THE ROLE OF ASTAXANTHIN IN ADIPOCYTE BIOLOGY

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Abstract

Adipose tissues play a critical role in the regulation of systemic health. Astaxanthin (Ast), a natural antioxidant and anti-inflammatory compound, has been suggested to influence adipose tissue health. However, its role in regulation of proliferation of human adipose-derived stem cells (hASCs) and their differentiation into adipocytes is not well understood. This thesis examined how Ast affected hASC proliferation, adipogenic differentiation, and underlying signaling pathways. We found that Ast alone did not exhibit any effects on hASC proliferation or pro-inflammatory signaling activities. However, Astaxanthin (Ast) blocked the actions of TNFα, a pro-inflammatory cytokine increased in obesity, thereby rescuing hASCs from its inhibition of proliferation and induction of proinflammatory signaling activity. At low doses (0.01 – 0.1 μM), Ast promoted adipogenesis in hASCs, indicated by upregulation of adipogenic protein markers and lipid accumulation. We showed that Ast enhanced adipogenesis through suppression of the anti-adipogenic Wnt/β-catenin signaling pathway, which led to the induction of key adipogenic transcription factors, C/EBPα and PPARγ. However, higher doses of Ast (2 – 10 μM) reduced adipogenesis and exhibited possible cytotoxicity. Our data demonstrated that physiologically achievable levels of Ast not only blocked TNFα-suppression of proliferation in hASCs but also enhanced their differentiation into adipocytes. These findings suggest that Ast may support hyperplastic growth of adipose tissues, which could ameliorate adipose tissue dysfunctions in obesity, providing protective effects against obesity-associated cardiometabolic disorders.

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List of Abbreviations

- Ast – Astaxanthin
- ASC – Adipose stem cells
- hASCs – Human adipose stem cells
- CMDs – Cardiometabolic diseases
- CEBPα – CCAAT/enhancer-binding protein alpha
- PPARγ – Peroxisome proliferating-activated receptor gamma
- LPL – Lipoprotein lipase
- PLIN1 – Perilipin 1
- ADPN – Adiponectin
- NSCs – Neural stem cells
- MSCs – Mesenchymal stem cells
- TNFα – Tumor necrosis factor-alpha
- IL – Interleukin
- qPCR – Quantitative real-time polymerase chain reaction
- NRF2 – Nuclear factor erythroid 2-related factor 2
- NF-κB – Nuclear factor kappa B
- MAPK – Mitogen activated protein kinase
- ROS – Reactive oxygen species
- SOD – Superoxide dismutase
- GPX – Glutathione peroxidase
- MDA – Malondialdehyde

Chapter 1: Introduction

Cardiometabolic diseases and obesity

Cardiometabolic diseases (CMDs) such as obesity, severe obesity, dyslipidemia, hypertension, prediabetes, diabetes, chronic kidney diseases, nonalcoholic fatty liver disease, and metabolic syndrome, are an interconnected group of conditions that are associated with cardiovascular system and metabolism (1). CMDs have been increasing in the past years as the major cause of morbidity and mortality around the world (2,3). Oxidative stress and increased inflammation are markers of CMDs, which are observed in adipose tissues, liver, pancreas and intestinal lining (4).

Obesity is a significant contributing factor in CMDs and it is also defined as a characteristic of CMDs (5). Obesity occurs from excess accumulation of fat that is beyond acceptable range that results from impairment in energy balance mechanisms in the body. While dietary intake is a major contributor, other physiological mechanisms also play roles in the pathogenesis of obesity and its associated diseases (6).

Adipose tissues

Adipose tissues are metabolically active endocrine organs and play crucial roles in maintaining systemic health. Multiple cell types are present in adipose tissues, including adipocytes, adipose stem cells (ASCs), and various immune cells (7). Adipocytes are the major storage site of energy, where energy is stored as triacylglycerol (TAG) in lipid droplets (7). When systemic energy demands increase during fasting or exercise, TAG is hydrolyzed through lipolysis and fatty acids and glycerol are released into circulation from adipocytes, providing energy sources to other organs (8). Adipocytes and other cell types secrete peptide hormones and cytokines, which are collectively referred to as adipokines (8). With development of obesity, adipose tissue becomes dysfunctional, characterized by hypoxia, fibrosis, and inflammation. Higher mass of dysfunctional adipose tissues in obesity release elevated levels of fatty acids and proinflammatory cytokines while secreting reduced levels of adiponectin and omentin (9,10). These alterations in adipose derived metabolic and endocrine factors negatively impact functions of other metabolic organs, including liver and skeletal muscle, by inducing ectopic fat deposition, insulin resistance, and proinflammatory signaling activities, which contribute to the risk of CMDs (9).

To cope with increased energy storage, adipose tissue expands through hypertrophy (increase in cell size) and or hyperplasia (increase in cell numbers) and hyperplasia is advantageous as it helps to maintain the tissue function (10,11). Limited hyperplastic growth is associated hypertrophy of adipocytes, leading to dysfunctions in adipose tissues (12). Hyperplastic growth occurs through recruitment of ASCs and their differentiation into adipocytes.

In adipose tissues, the stromal vascular fraction constitutes of ASCs, preadipocytes committed to adipogenesis, and other cells (10). ASCs are a type of mesenchymal cells (MSCs) and have multilineage fates, including adipocytes, osteocytes and myocytes. ASCs proliferate to main their populations and several factors, including proinflammatory cytokines and oxidative stress, are known to inhibit their proliferation ((13)) **(Figure 1.1)**. ASCs are also differentiated into adipocytes through adipogenesis.

The adipogenic program

Adipogenesis is the process by which ASCs are committed into adipogenic lineage followed by differentiation into lipid laden mature adipocytes **(Figure 1.1)** (7). This transition is regulated by the complex array of transcription factors and numerous factors known to affect adipogenesis by controlling CCAAT/enhancer binding protein-alpha (C/EBPα) and peroxisome proliferator–activated receptor-gamma γ (PPARγ). C/EBPα is commonly expressed in adipose tissues and it is part of the family of leucine zipper transcription factors that include C/EBPβ (14). Upon adipogenic induction, C/EBPβ is temporally increased leading to stimulation of C/EBPα and PPARγ expression (15). C/EBPα itself cannot facilitate adipogenesis without PPARγ, but it is necessary to maintain expression levels PPARγ, the master regulator of adipogenesis. Two isoforms of PPARγ, PPARγ1 and PPARγ2, are generated through alternative splicing and the γ2 isoform is more specifically expressed in adipose tissues and is known to be a more potent pro-adipogenic isoform than γ1 (16). PPARγ is the adipogenic transcription factor that induce expression of adipocyte-specific proteins, including fatty acid binding protein 4 (*aP2*), adiponectin (ADPN), leptin, lipoprotein lipase (LPL) and perilipin 1 (PLIN1) (17,18). Leptin is a satiety hormone that regulates energy intake and expenditure and ADPN is an adipokine that promotes insulin sensitivity (19). PLIN1 is a lipid droplet-associated protein that regulates both TAG synthesis and lipolysis.

Several pathways are involved in the adipogenic commitment step, including the hedgehog, tumor growth factor-beta, and Wnt/β-catenin signaling pathways (14). The Wnt/β-catenin signaling pathway inhibits adipogenic differentiation while promoting osteogenesis (20,21). When the Wnt/β-catenin is activated, key adipogenic transcription factors, CEBPα and PPARγ, are downregulated, thereby maintaining ASCs in an undifferentiated state (20,22). Multiple Wnt family, including Wnt3, Wnt5, and Wnt10b downregulated C/EBPα and PPARγ and promotes osteogenic genes in MSCs (12,21,23). These indicate that the Wnt/ β-catenin signaling plays biphasic roles in lineage commitment of MSCs, reducing adipogenic commitment while promoting osteogenic commitment.

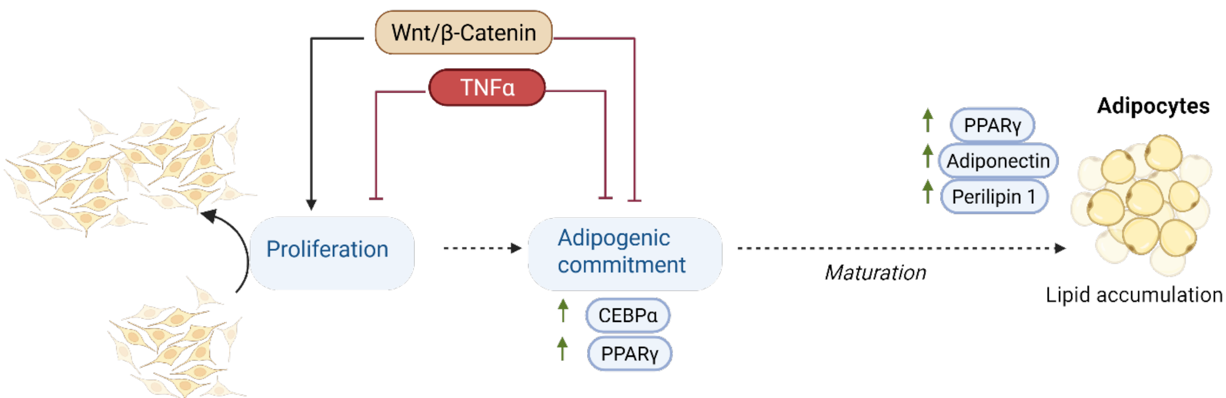


Figure 1.1. Regulation of proliferation of adipose progenitors and their differentiation into adipocytes.

Astaxanthin

Astaxanthin (Ast) has the chemical formula of $C_{40}H_{52}O_4$ and a molecular weight of 596.841 g/mol. It is a fat-soluble xanthophyll and an oxygenated carotenoid. Astaxanthin is abundantly sourced from the microalga *Haematococcus pluvialis* (*H. pluvialis*). Other sources include *Chlorella zofingiensis* and *Phaffia rhodozyma* (24). *H. pluvalis* produces Ast under high stress conditions such as high salt/nitrogen deficiency or low light intensity/ high temperature as a form of response (24). The microalgae are often fed upon by aquatic creatures such as salmon, trout, shrimp, and crabs, and Ast cumulates in their bodies, imparting the vibrant red-orange color (24). Humans can obtain Ast by consuming seafoods and commercially available supplements (**Figure 1.2**). Of all seafood, wild salmon is one of the rich sources of Ast providing 21.8 µg or up to 38 µg of Ast per gram of salmon (25). The Ast composition in marine organisms is influenced by multiple factors, including the primary source of Ast in their diet, their trophic level within the food chain, and their metabolic efficiency in converting precursor compounds to Ast (26). The FDA approves a dosage of 12 mg/day or 24 mg/day for up to 30 days of Ast in the form of dietary supplements (27).

Figure 1.2. Humans acquire astaxanthin through ingesting aquatic animals or supplements.

The defining feature of Ast is its central nonpolar polyene chain composed of nine conjugated double bonds that provide light-absorbing properties and thus, giving it its orange-red color. The conjugated bonds also enable Ast to act as an antioxidant by readily accepting and stabilizing high-energy electrons (24) (**Figure 1.3**). At the both ends of the polyene chain, astaxanthin has ionone rings with keto-groups and hydroxyl groups, which provide hydrophilicity. Thus, Ast has amphiphilic nature, and this unique structure allows Ast to span through the lipid bilayer of cell membranes (24,28). Ast, like other carotenoids exist in *cis-* and *trans*-isomeric forms, with the *cis*-configuration to be more stable. The carotenoid can also be esterified with one or two fatty acids (29).

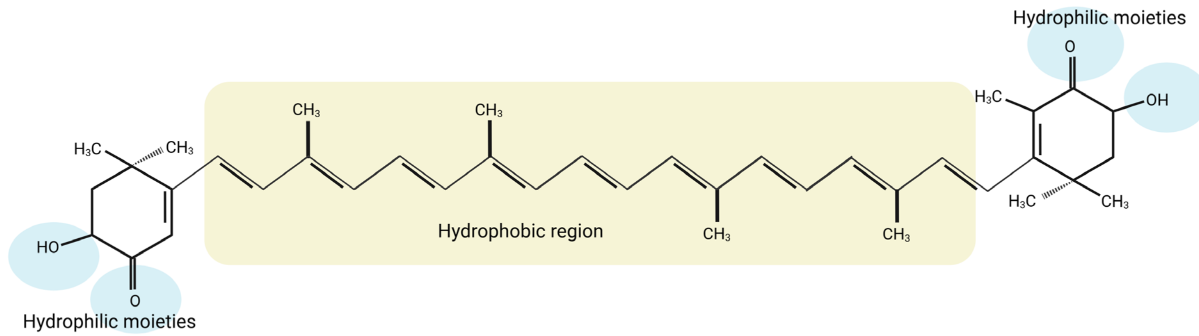


Figure 1.3. Structure of astaxanthin.

Astaxanthin, an antioxidant carotenoid

Antioxidant nutrients including carotenoids (beta-carotene, lycopene, and Ast) show promise in metabolic health by mitigating oxidative stress and inflammation, and maintaining cellular integrity (30). Ast is known to be a strong antioxidant providing protective effects against oxidative damage and oxidative stress, which is considered one of the major etiological factors in numerous degenerative diseases, including cardiometabolic diseases (29).

In vitro studies observed a protective effect of Ast in reducing reactive oxidative species (ROS) levels in human neuroblastoma cell line, MSCs, chondrocytes, and lens epithelial cells (31–34). Ast treatment increased expression genes involved in oxidative defense, including nuclear factor erythroid 2-related factor 2 (NRF2), glutathione peroxidase (GPX) 4, and superoxide dismutase (SOD) and proving protective effective against cellular cytotoxicity. Additionally, in pancreatic β-cells, Ast reduced oxidative stress, mitochondrial dysfunction, DNA damage, and apoptosis against high glucose-induced toxicity in transformed mouse cell (35).

The antioxidant effects of Ast has been tested in a variety of rodent models and Ast significantly lowered markers of oxidative stress, including malondialdehyde (MDA) and myeloperoxidase levels as well as enhanced catalase and superoxide dismutase (SOD) activity in kidneys and intestinal tissues (36–38). Ast feeding upregulated *Nrf2*, a key regulator of antioxidant responses, as well as increased the activity of antioxidant enzymes heme oxygenase-1 and GPX (39,40). Further, Ast provided protective actions against mitochondrial damage, liver injury, ischemic perfusion, and diabetes (31,40–42).

Few studies examined effects of Ast on oxidative stress and metabolic diseases in humans. Ast supplementation at doses of 11 – 13 mg/day effectively reduced oxidative stress markers, including MDA and serum isoprostanes in human clinical trials (43). Similarly, Ast supplementation to women with elevated oxidative stress revealed an increase in antioxidant capacity following 8 weeks of 12 mg/day Ast supplementation (44). Wu et al. suggested that the effectiveness of Ast in reducing oxidative stress is attributed to its ability to reduce lipid peroxidation, including MDA and isoprostanes (45).

Astaxanthin, an anti-inflammatory carotenoid

Chronic unresolved inflammation in metabolic tissues is a characteristic of CMDs and is often interconnected with oxidative stress, contributing to the pathogenesis of CMDs (46). Ast, in addition to its role as an antioxidant, also exerts anti-inflammatory effect as evidenced by in vitro studies, different rodent models, and human trials (47). In human hepatocellular carcinoma cells cultured in vitro, Ast has been shown to negatively regulate the proinflammatory signaling pathways such as $ERK_{1/2}$ mitogen activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) (48). Farrugia et al. found similar effects in cultured RAW 264.7 mouse macrophage cell lines, whereby Ast decreased interleukin (IL)-6, IL-1β and TNFα, and inhibited the NF-κB translocation to nucleus (40). Various isomers of Ast also significantly reduced TNF-α and IL-8 and other inflammatory markers, TNFα and COX-2 in Caco-2 cells (49).

The anti-inflammatory roles of Ast have been invested in rodent models. Ast supplementation of 10 mg/kg reduced expression levels of TNFα, IL-1, IL-6, and other proinflammatory markers in intestinal tissue of ischemic mice models (36). Lee at al. observed that feeding Ast (40 mg/kg) decreased inflammatory markers in peritoneal macrophages in lipopolysaccharide-induced sepsis in mice (50). Administration of Ast to high-sucrose and high-cholesterol induced hepatic steatosis model, 0.02% for 10 weeks, reduced insulin resistance and hepatic inflammation, mainly through the inhibition of MAPK and NF-κB pathways and fibrosis (51). The study also showed that the anti-inflammatory effect of Ast was more potent than vitamin E (51).

Limited number of clinical studies investigated anti-inflammatory roles of Ast and provided inconsistent findings. A systematic review and meta-analysis in 2022 found that Ast supplementation did not have significant effects on serum levels of IL-6 and TNFα (43). However, in diabetic patients, Ast reduced serum IL-6 levels, indicating its anti-inflammatory actions (43). Ast administration, 12 mg/day for 8 weeks to patients with coronary artery diseases, did not affect inflammatory markers (52)

In human and rodent models, Ast exhibited beneficial impacts on cardiometabolic health. Ast supplementation of 20 mg/day for 12 weeks supported weight management in subjects with obesity without affecting BMI and improved lipid profiles (53). Ast provided protective actions against high-fat diet induced weight gain and visceral adiposity in mouse models (54). Other rodent studies showed no differences on weight, although Ast exhibited antioxidant and anti-inflammatory effects (55,56).

Effects of Ast on adipocyte biology

Ast regulation of adipocyte biology is still a growing area of study. Ast has been shown to regulate both proliferation and adipgoenic program in various cell culture models and depending on cell types, concentrations, and experimental designs, contradictory results are reported.

Ast regulation of ASC proliferation

The regulation of adipose stem cell proliferation is essential in maintaining a pool of stem cell population. Ast treatment (2 – 10 μM) promoted proliferation and cell viability in human ASC (hASCs) (57) and a similar finding was observed in 3T3-L1 cells, a pre-adipogenic cell line, at concentrations of 2.5 and 5 μg/mL of Ast-ester (4.19 and 8.38 μM) (58). However, greater than 20 μM decreased proliferation and induced cytotoxicity in hASCs (57). Low doses (10 ng/ml, 6.8 nM) of Ast increased proliferation of neural stem cells (NSCs) (59), while another study showed that 3.7 ng/μL (6.8 nM) of Ast did not affect proliferation of neuroblastoma stem cells (SHSY-5Y) (60) (**Figure 1.4**). Additionally, Ast provided protection against oxidative stress-inhibition of ASC proliferation (57) and enhanced proliferation of ASCs derived from metabolically unhealthy horses, but not in healthy horses (57). These results suggest that depending on doses, cell types, and experimental condition, Ast has different effects on cell proliferation. Limited information is available for dose-dependent actions of Ast in proliferation of hASCs. Further, whether Ast provide protective effects against TNFα-mediated inhibition of hASCs have not been investigated.

Table 1.1 Studies on the effects of Ast on proliferation and differentiation in cells.

Studies	Cell types	Concentrations	Effects
Effects on Proliferation	hASCs (57)	**2 – 10 μM** **> 20 μM**	↑ ↓
	3T3-L1 cells (58)	2.5 & 5 μg/ml **(4.19 & 8.38 μM)**	No effect
	Human neural stem cells (59)	10 ng/ml (**6.8 nM**)	↑
	SHSY-5Y cells (60)	3.7 ng/μL (**5.61 μM**)	↓
Effects on Adipogenesis	3T3-L1 cells (58)	2.5 & 5 μg/ml (**4.19 & 8.38 μM**)	↓
	Human neural stem cells (59)	10 ng/ml (**6.8 nM**)	↑
	3T3-L1 cells (61)	Free Ast 24.03, Ast Ester 187.76 μg/g	↓

Ast regulation of adipogenesis

There are very limited studies conducted on the effect of Ast on adipogenesis (**Table 1.1**). Kim et al. reported that Ast feeding increased PPARγ expression in mouse adipose tissues, suggesting that Ast may enhance adipogenic potential of stem cells (59). In contrast, relatively high concentrations of Ast-esters, 2.5 and 5 μg/ml (4.19 – 8.38μM), exhibited anti-adipogenic effects in 3T3-L1 cells (58). Phadtare et al. also observed anti-adipogenic effect of shrimp oil extracts containing Ast-esters in 3T3-L1 cells (61). Ast at 10 ng/ml (6.8 nM) increased adipogenesis and its biomarkers such as peroxisome PPARγ in mouse derived NSCs (59). No prior studies, to the best of my knowledge, effects of dose-dependent effects of Ast on adipocyte differentiation in hASCs.

Research Question: What are the effects of astaxanthin on proliferation and adipogenesis of human adipose stem cells?

Research Goal: To study the effects of astaxanthin on adipocyte biology

Hypothesis: Astaxanthin has a beneficial effect on human adipocyte biology by maintaining pool of adipose progenitors and promoting adipogenesis

Research Objectives:

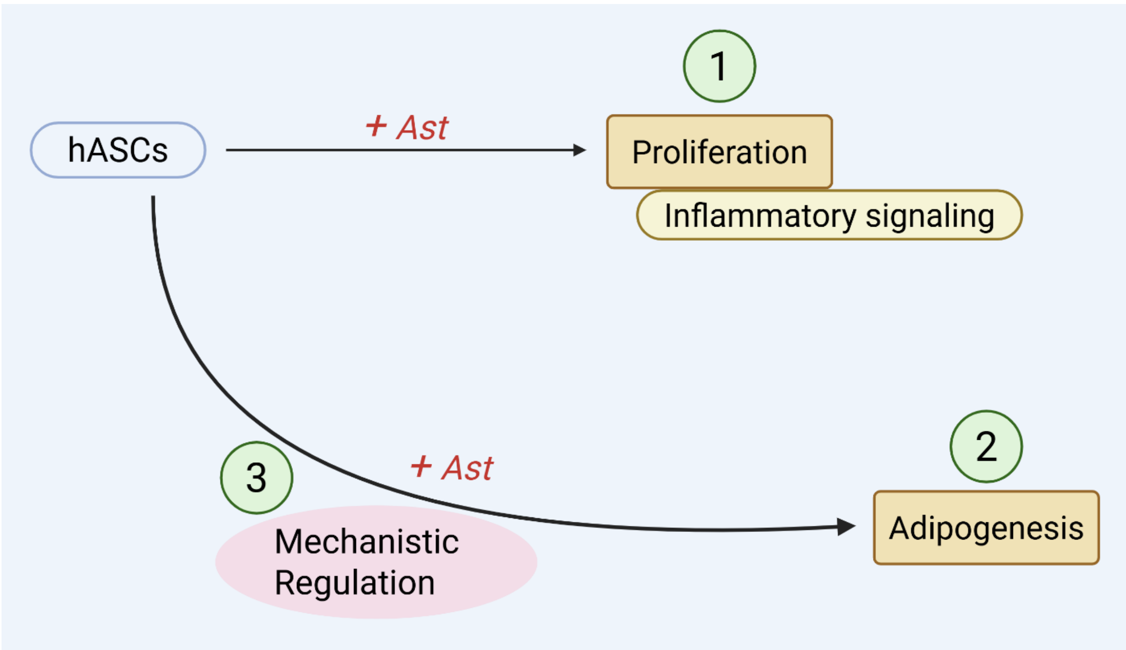
1. To assess effects of astaxanthin on proliferation of human adipose stem cells
   Hypothesis: *Astaxanthin promotes proliferation of human adipose stem cells*

2. To investigate effects of astaxanthin on adipogenesis of human adipose stem cells and underlying mechanisms

   Hypothesis*: Astaxanthin promotes adipogenesis by suppressing the anti-adipogenic Wnt/β-catenin pathway in human adipose stem cells*

Research conceptual framework

The effects of Astaxanthin on adipocyte biology



Chapter 2. Astaxanthin promotes proliferation and adipogenesis in human adipose stem cells.

ABSTRACT

Adipocytes not only store energy as neutral lipids but also function as endocrine cells. Making new adipocytes through recruitment of adipose stem cells and their differentiation into adipocyte through adipogenesis is essential for systemic metabolic health. Astaxanthin (Ast), enriched in seafoods, is getting more recognized in the bioactive compound family for its anti-inflammatory and antioxidative properties. However, limited information is available for astaxanthin regulation of proliferation and adipogenesis, especially in human adipose progenitors. This study examined the effects of Ast on human adipose stem cell (hASCs) proliferation and differentiation. hASCs were proliferated and differentiated in the presence of astaxanthin (0, 0.01, 0.1, 1, 2, and 10 μM) in the absence or presence of TNFα, following standard protocols. Proliferation rates were measured with MTT assay and adipogenesis was determined by measuring markers of adipocytes as well as lipid accumulation. Ast had no effects in the basal condition, but blocked TNFα-mediated inhibition of proliferation and induction of proinflammatory signaling pathway in hASCs. Lower concentrations of astaxanthin (0.01 and 0.1 μM) increased adipogenesis, while higher concentrations (2 μM and 10 μM) inhibited adipogenesis. Ast induced adipogenesis by suppressing the anti-adipogenic Wnt/β-catenin and adipogenic transcription factors, C/EBPα and PPARγ, during early periods of differentiation. Our results suggest that astaxanthin has beneficial impacts on adipose tissues by maintaining pool of adipose progenitors and promoting their differentiation into adipocytes.

1. INTRODUCTION

The prevalence of obesity has escalated substantially over the past several decades and is an alarmingly increasing global public health issue (62). Obesity is a significant risk factor for various diseases such as type 2 diabetes, dyslipidemia, cancer, and coronary heart disease, contributing to increased morbidity and mortality (63). Obesity is defined as the excessive accumulation of fat and expansion of adipose tissues, with a body mass index of > 30 kg/m^2 (6). The adipose tissue is a metabolic and endocrine organ that plays a crucial role in maintaining systemic health. In obesity, adipose tissues are dysfunctional, characterized by hypertrophy of adipocytes, hypoxia, fibrosis, oxidative stress and inflammation (9). Dysfunctional adipose tissues results in the increase of reactive oxidative species (ROS) and free radicals, causing oxidative stress and chronic inflammation (9,64). Consequently, these factors affect systemic health, increasing the risks of cardiometabolic diseases (CMDs).

Adipose tissue expands through hypertrophy and or hyperplasia. Hypertrophic growth occurs through increase of cell size, while hyperplastic expansion is a way for adipose tissue to remodel through recruitment from resident adipose progenitor or stem cells in the stromal vascular fraction of adipose tissue as well as their differentiation into adipocytes. Hyperplastic growth is thought to be beneficial preventing adipose tissue dysfunctions and thus, providing protection against obesity-associated diseases (11,12). In obesity and its associated metabolic diseases, the recruitment and or differentiation of adipose stem cells (ASCs) into adipocytes is limited, leading to the hypertrophy of existing adipocytes, a characteristics of a dysfunctional adipose tissues (8,12). Multiple factors, including elevated levels of oxidative stress and inflammation, have been shown to regulate proliferation as well as differentiation of ASCs. Tumor necrosis factor-alpha (TNFα), an inflammatory cytokine, is associated with reduced adipose stem cell differentiation and hyperplasia, resulting in the hypertrophy (57,65).

Bioactive compounds, particularly carotenoids are known to prevent dysfunctionality of adipose tissues (66,67). They positively influence key aspects of adipose tissue biology, including proliferation of ASCs and their differentiation into adipocytes, fatty acid oxidation capacity, thermogenesis, and secretory functions (12,66). Astaxanthin (Ast) is a xanthophyll carotenoid that is naturally produced by a variety of bacteria, microalgae, and yeasts (25). It is an oxygenated, non-provitamin A carotenoid, and is known for its potential biological functions such as antioxidant, anti-inflammatory, and antineoplastic activities (68). Due to its unique chemical properties and the molecular structure, Ast is considered as one of the most potent antioxidants providing greater antioxidant activities than other carotenoids (25,69).

The potential health effects of Ast against diseases have been extensively studied, including its anticancer and antidiabetic properties. Ast has been studied in mouse models and human trials to have decreased visceral adiposity, improved lipid profiles, and attenuated inflammation and fibrosis (69–72). Recent research has also highlighted its role in adipose tissue biology, showing that Ast can improve metabolic profiles by reducing lipid accumulation, enhancing fatty acid oxidation, and inflammation (69,70).

Previous studies on the direct effects of Ast on proliferation yielded inconsistent results depending on the cell types, concentrations, and experimental conditions used. While low doses of astaxanthin (0.4774 ng/mL or 0.8 nM) enhanced proliferation, concentrations higher than 20 μM suppressed proliferation in human ASCs (hASCs), and similarly, 500 ng/mL (~0.8 μM) of Ast decreased proliferation in human ASCs (73,74). On the contrary, Ast increased proliferation at 10 ng/ml (16.8 nM) in neural stem cells (NSC) (59), while another study showed that 3.7 ng/μL (6.8 nM) of Ast had no effects on the proliferation of neuroblastoma stem cells (SHSY-5Y) (60). Additionally, Ast provide protection against oxidative stress-mediated inhibition of ASC proliferation (57).

Few studies have examined the effects of Ast on adipogenesis. Treatment of Ast at a concentration of 25 μg/mL (~42 μM) significantly inhibited adipogenesis and lipid accumulation in 3T3-L1 cells, a widely used preadipocyte model derived from mouse embryos, while no inhibition was observed at lower concentrations, 1 - 10 μg/mL (~1.68 – 16.74 μM) (75). In contrasts, in NSCs, Ast at 10 ng/ml (6.8 nM) increased adipogenesis and its biomarkers such as PPARγ (59). Limited data is available for Ast-regulation of adipogenesis in hASCs.

In the current study, we examined dose-dependent effects of Ast on proliferation of hASCs in the absence or presence of TNFα, a proinflammatory cytokine increased in adipose tissues in obesity and its associated metabolic diseases (10). We also tested dose-dependent effects of Ast on adipogenesis and underlying cellular and molecular mechanisms involved in the Ast regulation of adipogenesis in hASCs.

2. MATERIALS AND METHODS

Study Sample and Experimental Design

- We used hASCs derived from different donors, without pooling.
- All experiments were repeated at least 3 times.

Materials

Ast used in this study is a free form of *H. pluvialis* that was purchased from Sigma-Aldrich. Working concentrations for Ast are achieved through serial dilutions using DMSO as the vehicle. All other reagents and materials were purchased from Sigma-Aldrich, Fisher Scientific or Thermo-Fisher Scientific.

hASCs culture

hASCs were derived from abdominal subcutaneous adipose tissues from human donors without known endocrine or metabolic disorders based on medical history, and were cultured and differentiated following published papers (76). Human adipose-derived stem cells (hASCs) were cultured in 10 cm tissue culture dishes with growth media (alpha-Minimal Essential Media with 10% fetal bovine serum and penicillin/streptomycin) at 37 °C in a 5.5% CO_2 incubator.

Effects of Ast on proliferation and inflammatory signaling activity in hASCs

To determine cell viability and proliferation, MTT assay were performed. hASCs were cultured in 10 cm tissue culture dishes until pre-confluence and then, seeded into 96-well plates at 2,500 – 3,000 cells/cm^2. After baseline measurement, cells were treated with Ast (0, 0.01, 0.1, 1, 2, and 10 μM) in growth medium for 24, 48, and 96 hours. After incubation, MTT solutions were added, and absorbance were be measured at 565 nm using a microplate reader following DMSO extraction. Data are presented as relative to the control without Ast at 24 hrs (**Figure 2.1**).

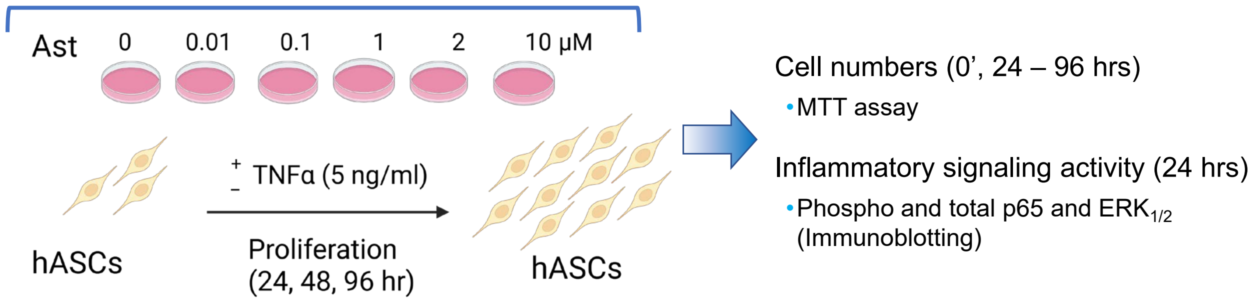


Figure 2.1. Experimental procedures to test effects of Ast on proliferation and inflammatory signaling pathway in hASCs.

For experiments involving TNFα, hASCs were seeded in 96-well plates at 2,500 – 3,000 cells/cm^2 and treated with Ast (0, 0.01, 0.1 and 1 μM) in the presence or absence of recombinant human TNFα (5 ng/ml) for 24 hours, followed by MTT essays at baseline and 24 hours (**Figure 2.1**). Cells were plated into 12-well plates at 5000 – 10,000 cells/cm^2 and treated with combinations of Ast and TNFα for 24 hrs, followed by harvest for measurement of inflammatory signaling activity with immunoblotting.

Effects of Ast on hASC differentiation

Cells were seeded into 12-well plates at 5000 – 10,000 cells/cm^2 and grown in the growth media. 2 days after achieving confluence, cells were differentiated with complete differentiation media (DMEM/F12 with IBMX, insulin, dexamethasone, rosiglitazone) containing varying concentrations of Ast (0, 0.01, 0.1, 1, 2, and 10 μM), following previously published protocol (76). Following a 3-day induction period, the media were be replaced with maintenance media (DMEM/F12 with insulin and dexamethasone) containing the same Ast concentrations. Cells were refed with Ast in the previously mentioned concentrations every 2 to 3 days and harvested on different time points during 13 to 14 of differentiation periods (**Figure 2.2**).

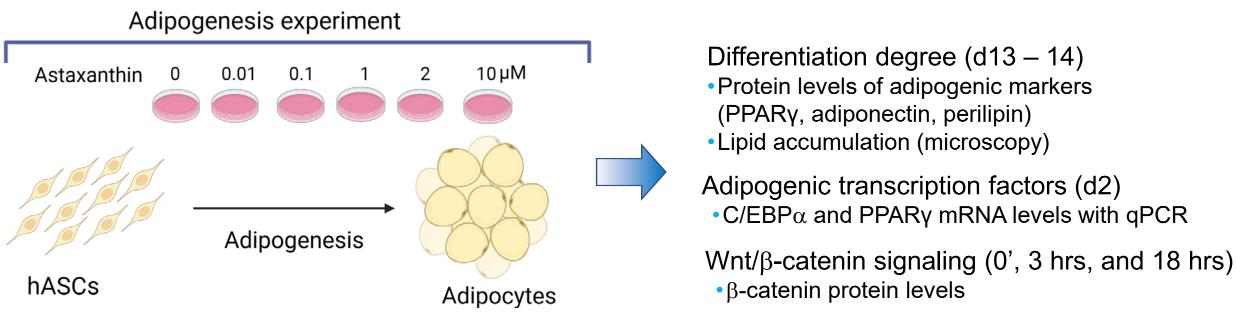


Figure 2.2. Experimental procedures for testing effects of Ast on adipogenesis in hASCs.

Testing effects of Ast on 3T3-L1 differentiation

3T3-L1 preadipocytes were cultured in 10 cm tissue culture dishes with DMEM/10% FBS/ at 37 °C in a 5.5% CO_2 incubator until pre-confluency. Cells were plated into 6-well plates and grown in DMEM/10% FBS and differentiation was initiated after 2 days of reaching confluency. Briefly, cells were induced to adipogenesis with a medium containing 10% FBS/DMEM, insulin, IBMX, and dexamethasone with Ast (0, 0.01, 0.1, 1, 2, and 10 μM) for 2 days. The differentiation medium was then replaced with insulin-containing DMEM/10% FBS and maintained for 24 hours. Subsequent media changes to fresh DMEM/10% FBS occur every 2-3 days, with cells harvested on day 7 of differentiation.

Microscopy imaging

After differentiating cells with Ast (0, 0.01, 0.1, 1, 2, and 10 μM) for 13 – 14 days, neutral lipids were stained with LipidTox-Green for 1 hr and then, mounted on glass slides with DAPI mounting media. Florescence microscopy was performed using an Olympus X45 microscope. Bright field images were also taken with a Nikon TE microscope.

Immunoblotting

Cells lysed in lysis buffer with 5% sodium dodecyl sulfate (SDS). After sonication and centrifugation at 1,000 rpm for 6 minutes at 25 °C, equal amounts (10-15 μl) of protein extracts were be subjected to 10% SDS-PAGE and transferred to Immobilon-FL PVDF membranes (Millipore). Membranes were incubated overnight at 4°C with primary antibodies against PPARγ (Cell Signaling Technology, 1:1000), perilipin 1 (1:3000), adiponectin (BD Biosciences, 1:5000), HSP90 (Santa Cruz Biotech, 1:2000), β-catenin (Santa Cruz Biotech, 1:2000), phospho and total p65 NF-κB and ERK1/2 (Cell Signaling Technology, 1/1000), and Lamin A/C (Protein Tech, 1:5000). Following incubation with appropriate secondary antibodies conjugated with horse radish peroxidase (Cell Signaling Technology, 1:5000), chemiluminescence signals were be detected with West-Pico and West-Femto chemiluminescence substrates (Thermo Fisher Scientific) and iBright FL1500 (Thermo Fisher Scientific), and relative band intensities were analyzed using iBright analysis software.

RNA extraction and measurement of mRNA expression by qPCR

Total RNA was extracted from cells after 2 or full differentiation using Trizol. After checking quality and quantity of RNA with a Nano-Drop (Thermo Scientific), 500 to 1000 ng of total RNA was used to make cDNA using Transcriptor First Strand cDNA synthesis kit (Roche). Quantitative real-time polymerase chain reaction (qPCR) was performed using SYBR Green and TaqMan probe assays in 96-well plates to analyze target gene expression for adipogenic genes (PPARγ, CEBPα and aP2). Cyclophilin A (PPIA) as a reference gene and relative expression levels were calculated using $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data are expressed as mean ± mean standard errors and analyzed using appropriate statistical methods. One-way or two-way ANOVA was employed to analyze differences among groups, with post-hoc tests for pairwise comparisons. Statistical significance was set at $p < 0.05$. Data analysis was conducted using Graphpad prism.

3. RESULTS

3.1 Ast blocked the anti-proliferative effects of TNFα in hASCs

ASCs have the capacity for self-renewal/proliferation to maintain pool of adipose progenitors, which supports the regeneration and differentiation of adipocytes (74). To investigate whether Ast influenced their proliferation rates, hASCs were grown with various concentrations of Ast (0, 0.01, 0.1, 1, 2, and 10 μM) for 24, 48, and 96 hrs and cell numbers were assessed with MTT assay. Ast did not affect proliferation rates nor cell viability in hASCs at any concentrations up to 96 hrs **(Figure 3.1)**.

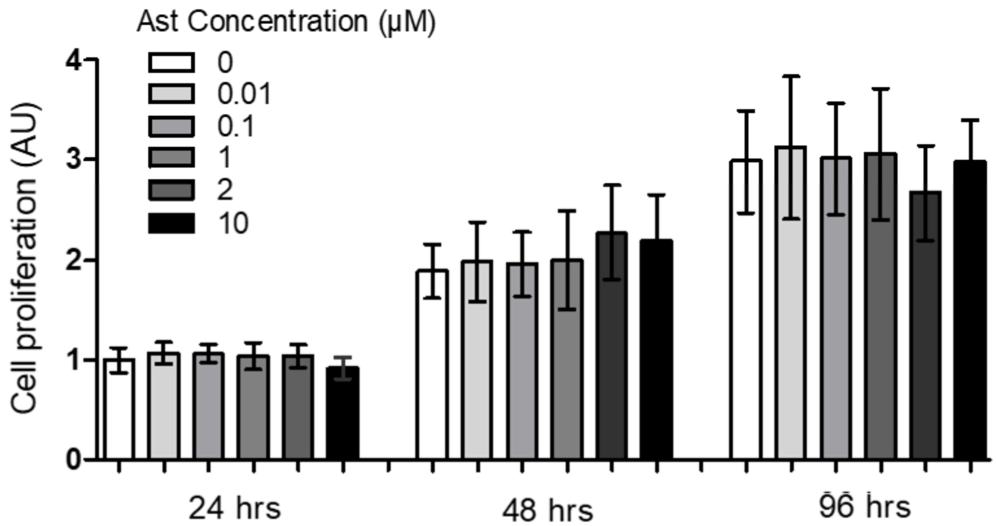


Figure 3.1. Ast did not affect proliferation rates in human adipose stem cells.
Cells were grown in the presence of different concentrations of Ast (0 – 10 μM) for 24, 48, and 96 hrs, followed by MTT assay to measure numbers. Data were analyzed by one-way ANOVA at each time points, n = 3. AU, arbitrary unit.

TNFα, a proinflammatory cytokine is increased in obesity and known to inhibit cell proliferation and viability in ASCs (12,77). Since Ast has been previously implicated as having anti-inflammatory characteristics (34,37), we next investigated whether Ast blocked effects of TNFα in hASCs. We conducted an overnight treatment of hASCs with different concentrations of Ast (0 –1 μM) in the absence or presence of TNFα (5 ng/ml), followed by MTT assay to assess cell numbers. Similar to previous findings (78), TNFα inhibited proliferation rates in hASCs ($p<0.01$, n=6) **(Figure 3.2)**. Ast (0.01 μM – 1 μM) blocked the anti-proliferative effects of TNFα.

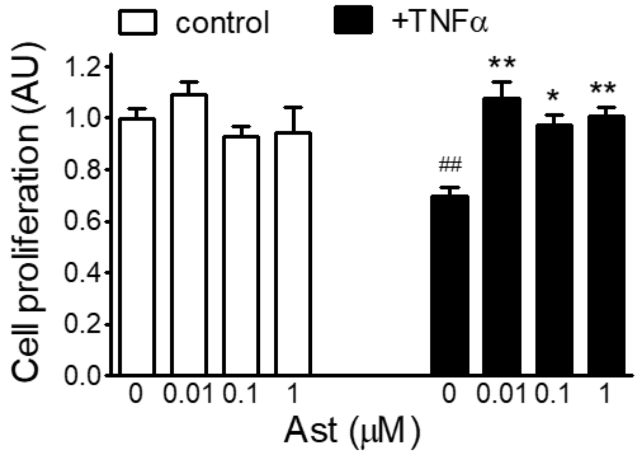


Figure 3.2. Ast blocked TNFα-inhibition of proliferation in hASCs.

Cells were grown overnight with or without Ast (0 – 1 μM) and TNFα (5 ng/ml) for overnight, cell proliferation rates were measured with MTT assay. Data were analyzed by two-way ANOVA and Bonferroni post t-tests. *p<0.05 and **p<0.01; effects of Ast, ##p<0.01; effects of TNFα, n=6. AU, arbitrary unit.

3.2 Ast blocked TNFα-induction of proinflammatory signaling activity in hASCs

TNFα activates proinflammatory signaling pathways, including mitogen activated protein kinase (MAPK) and p65 NF-κB (79). We next determined if Ast blocked the proinflammatory actions of TNFα in hASCs. After treatment with combinations of Ast (0 – 1 μM) and TNFα (5 ng/ml) for 24 hrs, phosphorylated p65 NF-κB and $ERK_{1/2}$ levels were measured with immunoblotting. As expected, TNFα increased phosphorylated p65 and $ERK_{1/2}$ levels by 2 to 3 folds in hASCs (**Figure 3.3**). Ast alone did not have any significant effects on the proinflammatory signaling activities. However, Ast effectively blocked the TNFα-induced upregulation of p65 and $ERK_{1/2}$ phosphorylation in a dose-dependent manner.

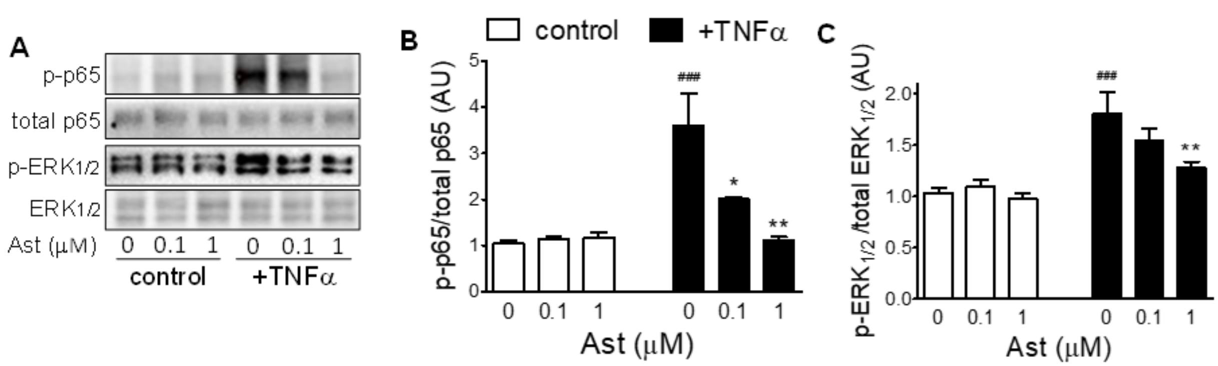


Figure 3.3. Ast blocked TNFα-induction of inflammatory signaling pathways in hASCs. After treating cells with TNFα (5 ng/ml) ± AST (0 – 1 μM) for 24 hrs, protein levels of phospho and total $ERK_{1/2}$ and p65 NF-κB were measured with immunoblotting. Representative blots are presented in A and relative expression levels of phospho to total $ERK_{1/2}$ and p65 NF-κB are shown in B and C. Data were analyzed by two-way ANOVA and Bonferroni post t-tests. $^{\#\#}p<0.01$; effects of TNFα, $^{*}p<0.05$ and $^{**}p<0.01$; effects of Ast, n=6. AU, arbitrary unit.

3.3 Dose-dependent effects of Ast on adipogenesis in hASCs

Previous studies have found inconsistent results on the effects of Ast on adipocyte differentiation, depending on doses of Ast and cell types used (14,58,61). Further, there is very limited understanding on the effects of Ast in hASCs. We tested effects of varying concentrations (0 – 10 μM) of Ast on the differentiation of hASCs following a standard protocol for 13 to 14 days (76) and adipogenesis was determined by measuring expression levels of adipogenic proteins and lipid accumulation. Low doses of Ast (0.01 - 0.1 μM) increased the expression of adipogenic biomarkers, PPARγ, PLIN1 and ADPN, indicating enhancement of adipogenesis (**Figure 3.4A-D**). However, high doses of Ast (2 and 10 μM) had inhibitory effects on the expression of all three protein markers (**Figure 3.4A-D**). Lipid accumulation serves as a key indicator of adipogenesis, reflecting the extent of differentiation of hASCs into mature adipocytes. Similar to expression levels of adipogenic makers, lower concentrations of Ast (0.01 and 0.1 μM) promoted lipid accumulation, while a high dose (10 μM) of Ast decreased (**Figure 3.5**). Collectively, these findings indicate dose-dependent effects of Ast on adipogenesis such that low concentrations of Ast promotes while high concentrations inhibit adipogenesis compared to basal level implying that Ast supports hyperplastic growth at certain levels however it proves toxic and can impair this process at higher concentrations adipogenesis in hACs.

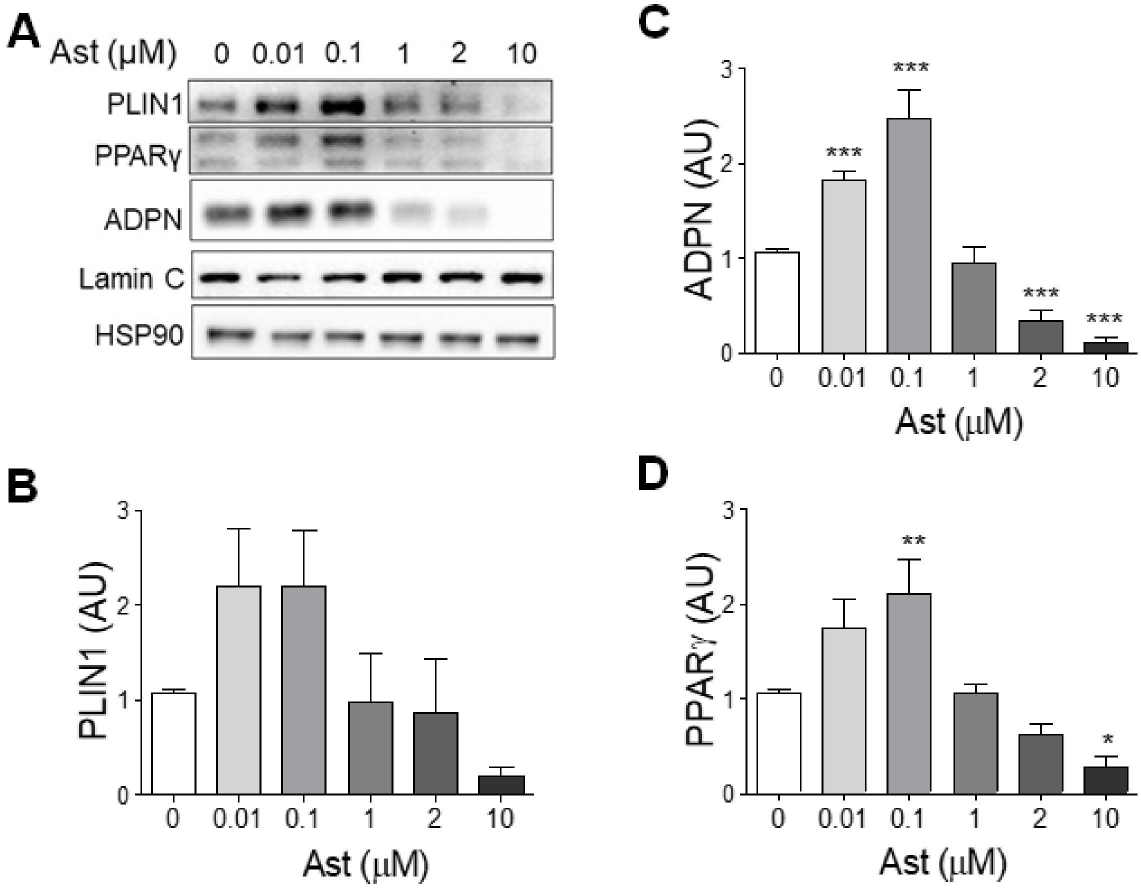


Figure 3.4. Ast has a dose-dependent effect on differentiation of hASCs.

hASCs were differentiated in the presence of Ast (0 - 10 μM) and adipogenesis was determined by measuring protein levels of adipogenic markers [peroxisome proliferator-activated receptor (PPARγ), adiponectin (ADPN), perilipin 1 (PLIN1)] and lipid accumulation after d13 – 14 of differentiation. A. Representative images of immunoblotting. B – D. Relative expression levels to HSP90, a loading control. *p<0.05, **p<0.01, and ***p<0.001, compared to the control, n = 4 to 9. AU, arbitrary unit.

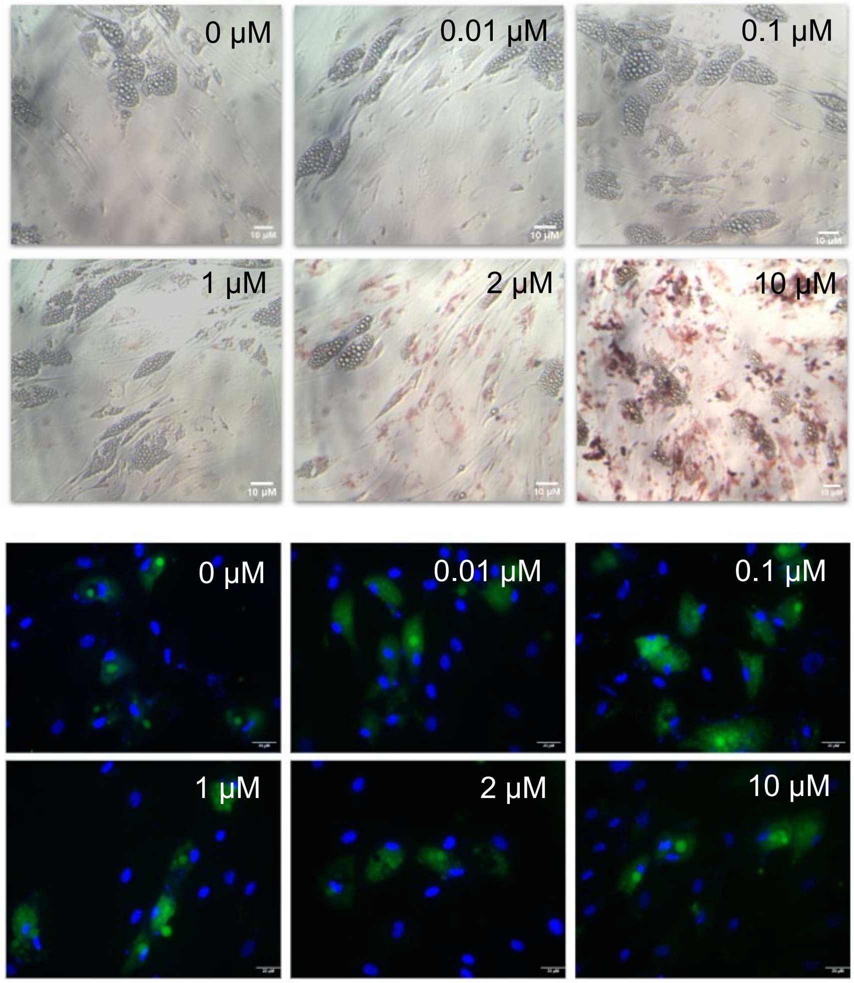


Figure 3.5. Ast has a dose-dependent effect on lipid accumulation in hASCs.
hASCs were differentiated in the presence of Ast (0 - 10 µM) and adipogenesis was determined by measuring lipid accumulation. Top panel: Bright field images. Bottom panel: Fluorescence microscopy. Green indicates LipidTox staining of neutral lipids and blue indicates DAPI staining of nucleus. White bars = 10 microns.

3.4 Dose-dependent effects of Ast on differentiation of 3T3-L1 cells

A previous study found an inhibitory effects Ast on lipid accumulation in 3T3-L1 cells (75), a well-established and widely used preadipocyte cell line derived from mouse embryos (80). However, they used a high concentration of Ast, 25 g/mL (41.9 µM), which is known to inhibit cell proliferation and exhibit cytotoxic effects (57). Therefore, we next tested dose-dependent effects of Ast on differentiation of 3T3-L1 cells. Similar to the data in hASCs, lower doses (0.01

– 0.1 μM) of Ast promoted adipogenesis, as indicated by higher expression levels of adiponectin (**Figure 3.6**).

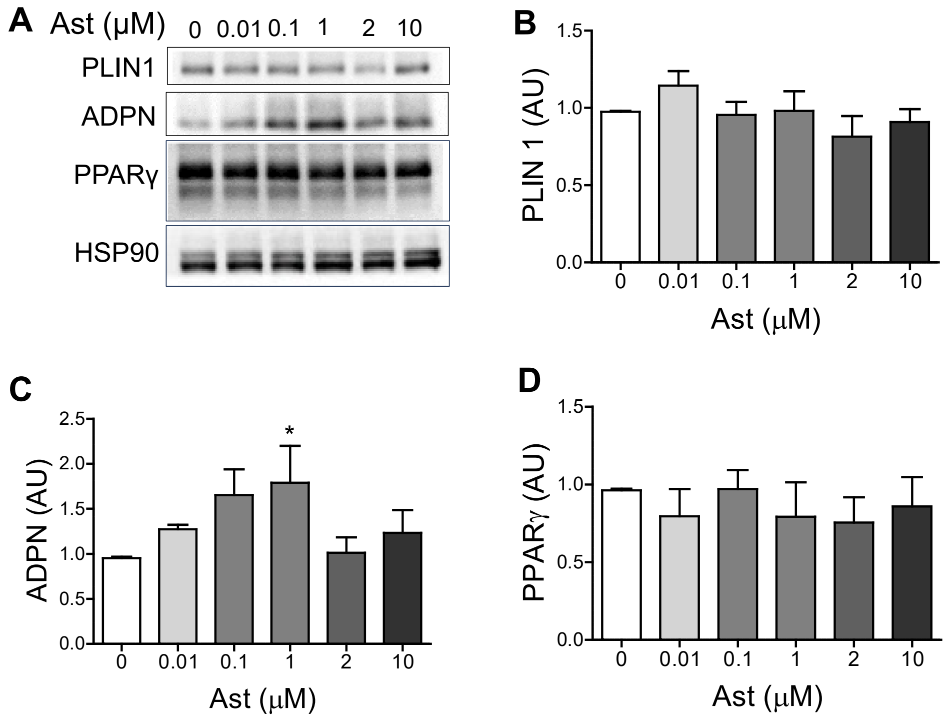


Figure 3.6. Dose-dependent effects of Ast on differentiation of 3T3-L1 cells.
3T3-L1 cells were differentiated in the presence of Ast (0 – 10 μM) and adipogenic markers [perilipin 1 (PLIN1), adiponectin (ADPN), and peroxisome proliferator-activated receptor (PPARγ)] were measured with Immunoblotting on d7 of differentiation. Representative images are shown in A and relative expression levels of PLIN1, ADPN, and PPARγ to HSP90 (a loading control) are presented in B, C, and D. *p<0.05 compared to the control, n = 4 to 5. AU, arbitrary unit.

3.5 Ast promoted expression levels of adipogenic transcription factors in hASCs

Adipogenesis involves a comprehensive network of transcription factors that regulate expression of adipocyte-specific proteins. At the onset of adipogenesis, CCAAT/enhancer binding protein α (CEBPα) and PPARγ, the master regulators of adipogenesis, are activated and are able to induce downstream adipogenic proteins such as fatty acid binding protein 4 (aP2), adiponectin, and perilipin 1 (14,17). We therefore assessed the effects of Ast on gene expression levels of these key regulatory transcription factors on day 2 of differentiation. Low doses of Ast upregulated both PPARγ and CEBPα with significant effects observed at 0.1 μM ($p<0.01$) (**Figure 3.7A & B**). Ast did not significantly affect *aP2* (**Figure 3.7C**), possibly due to its role as a late stage adipogenic marker. These findings indicate that Ast promotes adipocyte differentiation by upregulating the key adipogenic transcription factors.

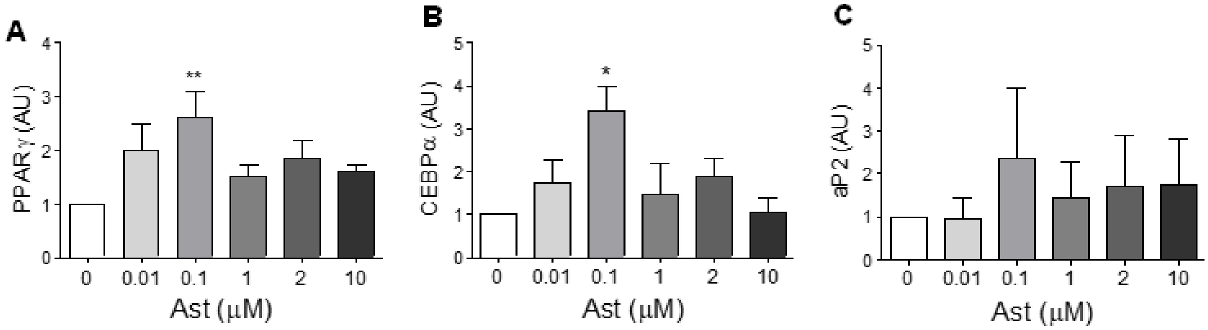


Figure 3.7. Ast had dose-dependent effects on adipogenic transcription factors. After differentiation of hASCs in the presence of varying concentrations of Ast (0 – 10 μM), mRNA expression levels of *PPARγ, CEBPα* and *aP2* were measured with RT-qPCR. *$p<0.05$ and **$p<0.01$, compared to the control, n = 4. AU, arbitrary unit.

3.6 Ast suppressed expression levels of β-catenin in hASCs

The adipogenic commitment of progenitor cells is known to be controlled by the Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling inhibits adipogenesis by blocking the expression of transcription factors CEBPα and PPARγ (22,81). We therefore assessed whether Ast improvement of adipogenesis is mediated through inhibition of the Wnt/β-catenin signaling by measuring expression levels of β-catenin before and 3 and 18 hrs of differentiation with or without Ast (0.1 μM). As expected, β-catenin expression levels declined after 18 hrs of adipogenic induction in hASCs compared to time zero ($p = 0.04$) (**Figure 3.8**). Ast decreased β-catenin expression at both 3 and 18 hrs of adipogenesis ($p<0.05$ at both 3hr and 18hr). This finding indicates that Ast blocks anti-adipogenic, Wnt/β-catenin signaling, thereby enabling the key events initiating adipogenesis to be switched on.

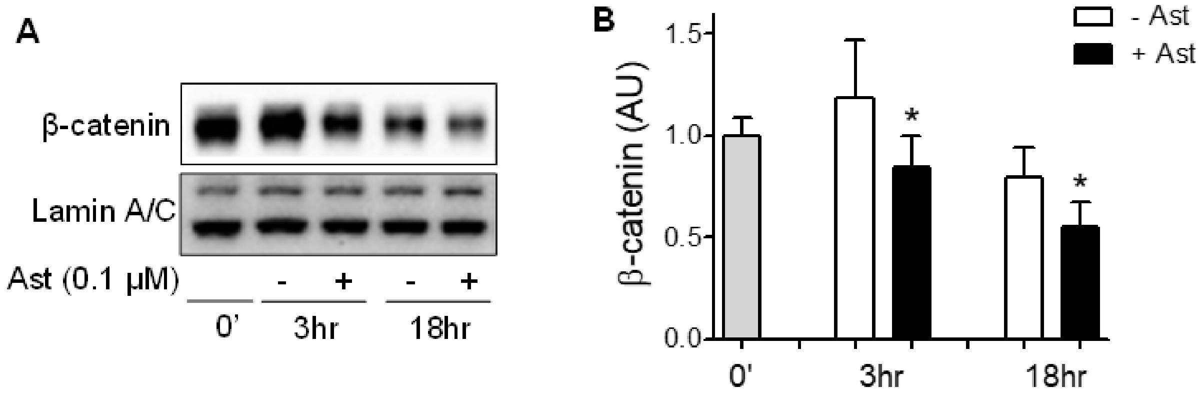


Figure 3.8. Ast decreased β-catenin expression in hASCs.

After differentiation of hASCs with or without Ast (0.1 μM), expression levels of β-catenin protein levels were measured with immunoblotting at 0, 3 and 18 hrs of adipogenesis. A. Representative blots. B. Relative expression levels of β-catenin to lamin A/C. *$p<0.05$ compared to the control at each time point, n = 5. AU, arbitrary unit.

4. DISCUSSION

Recruitment of adipose progenitors and their differentiation into adipocytes are essential for the proper metabolic and endocrine functions of adipose tissues, and systemic metabolic health. Ast, an anti-inflammatory and antioxidant carotenoid, has been suggested to regulate adipocyte biology providing beneficial impacts on cardiometabolic diseases (82). However, very limited numbers of studies have explored its effects on hASCs. In this study, we examined the impact of Ast on hASC proliferation, differentiation, and the underlying molecular mechanisms involved. Our findings reveal that Ast effectively blocked effects of TNFα on inflammatory signaling activity and proliferation in hASC, suggesting its role in maintenance of adipose progenitor pool. Moreover, Ast treatment promoted adipogenesis in a dose-dependent manner. Further investigation into the Wnt/β-catenin signaling pathway revealed that Ast suppressed this anti-adipogenic signaling pathway, thereby enhancing the key adipogenic transcription factors, C/EBPα and PPARγ, during the early periods of differentiation. Our results collectively suggest that Ast support the maintenance of a functional adipocyte pool, which could provide protective effects against obesity-associated cardiometabolic health.

TNFα, a pro-inflammatory cytokine increased in obesity, has been shown to impair adipose tissue function by inducing insulin resistance, suppressing lipoprotein lipase activity, and inhibiting ASC self-renewal (57,83). TNFα signaling through the canonical NF-κB pathway and MAPKs to increase proinflammatory cytokines, including TNFα, IL-1β, and IL-6 (84,85). We found that Ast blocked TNFα-stimulation of p65 NF-κB and $ERK_{1/2}$ MAPK phosphorylation without affecting basal levels in hASCs. Similarly, previous studies using hASCs and mice models show that Ast treatment/supplementation reduced TNFα in adipose derived cells and kidney tissues (38,57). Nawaz et al. previously reported that dietary Ast supplementation (0.02%) provided protective effects against high-fat diet-induced adipose tissue inflammation (reduced expression levels of IL-1β, IL-6, and TNFα and macrophage infiltration) and oxidative stress in murine adipose tissues (72). Yang et al. also showed that Ast blocked an oxidative stress-induced cytokine expression, IL-6 and TNFα in hASCs (57). Overall, these data support the anti-inflammatory actions of Ast. ASCs from individuals with obesity exhibit more fibrotic and proinflammatory phenotypes (10) and Ast may provide beneficial impacts by suppressing inflammation (86).

Maintaining a functional ASC population is essential for supporting hyperplastic growth and sustaining adipogenesis. The numbers of ASCs and their morphological characteristics, proliferation rates, and adipogenic potential are affected by obesity, ageing and metabolic status, and inflammation and oxidative stress have been shown to limit the self-renewal capacity of ASCs (32,52,53,57). We found that Ast (0.001 – 10 µM for up to 96 hrs) did not exhibit any effects on proliferation in hASCs. Results from previous studies on Ast-regulation of proliferation have been inconsistent. In contrast to our results, 0.01- 1 µM of Ast promoted proliferation in hASCs after 24 and 48 hrs (57). Further, Ast (10 ng/ml or ~ 8.38 µM) treatment promoted proliferation in neural progenitor cells after 24, 48 and 72 hrs (87). However, Mularczyk et al. found no effect of Ast on the proliferative capacity in ASCs derived metabolically healthy horses (78), similar to our results.

Although Ast did not affect basal rates, it blocked TNFα-mediated suppression of hASC proliferation rates. Consistent with these, Mularczyk et al. showed that Ast (~16.8 and 33.5 µM) increased proliferation rates of ASCs derived from horses with metabolic syndrome, which exhibited reduced proliferation than cells derived from normal horses (78). Yang et al. also showed that Ast (10 μM) blocked the H_2O_2-mediated inhibition of hASC proliferation (57). Ast treatment reduced markers of oxidative stress while increasing NRF2, a transcription factor that plays a central role in anti-oxidant response (57,78), suggesting that anti-oxidant capacity of Ast may be involved in the protective effects in ASC proliferation and being protective against oxidative stress. Our results suggest that Ast also helps to maintain pool of adipose progenitors through suppression of inflammatory signaling that is increased in adipose tissue of obesity. Therefore, antioxidant and anti-inflammatory actions of Ast may explain the protective actions of Ast in maintaining pool size of adipose progenitors in epididymal adipose tissue in mice after high-fat diet (72,75).

ASCs in obesity exhibit low adipogenic capacity, which is associated with hypertrophy of adipocytes, leading to ectopic fat deposition and increased risk of insulin resistance, type 2 diabetes, and other cardiometabolic disorders (10). Further, impaired adipogenesis is associated with dysfunctional adipose tissues, including inflammation, oxidative stress and fibrosis (84,88). Therefore, we assessed whether Ast, an anti-oxidant and anti-inflammatory carotenoid, enhanced adipocyte differentiation in hASCs. Our study showed that low concentrations (0.01 and 0.1 μM) of Ast promoted adipogenesis, upregulating key adipogenic biomarkers and lipid accumulation. Consistent with these, a previous study showed that Ast (~0.017 μM) promoted adipogenesis in neural progenitor cells (59). However, a high concentrations of Ast (~42 μM) significantly inhibited adipogenesis and lipid accumulation in 3T3-L1 cells, while lower concentrations of Ast (~1.68 – 16.74 μM) did not affect (75). We also find that up to 10 μM of Ast did not suppress adipogenesis in 3T3-L1 cells. Overall, these indicate that low doses of Ast promoted adipogenesis while higher doses inhibit adipogenesis. Plasma levels of Ast are reported to be 55.2 ± 15.0 μg/L (0.09 μM) after single dose of 40 mg and 1.3 ± 0.1 mg/L (2.1 μM) after a single intake of 100 mg ((89,90). Therefore, the concentrations used in our study is achievable with Ast intake in humans.

A previous study showed that greater than 20 μM of Ast exhibited cytotoxicity (75) and we also observed that when hASCs were treated with high doses of Ast (greater than 2 μM) for more than one week, they started to accumulate Ast and had high levels of $ERK_{1/2}$ MAPK signaling activity, indicating potential cytotoxicity (FT, YY and MJL, unpublished observation and Figure 3.4E). Therefore, the anti-adipogenic effects of high doses of Ast on adipogenesis may be related to their cytotoxic effect.

ASCs are tissue-resident multipotent stem cells that have multi-lineage potential. ASC differentiation into adipocytes is regulated through complex network of signaling pathways and transcription factors. Pro-adipogenic processes are induced when key adipogenic transcription factors such as CEBPα and PPARγ are upregulated (14). The Wnt/β-catenin signaling regulates developmental processes (20). The Wnt/β-catenin also regulates the multi-lineage commitment of stem cells, where it can promotes osteogenic processes while inhibiting adipogenic lineage (21). When the Wnt/β-catenin is activated, key adipogenic transcription factors, CEBPα and PPARγ, are down regulated and adipogenesis is inhibited (20). In this study, we found that Ast suppressed the anti-adipogenic Wnt/β-catenin pathway during the early periods of differentiation (3 – 18 hrs) and stimulated the expression of CEBPα and PPARγ on day 2 of adipogenesis. While both transcription factors are critical for adipogenesis, C/EBPα itself cannot increase adipocyte without PPARγ, but maintains expression of PPARγ, the master regulator of adipogenesis increasing adipocyte specific proteins, including perilipin 1 and adiponectin (91). Our results indicate that Ast enhances adipogenesis by suppressing the Wnt/β-catenin signaling pathway that leads to induction of adipogenic transcription factors in hASCs.

Several studies have reported that Ast-suppression of the Wnt/β-catenin signaling pathway in other cell types. Ast supplementation (15 mg/kg) attenuated tumor formation by inhibiting the Wnt/β-catenin pathway in Syrian hamster buccal pouch carcinogenesis model (92). Studies have shown that inflammatory cytokines such as TNFα and IL-6 suppress adipogenesis through activation of the Wnt/ β-catenin signaling pathway (81). Ast (100, 200, and 300 μM) inhibited both the Wnt/β-catenin and NF-κB pathways in human hepatocellular carcinoma cells (48). We found that Ast exhibited inhibitory effects on TNFα-induced proinflammatory signaling activity in hASCs, which may have contributed to its suppression of the Wnt/β-catenin activity.

Our study primarily analyzed hASCs isolated from abdominal subcutaneous adipose tissue. Limited lipid storage capacity in this depot can lead to ectopic fat deposition, with visceral fat accumulation serving as a key marker of such dysfunction (12). However, we did not directly investigate the effects of Ast in hASCs derived from visceral depots. hASCs from visceral fat exhibit higher levels of inflammation and stress signaling activity and poor adipogenic capacity (93). Future studies assessing Ast regulation of adipogenesis in visceral hASCs are warranted.

Collectively, our findings suggest Ast has a beneficial role in hyperplastic growth of adipose tissues. The role that Ast plays in maintaining pool of adipose progenitors and promoting adipogenesis is important for cardiometabolic health (7). While many studies argue that Ast inhibiting adipogenesis is beneficial for addressing obesity, this process is necessary for formation of new adipocytes which contribute to maintaining systemic health (94,95). CMDs usually characterized by obesity (88) is a state of hypertrophy with dysfunctional adipocytes. In this state, adipocytes have limited expansion and impaired storage capacity (10), releasing chemokines and cytokines leading to inflammation and oxidative stress, driving CMDs (10,96). Ast plays a beneficial role in maintaining adipose progenitors and promoting adipogenesis, supporting hyperplastic growth, which helps to maintain adipose tissue function, providing protective effects against CMDs.

Chapter 3. Final Remarks

Ast has emerged as a bioactive compound of considerable interest due to its potent antioxidant and anti-inflammatory properties. Its unique chemical structure, featuring both polar and nonpolar regions, enables it to span lipid bilayers and exhibit an antioxidant role efficiently (24), thereby mitigating oxidative stress, a critical component in the pathology of numerous chronic diseases. While its efficacy has been demonstrated across various models, from in vitro systems to in vivo mouse models , the mechanisms by which Ast influences adipocyte biology, particularly adipogenesis in human adipose derived stem cells (hASCs), remain insufficiently characterized.

Adipose tissue plays a critical role in metabolic regulation, and maintaining a functional adipocyte pool through healthy adipogenesis is essential for cardiometabolic health. This study investigated the effects of Ast, a known antioxidant and anti-inflammatory compound, on (hASCs). We demonstrate that Ast attenuated TNFα-induced inflammation and suppression of proliferation in hASCs. We also showed that for the first time, to best of knowledge, that Ast promoted adipocyte differentiation in hASCs through modulation of the Wnt/β-catenin signaling pathways. These findings highlight potential Ast potential as a modulator of adipose tissue homeostasis and a candidate for future therapeutic strategies targeting obesity-related metabolic dysfunction.

Understanding how Ast modulates adipocyte biology at physiologically relevant concentrations is imperative. Several studies that have investigated the effects of Ast on adipogenesis remain contradictory. For example, while Ast has been observed to increase adipogenesis in neural stem cells at nanomolar concentrations, others have shown reduced adipogenic markers and lipid accumulation in adipocytes at higher doses (59,61). Our study found that Ast has a dose dependent effect on adipogenesis in hASCs. Lower concentrations increased adipogenic program by inhibiting the anti-adipogenic Wnt/β-catenin pathway and inducing the master regulators of adipgogensis, C/EBPa and PPARg in hASCs. However, greater than 2 μM Ast exhibited inhibitory effects on adipogenesis, potentially due to its cytotoxicity. The findings in this study highlight the nuanced effect of Ast depending on its dosage and cell type used. While the inhibition of adipogenesis may be advantageous in metabolic conditions such as hyperplastic obesity, excessive suppression of this process can also be detrimental (7). Healthy adipogenesis, particularly hyperplasia through ASC recruitment and differentiation, is critical for adipose tissue remodeling and systemic metabolic health (7) .

The role of Ast as an antioxidant is not just limited to regulation of adipogenesis. Previous studies have shown that in mature adipocytes, Ast also has an effect on lipid accumulation by reducing TAG levels in adipocytes (75). As an antioxidant, Ast reduced oxidative stress in the liver and epididymal white adipose tissue (72). Ast alleviated oxidative stress and inhibited apoptosis in pancreatic β-cells (16) and reversed lipid peroxidation, inhibiting ferroptosis in SHSY-5Y cells (60). Further, Ast suppressed proinflammatory pathways in liver injury-induced mice (55,97) and cardiovascular disease mice models (37). Oxidative stress and inflammatory characterize numerous chronic diseases including CMDs and therefore, Ast has potential to provide beneficial impacts through multiple mechanisms in addition to its promotion of adipogenesis.

Strengths and Limitations

Previous studies on Ast regulation of proliferation of adipose progenitors and their differentiation into adipocytes have reported inconsistent findings, depending on cell types and experimental conditions. We investigated the effects of astaxanthin on proliferation and adipogenesis using hASCs, which provide reliable models for translational medical research (98,99) and offer greater relevance to human biology compared to rodent models and 3T3-L1 cells (100,101). We also tested careful dose-dependent effects of Ast and found that low doses of Ast promoted adipogenesis. Our data also suggest that high concentrations of Ast inhibits adipogenesis by inducing cytotoxicity. We further investigated mechanistic pathways by which Ast regulates adipogenesis, further reaffirming our findings.

One limitation of our study is that we did not directly establish a causal relationship between inflammation or oxidative stress and the Wnt/β-catenin signaling pathway in the context of Ast regulation of adipogenesis. Additionally, although we observed that accumulation of Ast in cells when they were treated with greater than 1 μM for more than 5 to 6 days during adipogenesis, which may explain the inhibition of adipogenesis, we did not establish a causal relationship.

Future studies

Future investigations should aim to elucidate the specific interactions between inflammatory markers and this signaling pathway in response to Ast. While in vitro studies using hASCs provide valuable mechanistic insights, complementary in vivo studies are warranted to validate these findings and assess physiological relevance of Ast bioavailability and functionality across various tissues. Further studies also can be conducted to determine the healthy dosage of Ast for human beings, extending into identifying form of Ast that is consumed and bioavailability.

Implications

Collectively, our findings suggest Ast has a beneficial role in hyperplastic growth of adipose tissues. The role that Ast plays in maintaining pool of adipose progenitors and promoting adipogenesis is important for cardiometabolic health (7). While many studies argue that Ast inhibiting adipogenesis is beneficial for addressing obesity, this process is necessary for formation of new adipocytes which contribute to maintaining systemic health (94,95). CMDs usually characterized by obesity (88) is a state of hypertrophy with dysfunctional adipocytes. In this state, adipocytes have limited expansion and impaired storage capacity (10), releasing chemokines and cytokines leading to inflammation and oxidative stress, driving CMDs (10,96). Ast plays a beneficial role in promoting adipogenesis, supporting adipose tissue function against CMDs. Ast has been characterized widely as an antioxidant in other tissues of the body, the gut microbiome, liver and the brain (59,102,103) by modulating key inflammatory pathways (50), through which Ast exerts its protective effects. These findings suggest that Ast helps protect against CMDs by modulating oxidative stress and pro-inflammatory pathways, preserving the integrity of cardiometabolic organs. Our study further demonstrates that similar protective effects can be observed in adipocytes. Notably, as our findings are based on a hASC model, they offer significant translational potential for future therapeutic applications in human metabolic health.

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