, absorption can vary related to individual differences in anatomy as well as dietary components present in the gastrointestinal tract with naringenin.[83]

2.4.2 Plasma levels of naringenin in humans

U.S. adults are estimated to consume about 189 mg/day of flavonoids with 14mg of them consisting of flavanones mostly from citrus fruit juice intake.[85] Absorption and metabolism of naringenin varies between individuals and studies of plasma concentrations are ongoing. Estimation of intake is difficult due to the numerous variations in the content of foods, which is related to diverse growing conditions, storage, processing, and preparation.[83]

In one study, participants consumed either orange juice or grapefruit juice that had concentrations of naringenin of 151 and 1283 μM respectively. The peak plasma concentrations of naringenin were 0.6 ± 0.4 μM from orange juice and 6.0 ± 5.4 μM from grapefruit juice.[86] In a separate study, participants received 135 mg of naringenin, resulting in peak plasma concentrations ranging from 4.12 to 11.03 μM.[87] These studies show that naringenin levels vary by source, dose, and the individual differences and a level of 10 μM is physiologically achievable.

2.4.3 Assessment of the beneficial effects of naringenin: cell, animal, and human studies

Harmon and colleagues studied the effects of naringenin on proliferation of cultured 3T3-L1 cells. Proliferation of 3T3-L1 cells was inhibited by naringenin in a time and dose dependent manner, Naringenin also caused an increased lactate dehydrogenase (LDH) release from the cells at 100ug/mL, indicative of cytotoxic effects of naringenin at this level.[88] Therefore, it is important to use physiologically achievable doses.

Naringenin has been shown to be an agonist of PPARα and PPARγ. Concentrations of naringenin used ranged from 0 to 240 μM.[14] As noted above, PGC-1α is increased with cold stimulus increasing its ability to coactivate PPARγ at the UCP-1 promoter. NIH 3T3 cells were transfected with plasmids for PPARγ-Gal4 and Gal4- luciferase with naringenin for 24 hours. Naringenin dose dependently increased the luciferase activity, indicative of PPARγ activation.[89]

Rats fed a diet that contained 0.003, 0.006, or 0.012% naringenin for 6 weeks found significant reduction of triglycerides in adipose and liver tissue.[79]

Several animal studies using various mouse strains on diets containing naringenin reported reduced weight gain compared to the control diet. Ldlr-/- mice fed a high fat[90] or high cholesterol[78] diet that included 3% naringenin exhibited decreased adipocyte hypertrophy and resistance to diet induced weight gain than those that were not given naringenin. Three separate but similar studies utilized C57BL/6 mice that were fed a HFD[77] or ovariectomized[91] or that were ovariectomized and fed a HFD.[92] Out of the groups fed naringenin, ovariectomized and high fat diet mice saw reduced weight gain while all three studies had decreased intra-abdominal adiposity. This shows promising effects that naringenin may be able to treat obesity.

Further demonstrating the beneficial potential of naringenin for obesity treatment, some human studies have been completed. Two studies had participants consume one-half of a grapefruit or the equivalent amount of grapefruit juice before meals. In one study, overweight adults followed this regime for 6 weeks. Participants had decreased waist circumference but no significant weight loss.[93] Obese participants with metabolic syndrome that followed the same regime for 12 weeks in a randomized, double blinded study design, had a weight loss of 1.6kg and 1.5 kg (whole fruit vs. juice respectively).[94]

2.5 Overview of Indomethacin

Indomethacin (INDO) was discovered as a non steroid anti-inflammatory drug (NSAID) in 1963 to treat joint swelling from rheumatoid arthritis.[15] It works as a nonselective COX inhibitor for both COX1 and COX2. COX enzymes catalyze the rate limiting reaction that converts arachidonic acid and other omega-6 fatty acids to prostaglandins.[95] Since then, it is used for treatment of various conditions related to inflammation.

An in vivo study in mice demonstrated that INDO treatment was protective against a high sucrose and high fat diet-induced obesity and decreased insulin sensitivity. However, INDO treated mice showed increased hepatic glucose output and glucose intolerance.[96] It was reported that INDO promoted adipocyte differentiation in 3T3-L1 cells.[97] In recent years, INDO is often used for brown preadipocyte differentiation. One study reported INDO activated PPARγ and PPARα.[98] Interestingly, recent studies have reported that COX2, a rate-limiting enzyme in prostaglandin (PG) synthesis, is a downstream mediator of β-adrenergic signaling in WAT and is involved in induction of UCP1 expression in inguinal white adipocytes, but not in classic interscapular brown adipocytes.[99, 100] Inhibition of COX by INDO suppressed UCP1 expression in the recruited brown adipocytes.[99] This suggests that prostaglandin synthesis inhibited by INDO may not be required for classical brown adipogenesis but it may play a role in beige cell formation due to prostaglandins being a PPARγ ligand.[101] Further research to better characterize INDO's effects and mechanisms are warranted.

CHAPTER III
NARINGENIN PROMOTES UP-REGULATION OF PGC-1ALPHA IN THERMOGENIC ACTIVATION OF 3T3-L1

3.1. Introduction

Obesity continues to be a health problem in the US and is growing across the globe. Complications from the disease lead to increased health costs and decreases in quality of life for many.[102] White and brown adipose tissues are responsible for energy storage and adaptive thermogenesis, respectively. Inducible thermogenesis is of interest to increase overall energy expenditure to fight obesity.[3] Beige adipocytes are found in white adipose depots and are inducible to express UCP-1 for active thermogenesis.[25] These cells can respond to similar stimuli as brown adipocytes such as cold, hormones, and some pharmacological agents to take on a brown-like phenotype, this process is called browning.[103]

Naringenin is a citrus flavanone that has been shown to have beneficial health effects. It is found at the highest levels in grapefruit.[83] Positive effects on lipid metabolism, inflammation and resistance to diet-induced weight gain by naringenin have been demonstrated.[82] In mice, it has been shown that naringenin is protective against increased weight gain induced by a high fat or high cholesterol diet and in a similar study the levels of triglycerides were improved by naringenin.[78, 90, 92] In vitro naringenin has been shown to be a PPARγ agonist.[14] PPARγ can bind to the PPRE site on the UCP-1 promoter and is coactivated by PGC-1α.[28] Pharmacological PPARγ agonist rosiglitazone promotes browning in WAT cultures, leading to up regulation of UCP-1 and thermogenesis.[55, 104] However, it is unclear whether naringenin can promote the browning effect and what cellular mechanisms drive its beneficial effects.

As a coactivator for PPARγ and other transcription factors, PGC-1α is important in brown adipose tissue function and plays an important role in UCP-1 transcription, mitochondrial

biogenesis and oxidative metabolism.[62] We aim to study whether naringenin can increase browning and up regulate PGC-1α expression. We utilized 3T3-L1 adipocytes differentiated in the presence of varying physiologically achievable doses of naringenin and then stimulated cells with isoproterenol, a β-adrenergic receptor agonist.

3.2. Materials and Methods

3.2.1 Reagents

Murine 3T3-L1 cells were obtained from ATCC (Manassas, VA). Naringenin, dimethyl sulfoxide (DMSO), 3-Isobutyl-L-methylxanthine, dexamethasone, insulin, isoproterenol (ISO), and rosiglitazone (Rosi) were from Sigma Aldrich (St. Louis, MO). Calf serum (CS) was purchased from Hyclone (Logan, UT) and fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). UCP-1 antibody was purchased from Sigma Aldrich (St. Louis, MO), PGC-1α from Millipore (Temecula, CA), ERK1/2 antibody, and horseradish peroxidase-conjugated goat anti-rabbit were from Cell Signaling Technology (Danvers, MA).

3.2.2 Cell culture and treatment

Murine 3T3-L1 cells were cultured in DMEM supplemented with 10% CS and 1% penicillin and streptomycin at 37°C humidified atmosphere of 5% CO_2 until they reached confluence [designated as day 0 (D0)]. The cells were differentiated in DMEM containing 10% FBS, 1 μM dexamethasone, 0.5 mM, 5μM 3-isobutyl-1-methylxanthine (IBMX) and 10 μg/mL insulin for 3 days (D0-D3), followed by treatment with 10 μg/mL insulin in DMEM containing 10% FBS for an additional 2 days (D3-D5). The cells were then maintained in DMEM containing 10% FBS until fully differentiated at day 7 (D7). Naringenin doses 5, 10, 20 μM at start of differentiation and replaced with each media change. Rosi (1 μM) was used as a positive

control. On D7, the cells were stimulated with Isoproterenol (ISO, 1 μM) or the vehicle control for 6 h for RNA or 24 h for protein samples.

3.2.3 Western blot analysis

Total cell lysates were prepared using 1X RIPA buffer (Cell Signaling, Danvers, MA) and protein concentration were determined by the BCA assay kit (Thermo Scientific, Waltham, MA). Total cell lysates were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 20 mM Tris Base, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4) containing 5% nonfat milk. The membrane was immunoblotted with primary antibodies at 4°C overnight followed by secondary antibody conjugated with horseradish peroxidase for 1 h. Proteins were visualized using ECL western blot detection reagents (Pierce, Rockford, IL). Pixel intensities of immunoreactive bands were quantified using ChemiDocXRS+ imaging system with ImageLab software (Bio-Rad).

3.2.4 RNA, DNA preparation and semi-quantitative real-time PCR analysis

Total RNA was prepared using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Total RNA abundance was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was carried out using High capacity cDNA Reverse Transcription kit (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's instructions. mRNA expression of target genes and the housekeeping gene 36B4 was measured semi-quantitatively using Power Up SYBR master mix (Applied Biosystems, Austin TX). PCR reactions were run in a 96-well format using an ABI 7300HT instrument. Cycle conditions were 50°C 2 min, 95°C 10 min and

then 40 cycles of 95°C for 15 s, 60°C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, which normalizes with house keeping gene 36B4.

DNA was prepared using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions and total DNA concentration was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). COX II as well as 18S control gene were measured semi-quantitatively using Power Up SYBR master mix (Applied Biosystems, Austin TX). PCR reactions were run in a 96-well format using an ABI 7300HT instrument. Cycle conditions were 50°C 2 min, 95°C 10 min and then 40 cycles of 95°C for 15 s, 60°C for 1 min. Relative DNA content was calculated using the $2^{-\Delta\Delta Ct}$ method, which normalizes with 18s ribosomal DNA.

3.2.5 Statistical analysis

Data for mRNA and DNA experiments are presented as mean ± SEM. Measurements were performed in triplicates. Statistical analysis was performed using SigmaPlot 13.0 (Systat Software). One-way ANOVA followed by multiple comparisons test (Student-Newman-Keuls method) was performed to determine the differences of group mean between the treatment groups. The level of significance was set at $P<0.05$.

3.3 Results

3.3.1 Naringenin dose-dependently increases PGC-1α mRNA in isoproterenol stimulated 3T3-L1 adipocytes.

To demonstrate the effects of naringenin on white adipocytes, 3T3-L1 preadipocytes were differentiated in the presence or absence of naringenin (5, 10, 20 μM) for 7 days and then stimulated with isoproterenol (ISO) or the vehicle control for 6 hours to induce thermogenic

activation. Rosiglitazone was included as a positive control. At basal (non-ISO stimulated) conditions, thermogenic markers PGC-1α and UCP-1 were not increased by naringenin (Fig. 1A). However, under ISO stimulation naringenin dose-dependently increased UCP-1 and PGC-1α mRNA ($p<0.05$) (Fig. 1B). PPARγ showed no significant differences among naringenin doses, suggesting no differences in differentiation among treatments. Mitochondrial marker Nrf-1 and Tfam showed modest increases that were not significant under basal conditions. Under ISO stimulation, Nrf-1 was significantly increased by naringenin at 10 μM. SIRT1 had no significant differences for naringenin treatments under basal or ISO stimulated conditions.

Beige markers Tmem26 and CD137 showed no significant differences among naringenin treatments under basal conditions. However, Tmem26 was significantly decreased by naringenin under ISO stimulated conditions. No differences were shown in CD137 mRNA levels by naringenin. Brown adipocyte marker Meox-2 was significantly increased under basal conditions by naringenin at 5 and 10 μM; however, Meox-2 was significantly decreased when cells were stimulated by ISO with the exception of the positive control Rosi, which was increased by ISO (Fig. 1B).

3.3.2 Naringenin dose-dependently enhances UCP-1 and PGC-1α protein expression in ISO-stimulated 3T3-L1 adipocytes.

To determine if the increases in PGC-1α and UCP-1 mRNA lead to increases in protein expression we collected protein samples in a parallel experiment. 3T3-L1 cells were differentiated for 7 days with naringenin (5, 10, 20 μM) and then stimulated with or without ISO for 24 hours. Dose-dependent increases were seen under both basal and ISO stimulated conditions (Fig 2).

3.3.3 Naringenin increases the ratio of mitochondrial COXII/ nuclear 18S DNA in 3T3-L1 adipocytes.

To provide further evidence for naringenin's effects on PGC-1α, we compared mitochondrial DNA to the nuclear DNA by measuring a ratio of COX II (cytochrome c oxidase subunit II, a mitochondrial DNA coded gene) over 18S, (a nuclear DNA coded gene). At basal conditions, naringenin dose-dependently increased the ratio of COXII/18S and reached significance when used at 20 μM ($p<0.05$). As expected, the positive control Rosi significantly increased COXII/18S ($p<0.01$). (Fig. 3). This is indicative of increased mitochondrial DNA copy number and therefore increased mitochondrial biogenesis.

3.4 Discussion and Conclusions

Targeting beige adipocytes to increase thermogenesis and energy expenditure is a novel approach for obesity treatment and prevention. [25, 105] Naringenin, a citrus flavanone, has been previously shown to have beneficial effects on lipid metabolism and inflammation. We report that naringenin dose-dependently enhanced ISO-stimulated UCP-1 and PGC-1α mRNA expression. Consistently, naringenin dose-dependently increases UCP-1 and PGC-1α protein expression under both basal (non-ISO stimulated) and ISO stimulated conditions. Furthermore, at basal condition naringenin at 20 μM significantly increased the ratio of COXII/18S. Our study demonstrates that in addition to increased UCP-1 expression, naringenin promotes up regulation of PGC-1α and mitochondrial biogenesis in thermogenic activation of 3T3-L1 adipocytes.

To our knowledge we are the first to report that naringenin at a physiologically achievable dose, can enhance PGC-1α expression and increased mitochondrial DNA to nuclear DNA ratio in 3T3-L1 cells. It is conceivable that increased UCP-1expression under the ISO-

stimulated condition by naringenin, may be due to naringenin's ability to up-regulate PGC-1α expression as it is known that PGC-1α coactivates PPARγ on the UCP-1 promoter to increase UCP-1 transcription. Consistently, we show naringenin dose-dependently increased mitochondrial DNA as evidenced by the increased ratio of COXII/18S. Taken together, we demonstrate that naringenin promotes up regulation of PGC-1α, leading to increased mitochondrial biogenesis. These results suggest that naringenin may be beneficial for chronic conditions in which mitochondrial dysfunction is implicated, such as diabetes, endocrine dysfunction and Alzheimer's diseases.[106, 107]

We utilized Rosi, a known browning agent, as a positive control. We show that in addition to increased UCP-1 and PGC-1α expression, Rosi also significantly increased beige marker CD137 (under ISO-stimulated condition) and a novel brown marker Meox-2 (under both basal and ISO-stimulated conditions). Interestingly, Rosi seemed to suppress rather than to increase Tmem26 mRNA, another known beige marker. Wu etal found that Tmem26 was increased in beige culture except for one human sample, which showed a decrease in Tmem26.[25] However, our finding that Tmem26 was decreased by Rosi treatment was consistent with the findings by deJong and Garcia.[42, 43] While our data does not definitively show a beige phenotype by naringenin treatment, our finding of Tmem26 expression is consistent with other studies in thermogenic activated cells. This discrepancy suggests the need for a better understanding of beige cell origin and beige marker genes.

In conclusion, we have shown that naringenin dose-dependently increases PGC-1α expression and consequently mitochondrial biogenesis in ISO-stimulated 3T3-L1 adipocytes. Since PGC-1α is a major regulator of mitochondrial biogenesis and oxidative metabolism, future

studies of naringenin on diabetes, endocrine dysfunction and other chronic diseases in which mitochondrial dysfunction is implicated are warranted.

CHAPTER IV
INDOMETHACIN INDUCES BROWN ADIPOCYTE DIFFERENTIATION

4.1. Introduction

As obesity continues to persist in the US population and across the world, new interventions are needed to treat those currently impacted and prevent further spread of the disease. While lifestyle changes including diet, physical activity and behavior change can be used for treatment, they take a longer duration to implement and show slower improvements. Pharmacological agents that target adipose tissue have been used, but have dwindled due to harmful side effects, such as congestive heart failure by Rosiglitazone.[108] Other weight loss agents, such as the lipase inhibitor Orlistat, lead to undesirable malabsorption of lipids and lipid soluble vitamins.[109] Invasive interventions such as bariatric surgery are used but require major surgery, which can lead to post-surgery complications.

Indomethacin (INDO) is an FDA approved NSAID that is currently used to treat fever, pain and swelling related to inflammation. It acts as a non-selective COX inhibitor to inhibit prostaglandin synthesis.[95] Early research demonstrated that INDO activates PPARγ, a ligand-activated transcription factor playing a critical role in adipogenesis and promoted white adipocyte differentiation of a mesenchymal stem cell line C3H10T1/2.[110] Moreover, supplementation of INDO (HF/HS+INDO) prevented high fat/high sucrose-induced (HF/HS) weight gain, white fat increases and insulin resistance in obesity prone C57BL/6J mice.[16] White and brown differentiation share general characteristics and signaling pathways; therefore, we aim to study INDO on brown adipocyte differentiation. We modified our brown differentiation protocol to include insulin and T_3 but without the induction by dexamethasone and IBMX to decrease the basal differentiation and more clearly delineate INDO's effects.

4.2 Materials and Methods

4.2.1 Reagents

Indomethacin, Insulin, T_3, rosiglitazone (Rosi), and dimethyl sulfoxide (DMSO) are from Sigma Aldrich (St. Louis, MO). Primary antibodies for UCP-1 were from Sigma Aldrich (St. Louis, MO), PGC-1α from Millipore (Temecula, CA), ERK1/2 and horseradish peroxidase-conjugated goat anti-rabbit was from Cell Signaling Technology (Danvers, MA).

4.2.2 Cell culture and treatment

The murine brown fat cell line is a gift from Dr. Johannes Klein (University of Lubeck, Lubeck, Germany), who has generated the cell line from interscapular brown fat of newborn C57BL/6 mice (Klein, 2002). Brown fat cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) at 37 °C humidified atmosphere of 5 % CO2 in air, until they reached 100% confluence (designated as day 0). The cells were then induced to differentiate by treatment with differentiation media containing DMEM supplemented with 20% FBS, 1 nM T3, and 20 nM insulin every 2 days until day 4. To study the effects of INDO promoting differentiation in brown adipocytes, INDO (2, 5, 10, 20, 50 μM) or the vehicle control DMSO was added at D0 and replaced with each change of the media during the differentiation process.

4.2.3 Western blot analysis

Total cell lysates were prepared using 1X lysis buffer (Cell Signaling, Danvers, MA) and protein concentration were determined by the BCA assay kit (Thermo Scientific, Waltham, MA). Total cell lysates were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 20 mM Tris Base, 137 mM

NaCl, and 0.1% Tween 20 (pH 7.4) containing 5% nonfat milk. The membrane was immunoblotted with primary antibodies at 4°C overnight followed by secondary antibody conjugated with horseradish peroxidase for 1 h. Proteins were visualized using ECL western blot detection reagent (Pierce, Rockford, IL). Pixel intensities of immunoreactive bands were quantified using ChemiDocXRS+ imaging system with ImageLab software (Bio-Rad).

4.2.4 RNA, DNA preparation and semi-quantitative real-time PCR analysis

Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Total RNA was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was carried out using High capacity cDNA Reverse Transcription kit (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's instructions. mRNA expression of target genes and the housekeeping gene 36B4 was measured quantitatively using Power Up SYBR master mix (Applied Biosystems, Austin TX). PCR reactions were run in a 96-well format using an ABI 7300HT instrument. Cycle conditions were 50°C 2 min, 95°C 10 min and then 40 cycles of 95°C for 15 s, 60°C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, which normalized by house keeping gene 36B4.

DNA was prepared using TRI reagent according to the manufacturer's instructions and total DNA concentration was quantified using NanoDrop ND-1000 spectrophotometer. COX II as well as 18S control gene were measured quantitatively using Power Up SYBR master mix. PCR reactions were run in a 96-well format using an ABI 7300HT instrument. Cycle conditions were 50°C 2 min, 95°C 10 min and then 40 cycles of 95°C for 15 s, 60°C for 1 min. Relative DNA content was calculated using the $2^{-\Delta\Delta Ct}$ method, which normalized by 18S DNA.

4.2.5 Reporter gene assays

Brown preadipocytes seeded in 48-well plates were transiently transfected with PPRE-Luc reporters (PPRE X3-TK-luc was a gift from Bruce Spiegelman, Addgene plasmid # 1015) and β-galactosidase expression plasmid with Lipofectamine 2000 transfection reagent and Plus reagent (Thermo Fisher Scientific, Carlsbad, CA). Twenty-four hours post transfection the cells were then treated with INDO or the vehicle control DMSO for 18 h. The cell lysate was prepared and reporter luciferase and β-galactosidase activities were measured with GloMax Luminometer (Promega, Madison, WI). Relative luciferase activities were normalized by β-galactosidase activities.

4.2.6 Statistical analysis

Data for ORO, mRNA, DNA and reporter experiments are presented as mean ± SEM. Measurements were performed in triplicates. Statistical analysis was performed using SigmaPlot 13.0 (Systat Software). One-way ANOVA followed by multiple comparisons test (Student-Newman-Keuls method) was performed to determine the differences of group mean between the treatment groups. The level of significance was set at $P<0.05$.

4.3. Results

4.3.1 Indomethacin increases brown adipocytes differentiation as measured by Oil Red O stained cell morphology and absorbance.

After 6 days of differentiation, brown preadipocytes show morphological changes that include multilocular lipid formation, consistent with mature brown adipocytes. INDO dose-dependently increased lipid accumulation with significant increases at 20 and 50 μM ($p<0.05$) (Fig. 4), demonstrating its positive effects on brown adipocyte differentiation.

4.3.2 Indomethacin dose-dependently increases mRNA and protein expression of PGC-1α and UCP-1 in brown adipocytes.

To investigate the effect of INDO on brown adipocyte differentiation, brown preadipocytes were differentiated as described in the materials and methods section. INDO dose-dependently increased PPARγ mRNA and reached statistical significance from 5 to 50 μM ($p<0.05$) (Fig. 5). Moreover, INDO dose-dependently increased PGC-1α mRNA ($p<0.05$) (Fig. 5). Treatment with INDO at a dose of 10 μM or higher resulted in a significant increase in UCP-1 mRNA levels ($p<0.05$). Additionally, naringenin dose-dependently increased brown marker PRDM16 ($p<0.05$). Mitochondrial markers Tfam, and Nrf-1 were not significantly increased by INDO (Fig. 5). Furthermore, we show that INDO increased protein expression of PGC-1α and UCP-1 (Fig. 6), consistent with the changes in mRNA expression.

4.3.3 Indomethacin increases mitochondrial DNA in brown adipocytes.

To provide more evidence for indomethacin's induction of brown adipocyte differentiation, we isolated mitochondrial DNA and measured the ratio of mitochondrial COX II (cytochrome c oxidase subunit II) over 18S nuclear DNA. Significant increases in mitochondrial DNA/nuclear DNA were seen when cells were treated with INDO at a dose of 20 μM or 50μM ($p<0.001$) (Fig. 7).

4.3.4 Indomethacin activates PPRE reporter in brown preadipocytes.

INDO has previously been shown to bind and activate PPARγ. To explore the molecular mechanisms by which INDO promoted brown adipocyte differentiation, the PPAR responsive reporter gene assay (PPRE-Luc) was performed**.** INDO significantly increased PPAR responsive

luciferase reporter activity at 20 μM in brown preadipocytes, suggesting an increase in PPARγ activity ($p<0.05$) (Fig. 8). Rosiglitazone, a known PPARγ ligand, was used as a positive control.

4.4. Discussion and Conclusions

Increasing functional brown adipose activity has become a novel target for obesity treatment and prevention. We show here that indomethacin, a commonly used cyclooxygenase inhibitor dose-dependently promotes brown adipocyte differentiation, as revealed by increased lipid accumulation, increased expression of brown marker genes and increased mitochondrial biogenesis. We further demonstrate that INDO dose-dependently activates PPAR responsive reporter activities, consistent with a previous reports.[98] These results suggest that INDO promotes brown adipocyte differentiation possibly through activation of PPARγ. More research is needed to determine the role of other transcription factors that bind to the PPRE site such as PPARα.

To our knowledge, this is the first time to demonstrate that INDO is capable of inducing brown adipocyte differentiation in the absence of dexamethasone and IBMX. PPARγ signaling is considered to be critical for both white and brown adipocyte differentiation.[31] A synthetic PPARγ agonist rosiglitazone has been demonstrated to be a "browning" agent inducing formation of brown-like adipocytes in certain white fat depot.[44] Similarly, the finding that UCP-1 mRNA in inguinal WAT was higher in INDO treated (HF/HS+INDO) mice compared to the controls (HF/HS) suggests that INDO may promote browning in vivo. It was reported that INDO increased UCP-1 expression in the interscapular brown adipose tissue (iBAT) in INDO treated mice (HF/HS+INDO) compared to the controls (HF/HS); however, it was not significant, possibly due to the higher stimulation of UCP-1 mRNA by high fat/high sucrose diet.[16] It has been suggested that the ability of PPARγ agonists to induce brown differentiation and browning

are related to their abilities to induce expression of PGC-1α.[55, 111] We have shown that INDO dose-dependently increased both mRNA and protein expression of PGC-1α. Further studies are needed to define the role of PGC-1α in INDO's effects on brown adipocyte differentiation.

In conclusion, we demonstrate for the first time that INDO, a commonly used NSAID and COX inhibitor, dose-dependently promotes brown adipocyte differentiation in the absence of dexamethasone and IBMX. In light of the effects of INDO on white adipocyte differentiation and other detrimental effects on liver, pancreatic β-cells, and gastric integrity, targeted delivery of INDO to brown fat depot during development may be further explored to increase brown adipose tissue mass/function for obesity treatment and prevention in the future.

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APPENDIX

Figure 1. Effect of naringenin on mRNA expression of thermogenic, beige and brown markers with and without isoproterenol stimulation.
3T3-L1 preadipocytes were induced to differentiate in the presence or absence of increasing doses of naringenin (5, 10, 20 μM) for 7 days. On day 7, matured 3T3-L1 adipocytes were treated with isoproterenol (ISO) for 6 hours or the vehicle control DMSO. Target gene expression was normalized by 36B4 and relative fold changes are compared to the DMSO control. (A) mRNA expression without ISO stimulation. (B) mRNA expression with ISO stimulation. Rosiglitazone was used as a positive control. Data= mean± SEM (n=3). ***, $p<0.001$; **, $p<0.01$;*, $p<0.05$ compared to the DMSO group.

A

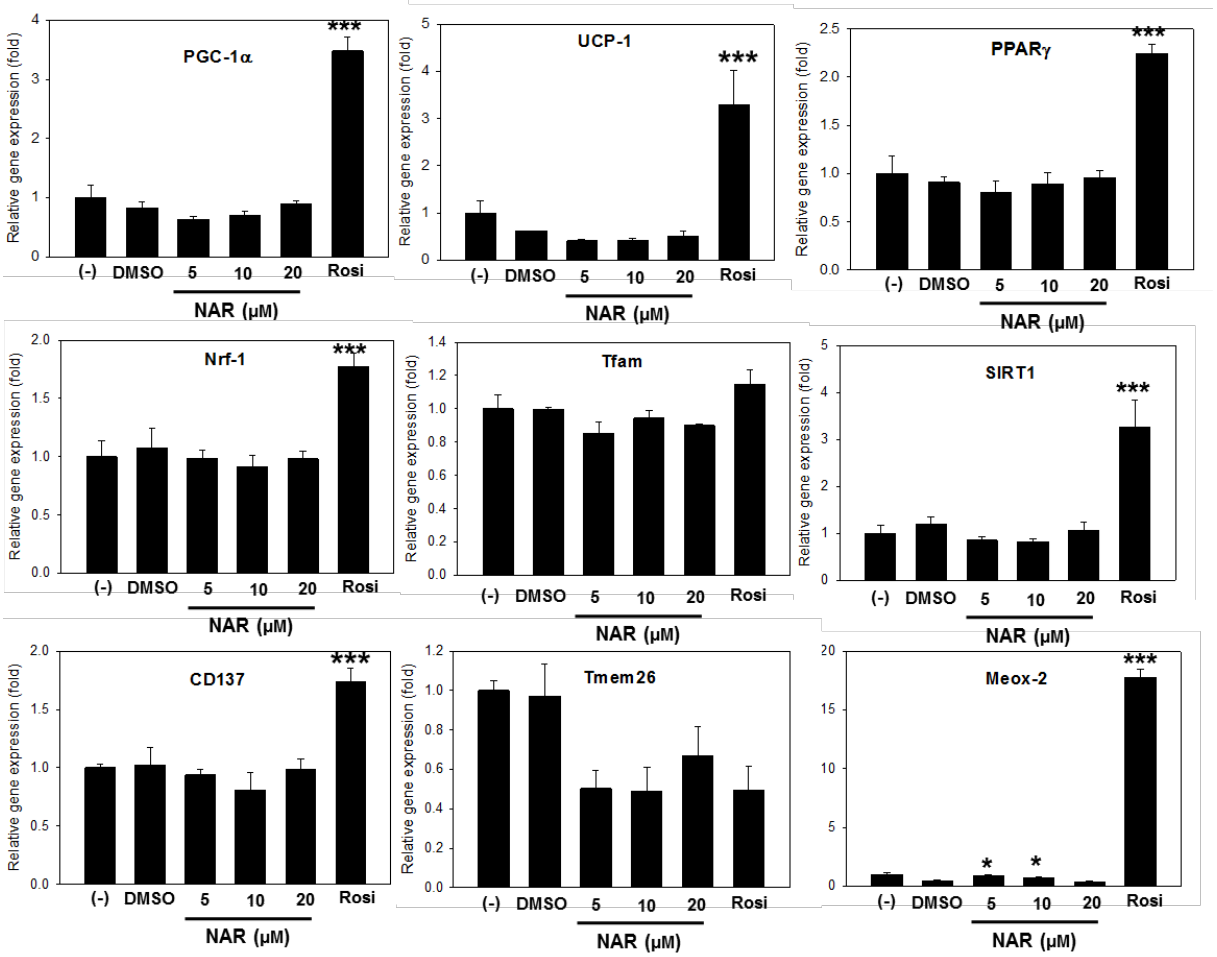

Figure 1. Continued

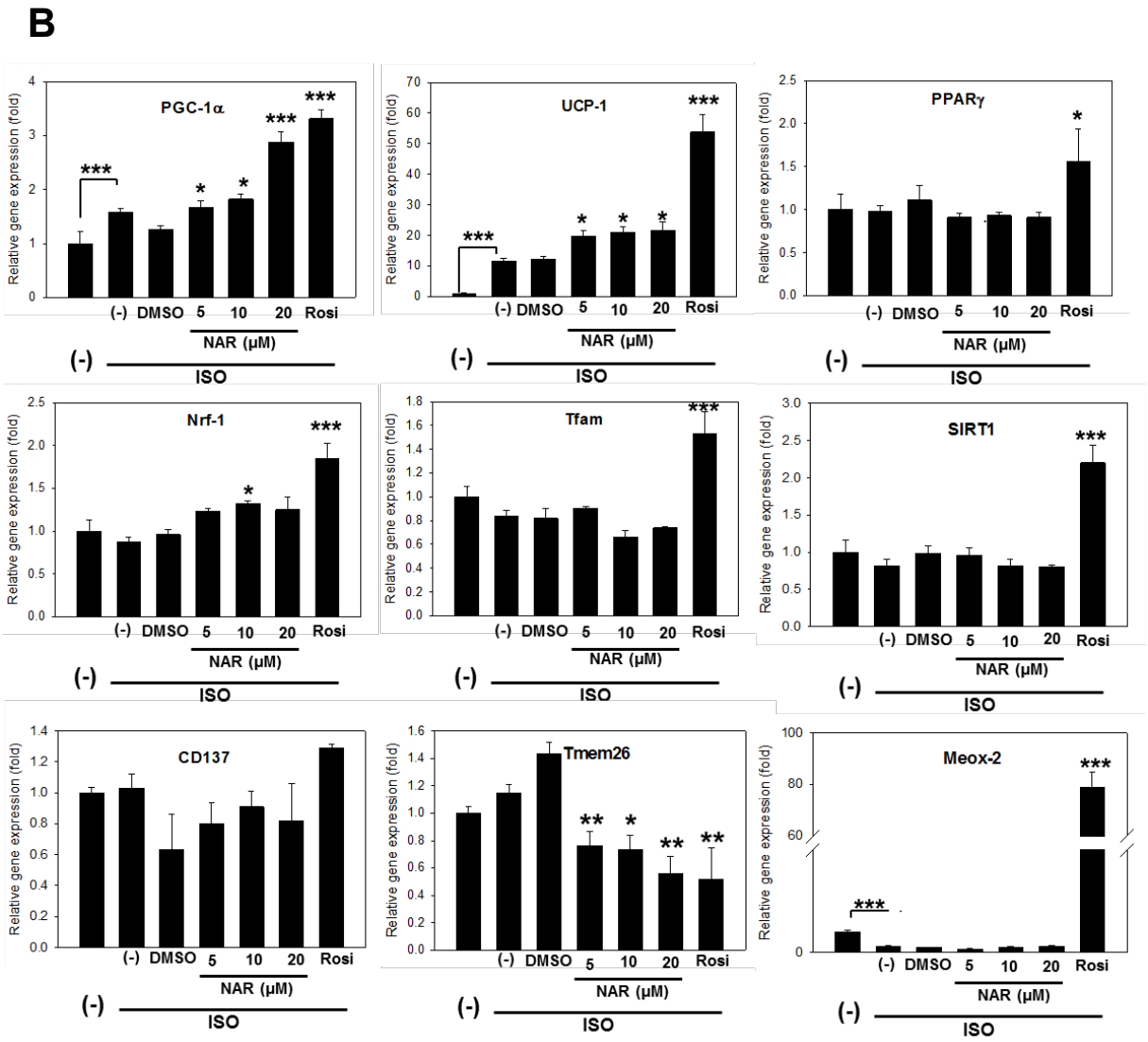

Figure 1. Continued

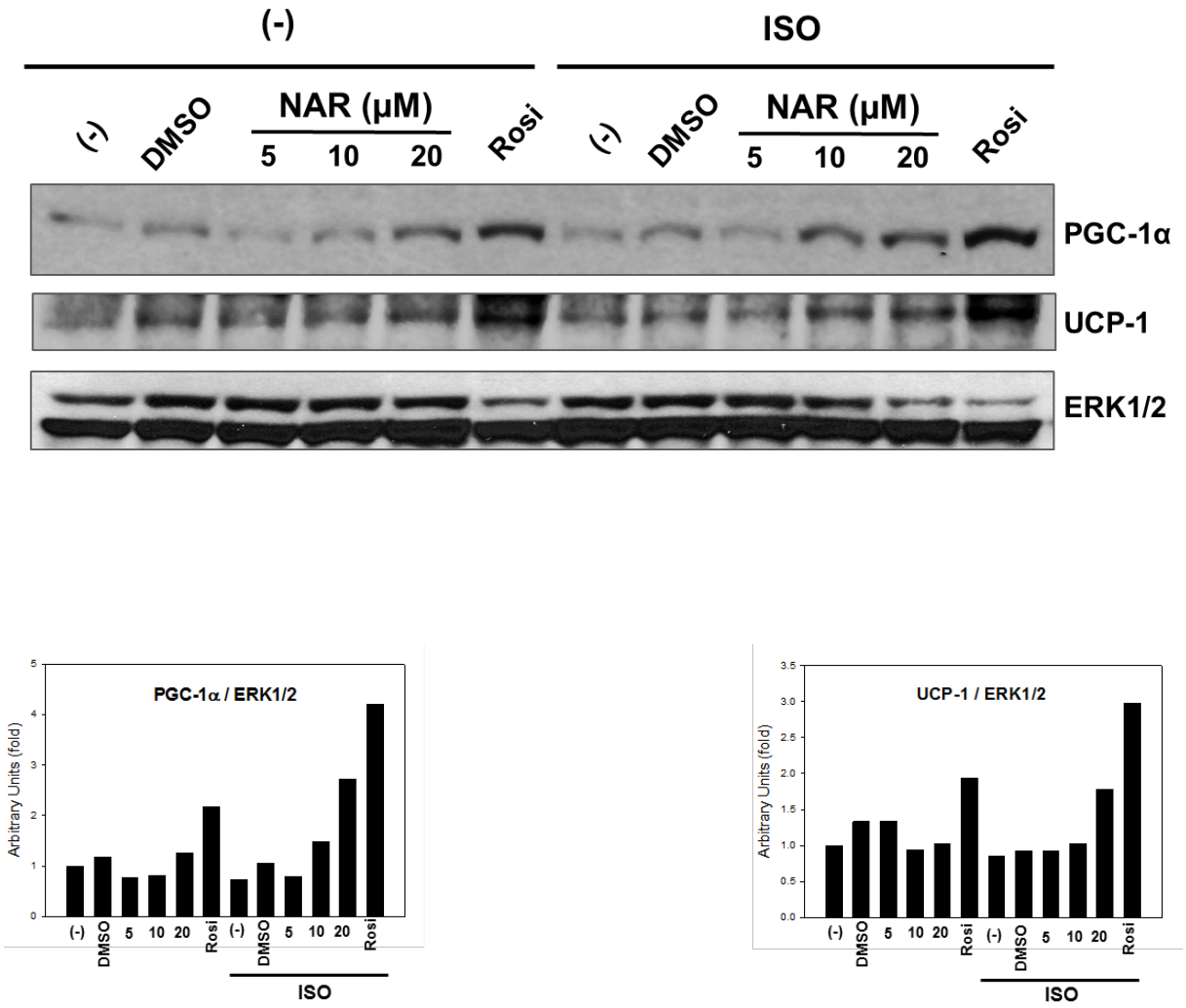

Figure 2. Effect of naringenin on PGC-1α and UCP-1 protein expression.
3T3-L1 cells were differentiated with increasing doses of naringenin (5, 10, 20 μM) until day 7 when they were treated with isoproterenol for 24 hours. Total cell lysates were prepared and analyzed by western blot. Densitometry was determined using ChemiDocXRS+ imaging system with ImageLab software. ERK1/2 was utilized as a loading control.

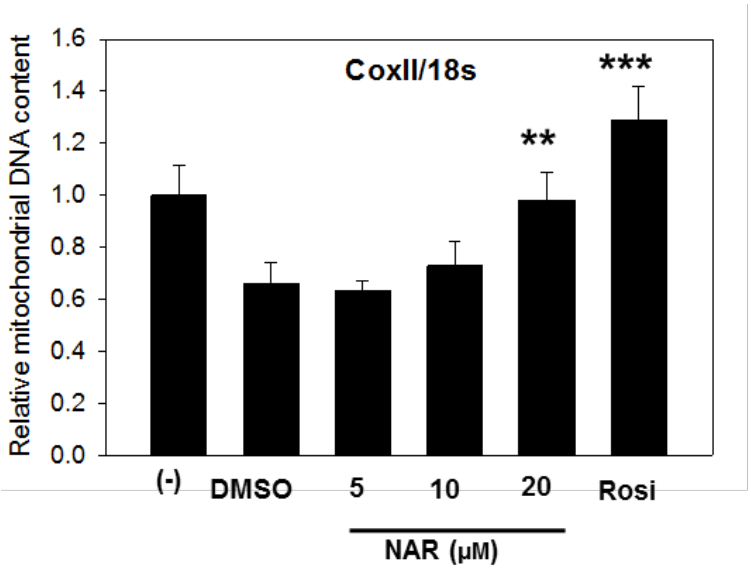

Figure 3. Naringenin increases the ratio of mitochondrial DNA marker CoxII over 18S DNA.

3T3-L1 cells were differentiated for 7 days in the presence or absence of Naringenin (5, 10, 20 μM). Total DNA was extracted and mitochondrial DNA content was quantified by the ratio of CoxII to 18S DNA. Relative changes are expressed as fold changes and compared to the DMSO control. Data= mean± SEM (n=3). **, ***, $p<0.01$ and $p<0.001$, respectively, compared to the DMSO group.

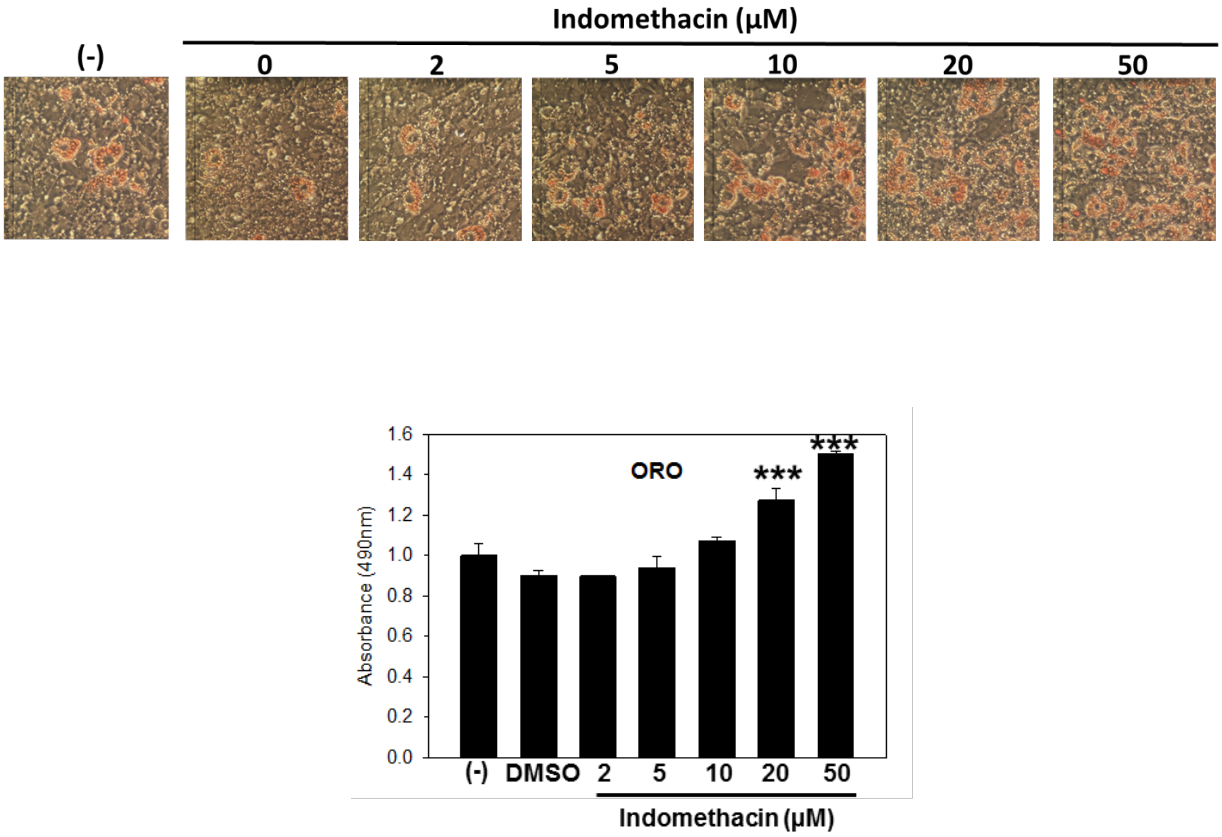

Figure 4. Indomethacin dose-dependently induces brown adipocyte differentiation. (A) Oil red O stained cell morphology at day 6 (B) Lipid accumulation measured by oil red O absorbance. Negative control cells were set at fold 1. Data= mean± SEM (n=3). Brown preadipocytes were differentiated in the presence or absence of increasing doses of INDO (2, 5, 10, 20, 50 μM) until day 6. ***, $p<0.001$ compared to the DMSO group.

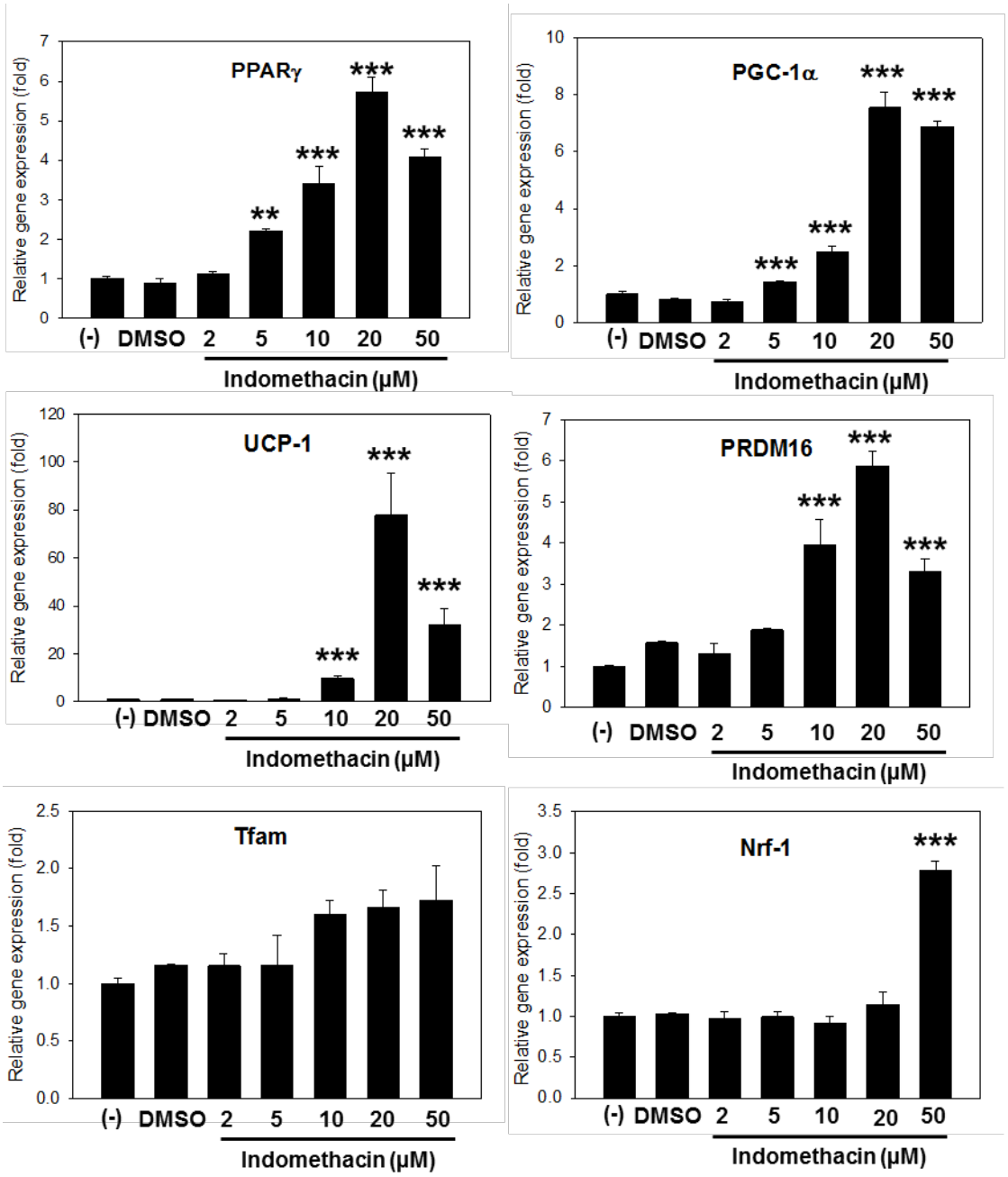

Figure 5. Indomethacin dose-dependently increases mRNA expression of brown adipocyte marker genes.
Brown preadipocytes were induced to differentiation in the presence or absence of increasing doses of INDO (2, 5, 10, 20, 50 μM) for 4 days. Target gene expression was normalized by 36B4 and relative fold changes are compared to DMSO control. Data= mean± SEM (n=3). ***, $p<0.001$; **, $p<0.01$;*, $p<0.05$ compared to the DMSO group.

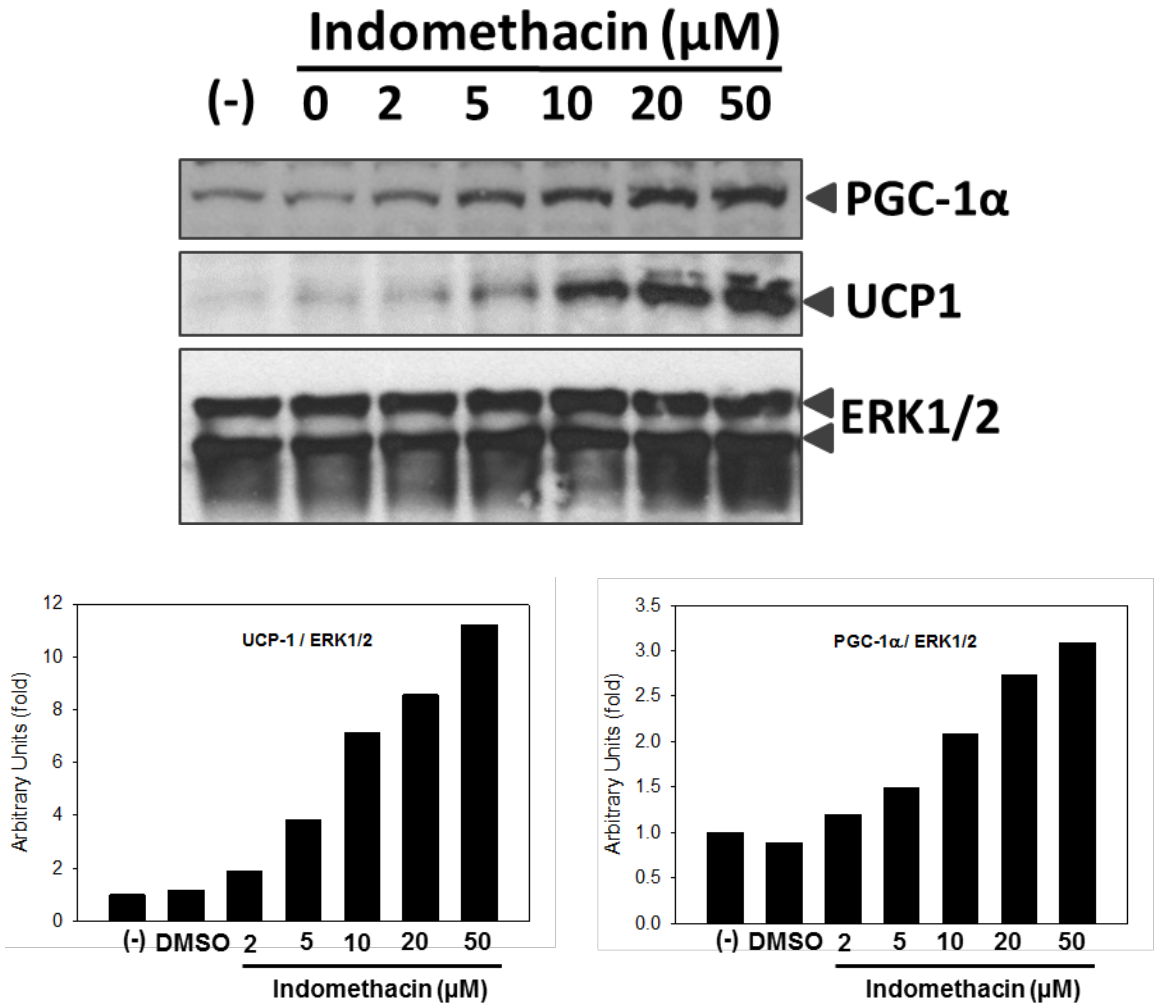

Figure 6. Indomethacin dose-dependently increases PGC-1α and UCP-1 protein expression.
3T3-L1 cells were differentiated in the presence or absence of increasing doses of INDO (2, 5, 10, 20, 50 μM) until day 4. Total cell lysates were prepared and analyzed using western blot (A). (B) Densitometry was determined using ChemiDocXRS+ imaging system with ImageLab software. ERK1/2 was utilized as a loading control.

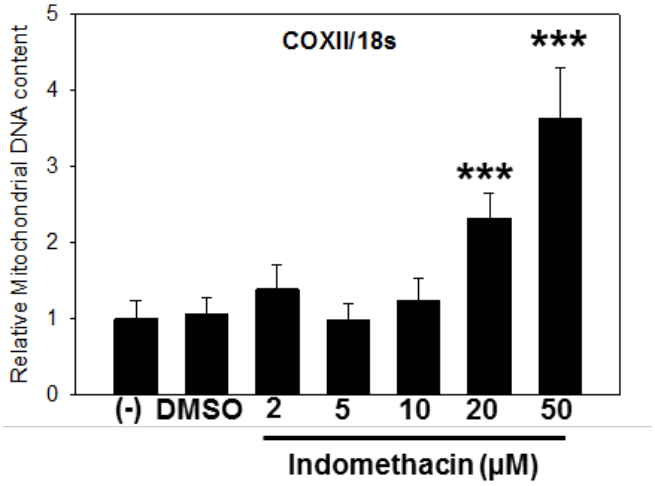

Figure 7. Indomethacin dose-dependently increases ratio of mitochondrial DNA marker CoxII over18s DNA.
Brown preadipocytes were differentiated for 4 days in the presence or absence of INDO (2, 5, 10, 20, 50 μM). Total DNA was extracted. Mitochondrial DNA content was quantified by the ratio of COXII to 18s ribosomal DNA. Relative changes are expressed as fold changes and compared to the DMSO control. Data= mean± SEM (n=3). ***, $p<0.001$ compared to the DMSO group.

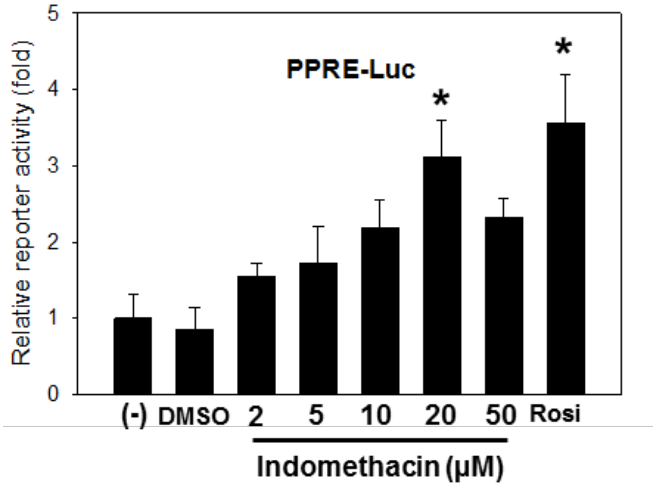

Figure 8. Indomethacin activates PPRE-Luc luciferase activity in brown preadipocytes.
Brown preadipocytes were transfected with PPRE-Luc plasmid and treated for 18 hours with or without INDO (2, 5, 10, 20, 50 μM). Luciferase activity was measured and normalized by β-galactosidase activity. Relative changes are expressed as fold changes and compared to the negative control (-). Data= mean± SEM (n=3). *, $p<0.05$ compared to the DMSO group.

VITA

Jamie Ann Kearns was born to Sheila Simpson Kearns and Warren Kearns in Torrington, Wyoming where she completed high school. She then attended the University of Wyoming graduating in 2014 earning her Bachelor of Science in Family Consumer Sciences with a concentration in Dietetics. While enrolled she worked as a nutrition aide for UW Dining services and participated in animal research. Upon graduation, she moved to Knoxville, Tennessee to complete a combined Masters/Dietetic Intern program at the University of Tennessee, Knoxville. During the program, she completed her thesis research under Dr. Ling Zhao for the Cellular and Molecular Nutrition concentration. She completed the dietetic internship with a clinical focus at several area hospitals. While at UTK she was also employed as a graduate assistant in the small animal facility. She defended her thesis on November 3rd, 2016. She intends to sit for the Commission of Dietetic Registration exam and has already accepted a position as a clinical dietitian at Rapid City Regional Hospital in South Dakota.