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Sodium Propionate and Sodium Butyrate Promote Fatty Acid Oxidation in HepG2 Cells under Oxidative Stress

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SODIUM PROPIONATE AND SODIUM BUTYRATE PROMOTE FATTY ACID
OXIDATION IN HEPG2 CELLS UNDER OXIDATIVE STRESS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Nutrition and Food Sciences

by
Kristina Jazmin Cook
B.S., Louisiana State University, 2014
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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of disturbances that includes simple steatosis, non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and liver cancer. NAFLD affects individuals that consume the typical Western diet consisting of high levels of fats and carbohydrates. The increase in circulating free fatty acids, palmitate and oleate, or lipopolysaccharides (LPS), induce oxidative stress and pro-inflammatory cytokine production in the liver, which all contribute to NAFLD progression. In this study, we are evaluating the mRNA expression of genes associated with fatty acid oxidation (FAO) and the protein expression of pro-inflammatory cytokines related to NAFLD using the HepG2 human liver hepatocellular carcinoma cells exposed to palmitate/oleate or LPS. The treatment of sodium butyrate (NaB) or sodium propionate (NaP) was used to relieve oxidative stress and inflammation in liver cells. The quantitative real-time polymerase chain reaction (qRT-PCR) results show that NaP or NaB, were able to promote FAO, regulate lipolysis, and reduce reactive oxygen species production by significantly increasing the mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α), peroxisome proliferator-activated receptor alpha (PPAR α), adipose triglyceride lipase (ATGL), carnitine palmitoyltransferase 1 alpha (CPT1 α), fibroblast growth factor 21 (FGF21), and uncoupling protein 2 (UCP2) in HepG2 cells. Together, NaP and NaB may produce synergistic effects by significantly increasing CPT1 α , PPAR α , and UCP2 mRNA expression in LPS-induced HepG2 cells and by significantly increasing CPT1 α and ATGL mRNA expression in palmitate/oleate-induced HepG2 cells. Only NaP treatment may

have the ability to reverse hepatic steatosis and increase whole-body energy expenditure by significantly increasing FGF21 mRNA expression in palmitate/oleate-induced HepG2 cells. The ELISA results reveal that only LPS significantly increased Tumor Necrosis Factor alpha (TNF- α) expression in HepG2 cells. At the same time, NaP alone or in combination with NaB significantly decreased TNF- α expression in LPS-induced HepG2 cells. The expression of IL-8 in both models showed no significant differences in all treatments. The Western blot analysis of CPT1 α protein expression increased by NaP alone or in combination with NaB in the palmitate/oleate model. In conclusion, this study shows promising results for the use of NaP and NaB as a potential new therapy in NAFLD.

CHAPTER 1. LITERATURE REVIEW

1.1. Non-Alcoholic Fatty Liver Disease Overview

The Western diet often consists of the high consumption of fats and carbohydrates, which leads to a caloric surplus and multiple metabolic diseases such as non-alcoholic fatty liver disease (NAFLD). This disease is the accumulation of fat build up in the liver with little to no consumption of alcohol and is the most common chronic liver disease around the world [1]. The liver is a multifunctional organ and is a principal regulator of lipids in the body [2]. However, when the liver's weight is 5-10% fat, it is considered a fatty liver which causes the increase of free fatty acids, oxidative stress, and subsequently inflammation and fibrosis. NAFLD affects around 35% of the general population and 76%-90% of disease-specific groups, such as obesity and diabetes [1]. The rising prevalence of NAFLD has made it the second most common cause of liver transplantation in the United States [3]. NAFLD is strongly associated with obesity, cardiovascular disease (CVD), including coronary heart disease and stroke, and metabolic syndrome, including insulin resistance, type 2 diabetes mellitus, hypertension, and dyslipidemia [4]. The continuum of NAFLD starts as fat accumulation in the liver, then inflammation and scarring that leads to non-alcoholic steatohepatitis (NASH), and lastly, cirrhosis occurs in which scar tissue replaces the liver cells.

The prevalence of NAFLD is difficult to diagnosis since patients are usually asymptomatic until they develop cirrhosis [5]. The diagnosis is often due to incidental elevations of serum alanine transaminase (ALT) and aspartate aminotransferase (AST) [5]. The gold standard for quantitating the stages of NAFLD is liver biopsy but can be costly, cause sampling error, and increases the chance of complications [5]. The main

pathologic classifications of NAFLD are Matteoni's, Brunt's, Kleiner's classification with the NAFLD Activity Score (NAS), Fatty Liver Inhibition of Progression (FLIP) algorithm with Steatosis Activity, and Fibrosis (SAF) Score [6]. Currently, NAS is the most commonly used examination in clinical trials [6]. However, there are many limitations, such as high variability among pathologists, poor correlation with metabolic risk factors, and the inability to predict fibrosis progression [7, 8]. Imaging methods are the most widely used non-invasive techniques for the diagnosis of NAFLD [6]. Ultrasonography is the most common method because of its safety, low cost, and ability to detect if a liver has more than 30% fat [6]. The main limitation of this method is the inability to differentiate steatosis from fibrosis [6].

1.2. Molecular Mechanisms in Non-Alcoholic Fatty Liver Disease

The liver is an essential organ in lipid metabolism and a central regulator of lipid homeostasis [9]. The liver is responsible for the synthesis of new fatty acids and their export and distribution to other tissues, as well as their use as energy substrates [9]. These processes are regulated by complex interactions between hormones, nuclear receptors, and transcription factors, which keep hepatic lipid homeostasis under control [10]. The disturbance of one or more of these pathways may cause the retention of fat within the liver, which can cause the development of NAFLD [9]. Hepatic fat build up results from an imbalance between lipid acquisition and lipid disposal, which includes four major pathways: uptake of circulating fatty acids, *de novo* lipogenesis (DNL), fatty acid oxidation (FAO), and export of lipids in very low density lipoproteins (VLDL) [9].

The uptake of circulating fatty acids by the liver is predominately reliant on fatty acid transport proteins (FATP), cluster of differentiation 36 (CD36), and caveolins located

inside the hepatocyte plasma membrane [11, 12]. The two main FATP isoforms found in the liver are FATP2 and FATP5 [12]. The fatty acid translocase protein, CD36, transports long-chain fatty acids and is controlled by peroxisome proliferator-activated receptor-gamma (PPAR γ), pregnane X receptor, and liver X receptor [9]. The caveolins are membrane proteins that contribute to lipid trafficking and the creation of lipid droplets [12]. After uptake of fatty acids, hydrophobic fatty acids must be transferred between different organelles by specific fatty acid-binding proteins (FABP) such as FABP1 because they cannot diffuse freely in the cytosol [11]. FABP1 is the predominant isoform in the liver and helps with the storage, transportation, and utilization of fatty acids and their acyl-CoA derivatives [11, 12].

The DNL pathway allows the liver to create new fatty acids from acetyl-CoA [9]. First, acetyl-CoA is turned into malonyl-CoA by acetyl-CoA carboxylase (ACC), and malonyl-CoA is changed into palmitate by fatty acid synthase (FASN) [9]. New fatty acids may undergo a series of desaturation, elongation, and esterification steps before being stored as triglycerides or distributed as VLDL particles [9]. Therefore, increased DNL can cause NAFLD since saturated fatty acids, such as palmitate, cause inflammation, and apoptosis [9].

The peroxisome proliferator-activated receptor alpha (PPAR α) controls the FAO pathway and occurs primarily in the mitochondria, which generates adenosine triphosphate (ATP), mainly when circulating glucose levels are low [9]. In mammalian cells, the peroxisomes, mitochondria, and cytochromes mediate FAO [13, 14]. In the outer mitochondrial membrane, carnitine palmitoyltransferase 1 alpha (CPT1 α) facilitates the entry of fatty acids into the mitochondria; however, mitochondria cannot oxidize very long

chain fatty acids and preferably metabolize via peroxisomal beta (β)-oxidation [15, 16]. The activation of PPAR α induces the transcription of a range of genes related to FAO in the mitochondria (medium- and long-chain acyl-CoA dehydrogenases), peroxisomes (acyl-CoA oxidase (ACOX) 1 and enoyl-CoA hydratase), and cytochrome-mediated (CYP4A1 and CYP4A3) [13, 16-18]. Oxidative damage and lipid oxidation to mitochondrial DNA diminish mitochondrial function, creating a vicious cycle to aggravate mitochondrial dysfunction and oxidative stress [16].

Oxidative stress causes the imbalance between the factors that generate reactive oxygen species (ROS) and those that protect the antioxidant system, which leads to structural modifications of biomolecules, loss of cell signaling and gene expression control, and apoptosis [19-22]. The induction of oxidative stress resulting from mitochondrial oxidation of fatty acids leads to lipid peroxidation, advanced glycation end products (AGEs), and chronic inflammation, which are involved in the progression of NAFLD [23]. AGEs are molecules produced by *in vivo* glycation and oxidation or can occur in foods that reach elevated temperatures, such as frying and grilling [24]. Glycation occurs when reducing sugars or oxidized lipids react with the epsilon (ϵ)-side of amino acids in proteins, amino phospholipids, or nucleic acids without enzymatic regulation and are the primary cause of internal and external protein damage [23]. The formation of unstable Schiff bases in glycation undergoes rearrangements, which generates Amadori products that make the structure more stable and are the first products of the Maillard reaction [23]. Amadori products give rise to AGEs due to their reactive carbonyl groups that condense with primary amino groups (Figure 1.1.) [23].

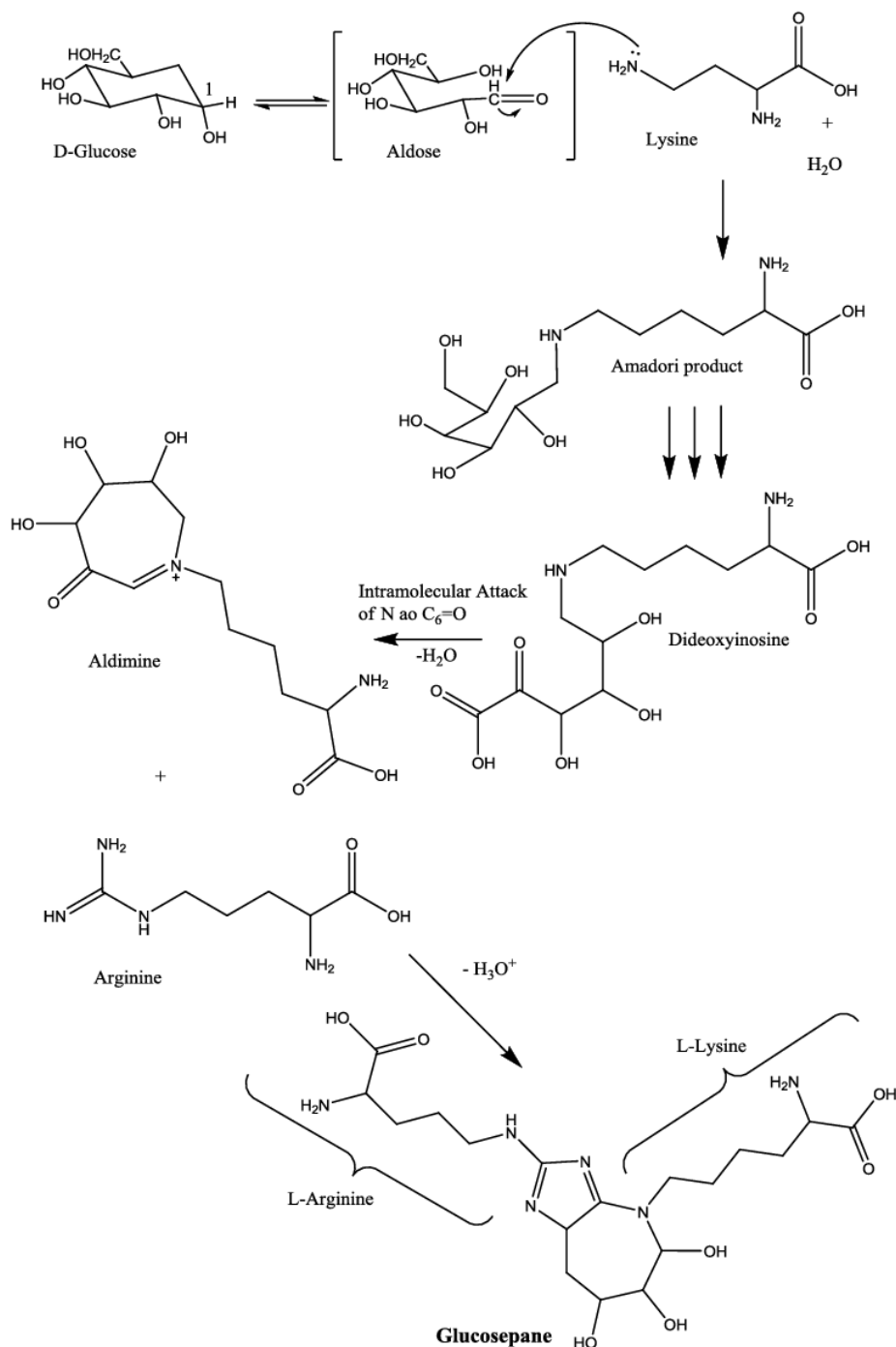


Figure 1.1. Formation of AGEs from reducing sugars. Amadori product formed from a nucleophilic attack on the anomeric carbon of the sugar by lysine. After consecutive displacements, the intermediate α -dicarbonyl dideoxyinosine will form. The α -dicarbonyl intermediate undergoes a nucleophilic attack on the carbonyl group by the ϵ -amine, giving aldimine, a precursor for cross-linking agent Lysine-Arginine, to form glucosepane. C-1, Anomeric carbon on sugar; epsilon (ϵ)-amine, of lysine.

Typically, glycation occurs slowly; however, under conditions of lipid peroxidation, the generation of AGEs increases drastically [23]. AGEs can cross-link with specific molecules by changing the chemical and biological properties or by interacting with matrix proteins and specialized receptors such as receptors for advanced glycation end products (RAGE) [23]. Oxidative stress stimulates AGEs/RAGE interaction by increasing the production of ROS and activating transcription factors, which lead to local tissue damage and higher inflammatory responses [23].

The liver has immune cells that try to cope with stress by recruiting cells such as macrophages or monocytes in response to injury by emitting pro-inflammatory signals, including cytokines, chemokines, and ROS [23]. The expression of inflammatory cytokines and chemokines plays a significant role in the pathogenesis of NAFLD [25]. Pathogen-Associated Molecular Patterns (PAMPs) refer to many bacterial products such as lipopolysaccharide (LPS), derived from the cell wall of gram-negative bacteria, and other molecules such as peptidoglycans, bacterial lipoprotein flagellins, bacterial RNA and DNA, which can reach the liver upon disturbance of the intestinal mucosal barrier and trigger innate immune cells, causing intracellular signaling cascades that intensify injury [26]. LPS binds to specific receptor-activating toll-like receptors (TLRs) such as TLR4 and TLR9 and can activate Interleukin (IL)-1 β , IL-18, IL-8, IL-6, IL-12, and TNF- α , which subsequently induce inflammation and fibrosis [26-29].

The last pathway is the export of triglycerides which is the only way to diminish hepatic lipid content [30]. Fatty acids are hydrophobic and must be packed into water-soluble VLDL particles along with apolipoproteins, cholesterol, and phospholipids before leaving the liver [9]. VLDL particles form inside the endoplasmic reticulum, where

apolipoprotein B100 (apoB100) is lipidated via the enzyme microsomal triglyceride transfer protein (MTTP) [9]. The developing VLDL particle must be further lipidated until it forms a mature VLDL particle, and this process occurs during transportation to the Golgi apparatus [31]. The apoB100 is required for VLDL export, while the triglyceride content varies [32, 33]. Both components, apoB100 and MTTP, are crucial in sustaining hepatic lipid homeostasis and hepatic VLDL secretion [9]. When the export does not occur, this results in hepatic lipid overload and intracellular lipid accumulation, which leads to steatosis, lipotoxicity, liver damage, and fibrosis [9].

1.3. Risk Factors for Non-Alcoholic Fatty Liver Disease

The primary risk factors for NAFLD include obesity, atherogenic dyslipidemia, hypertension, insulin resistance (IR) and glucose intolerance, CVD, genetics, and ethnicity [34]. Many rare genetic conditions can cause dysfunction of the standard processing of nutrients and lipids inside the liver [35]. Also, ethnicity plays a part in which Hispanics have the highest prevalence of NAFLD, followed by non-Hispanic whites and then African Americans [1]. The secondary risk factors include family history, gender, age, polycystic ovary syndrome, environmental toxins, medications, obstructive sleep apnea [34]. In terms of heritability, a fatty liver is significantly more common in siblings (59%) and parents (78%) of children with NAFLD [36]. Men have an increased prevalence of NASH, and women typically develop the disease later than men do [37]. The major contributors that cause these risk factors to develop are the Western diet that causes oxidative stress, inflammation, and gut microbiota changes.

1.3.1. Western Diet

The rapid increase in the prevalence of obesity, metabolic syndrome, and NAFLD all relate to the Western diet, which often consists of excessive caloric intake due to the increased consumption of elevated levels of sugar and fat with a sedentary lifestyle [38]. The liver is a vital organ for protein, fats, and carbohydrate metabolism, catabolism, and excretion of toxins [39]. Any functional impairment can affect the whole organism, which can lead to morbidity and mortality [39]. Globally, fructose consumption has increased over the last decade [39]. Fructose is a monosaccharide that is naturally present in fruit and honey and has a high sweetness over other sugars [40]. Fructose is also a significant component in sucrose also known as table sugar, which is a disaccharide of fructose and glucose, and in high fructose corn syrup (HFCS) which is a mixture of fructose and glucose monosaccharides [40]. Sucrose and HFCS are considered risk factors for NAFLD and obesity [39, 40]. Studies in humans show that fructose induces DNL and inhibits fatty acid oxidation in the liver [41-45]. Glucose is a crucial energy source for the entire body and is metabolized mainly by glucokinase or hexokinase [40]. Fructose metabolism occurs mostly in the liver and is principally metabolized by fructokinase [40]. The major isoform of fructokinase in the liver is fructokinase C, which phosphorylates fructose quickly and causes a reduction of ATP and intracellular phosphate [40]. The decrease in intracellular phosphate stimulates the enzyme, adenosine monophosphate (AMP) deaminase, that changes AMP to inosine monophosphate (IMP), resulting in the formation of uric acid [40]. The fall in ATP levels causes multiple reactions to occur, including a brief block in protein synthesis, an induction in oxidative stress, and mitochondrial dysfunction [46-48]. Fructokinase C is the main enzyme that metabolizes

fructose in the liver and is highly expressed in the small intestine [40]. The metabolism of fructose in the intestine results in an altered gut microbiome which increases gut permeability through the loss of tight junctions [40]. Consequently, endotoxin gets into the portal vein and triggers the formation of a fatty liver, leading to the progression of NAFLD [40].

In NAFLD, a fatty liver is the consequence of the excessive build up of various lipids [49]. Triglycerides are the most common type of lipids in the fatty liver [49]. Palmitic (C16:0) and oleic (C18:1) acids are the most common FFAs in liver triglycerides [50]. Fatty acids can come from the diet or synthesize in cells through metabolic pathways such as DNL [2]. Depending on the nutrient and energy environments, fatty acids can be quickly oxidized in peroxisomes and mitochondria to sustain the cellular bioenergetic homeostasis [51]. This ability to completely oxidize fatty acids to create energy occurs in most mammalian cells, but mature red blood cells depend only on glucose utilization to yield adequate amounts of ATP for survival [52]. There are different types of fatty acids with specific properties based on chain length and degree of saturation [53]. Palmitic acid, a saturated long-chain fatty acid, can be produced from the diet or endogenously from other fatty acids, carbohydrates, and proteins [54]. In autoimmune diseases, palmitic acid enhances the differentiation of naive T cells into T helper 1 (Th1) and T helper 17 (Th17) cells, promoting inflammation through activation of the p38 mitogen-activated protein kinases (MAPK) pathway [55]. Oleic acid, a monounsaturated long-chain fatty acid, is found naturally in numerous animal and vegetable fats and oils [55].

1.3.2. Gut Microbiota

A delicate balance between gut microbiota, intestinal epithelial cells, and gut mucosal system is vital to sustaining intestinal permeability and tissue homeostasis [56]. When the gut microbiota becomes imbalanced inside the body, this refers to gut dysbiosis. The gut-liver axis is the concept of gut bacteria affecting liver homeostasis from the interaction between the gastrointestinal tract and the liver [57]. The liver is the first organ to drain the gut through the portal vein and plays a vital role in host-microbe interactions [57]. The portal blood contains molecules and nutrients that cross from the gut to the blood, which makes the liver one of the most exposed organs to intestinal bacteria and bacterial-derived products (Figure 1.2.) [58]. The disturbances of the gut–liver axis include gut barrier disruption, bacterial translocation, inflammatory response in the liver, and changes in the composition of bacterial products [57]. Microbiome-derived compounds affect the hepatocytes by small molecules that lead to pro-inflammatory signaling, variations in gene expression, and modifications in metabolism and toxicity [57]. Bile acids in the small intestine promote the release of β -Klotho, a membrane protein, that affects hepatic synthesis [59]. β -Klotho binds to its receptor, farnesoid X receptor (FXR), inside the intestinal epithelial cells and travels to the liver through portal vein [57]. The β -Klotho binds to fibroblast growth factor receptor 4 (FGFR4) on the surface of hepatocytes and causes an altered metabolism [57]. Bile acids also stimulate the Takeda G-coupled receptor 5 (TGR5) on the Kupffer cells, which produce the secretion of pro-inflammatory cytokines that cause pro-inflammatory signaling to hepatocytes [57]. Peptidoglycan and LPS, bacterial pattern molecules, signal Toll-like receptor 2 (TRL2), and Toll-like receptor 4 (TRL4) [57]. The short-chain fatty acid, acetate, binds to its receptor G-protein coupled

receptor 43 (GPCR) GPR43 and alters metabolism, while butyrate acts as a histone deacetylase (HDAC) inhibitor and regulates gene expression [57]. Phenylacetate has an unknown mechanism that affects the expression of metabolic genes, FASN and lipoprotein lipase (LPL), which cause metabolic changes [57]. Lastly, the toxic derivative of ethanol, acetaldehyde, causes high oxidative stress on the hepatocytes [57].

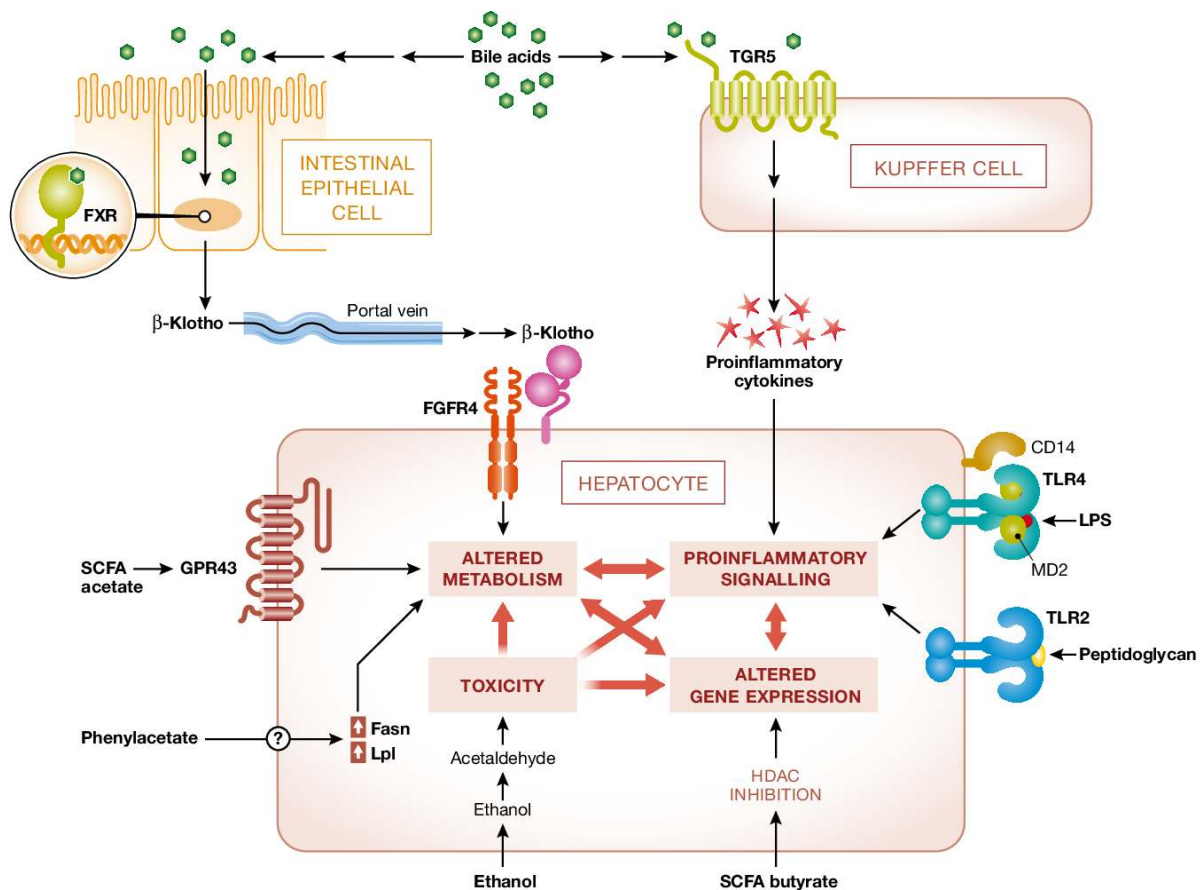


Figure 1.2. Gut microbiota-derived compounds affecting liver metabolism [57].

The healthy intestinal epithelium is an impenetrable physical barrier that keeps the host separate from the contents of the gut [57]. Tight junctions are essential regulators of intestinal permeability that prevent bacteria from entering the intestinal mucosa and

bloodstream under physiological conditions [60]. However, regulation becomes pathological with the disruption of tight junctions and extreme paracellular leakage of non-self antigens to the lamina propria, which leads to the progression of NAFLD and NASH [57].

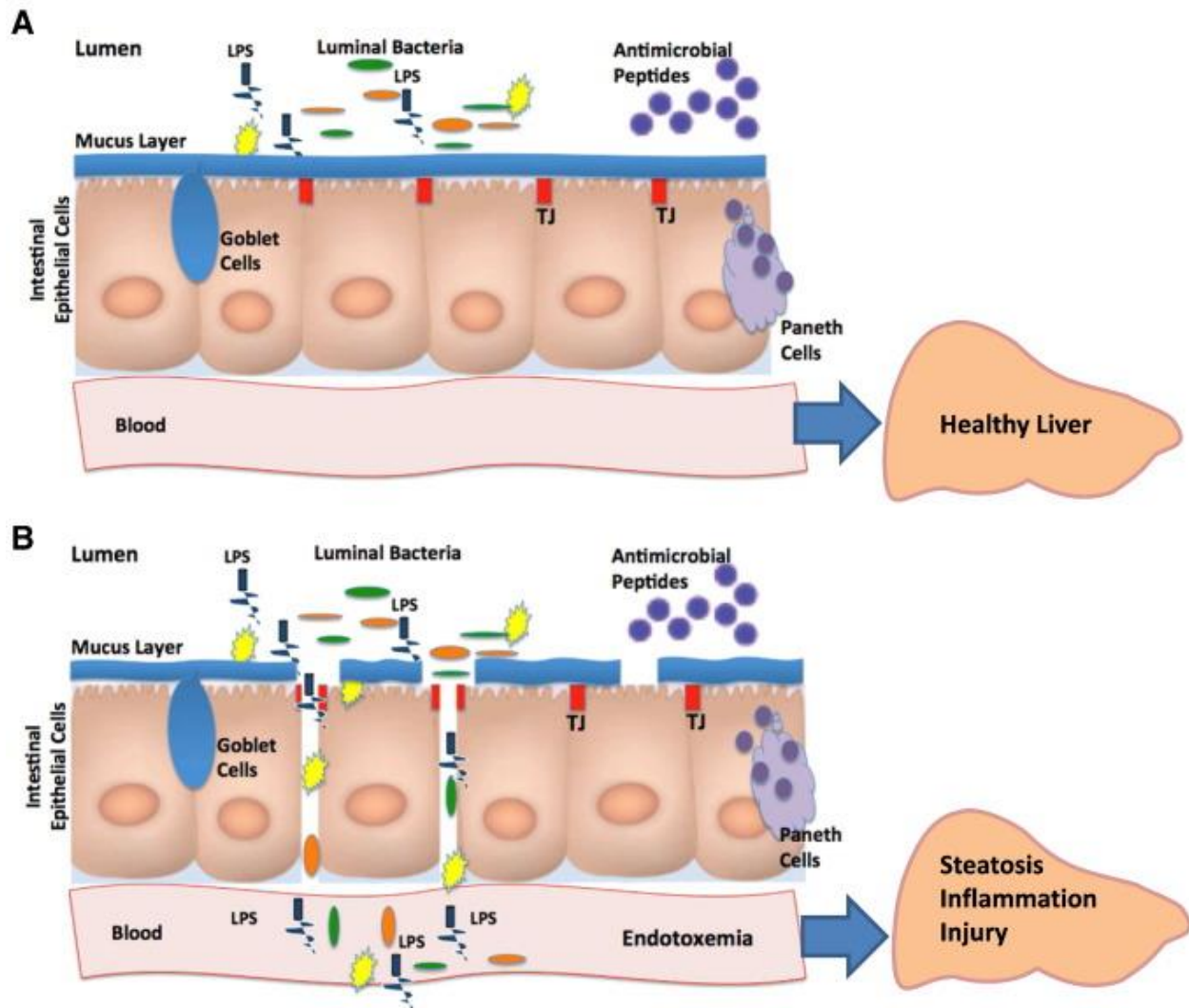


Figure 1.3. The intact (A) and disrupted (B) gut barrier.

The gut barrier consists of tight junctional complexes, a mucous layer produced by goblet cells, antimicrobial resistance facilitated by Paneth cells, and the complex network of innate and adaptive immune cell populations (Figure 1.3.) [57]. Gut barrier disruption causes the translocation of LPS from gram-negative bacteria to mucosa and circulation,

which initiates or enhances liver inflammation [57]. Multiple clinical and experimental data have confirmed that LPS significantly contributes to the development of obesity-related inflammatory liver diseases such as NAFLD and NASH [27, 61].

1.4. Treatment Options

The two main treatment options for NAFLD are dietary changes and lifestyle modifications. In some cases, body weight loss is not achievable. Therefore, a few recommendations include medications and supplements; however, there are multiple side effects. Unfortunately, the lack of understanding of this disease makes prevention a challenging option.

1.4.1. Therapeutic Approach

The most common antioxidant used in the treatment of NAFLD progression is Vitamin E [62]. However, the main concern for administering vitamin E as a treatment is the possibility of producing toxic doses and could potentially cause prostatic cancer or a hemorrhagic stroke [62]. Vitamin E treatment is beneficial for patients with biopsy-proven NASH who are non-diabetics and should receive a lower dose (300–400 mg/day) [63]. Pioglitazone treatment has been tested on NASH patients with prediabetes or type 2 diabetes and is confirmed to be safe and effective during a 3-year study [64]. The negative concerns for pioglitazone include prostate or pancreas cancer, fluid retention, weight gain, and cardiovascular events [62]. Other therapies investigate pentoxifylline (improves hepatic steatosis), obeticholic acid (improves hepatic steatosis, inflammation, and fibrosis), orlistat (improves insulin resistance), ursodeoxycholic acid, statins, omega-3 fatty acids, and glucagon-like peptide 1 receptor (GLP1R) agonists (improves hepatic steatosis) [65]. However, these therapies have shown to give varying and limited benefits.

1.4.2. Dietary Approach

New opportunities arise for bioactive compounds found in specific foods to address inflammation in NAFLD. Bioactive fatty acids include a variety of structures, from simple to complex fatty acids, each playing multiple roles in the body such as cell proliferation, metabolic homeostasis, and regulation of inflammatory progressions [66, 67]. Bioactive fatty acids also play a part in biological effects in cell signaling pathways by adjusting lipid composition [55]. The dietary fatty acid composition is a significant factor in NAFLD development since 15% of liver triglycerides come from the diet [68]. The diet can expose the liver to several types of lipids, such as fatty acids, cholesterol, and triglycerides [69].

1.4.2.1. Short-Chain Fatty Acids Significance

Humans lack the necessary enzymes to degrade dietary fibers. These nondigestible carbohydrates pass into the upper gastrointestinal tract unchanged and are fermented by anaerobic microbiota in the cecum and the large intestine. The bacterial fermentation produces multiple groups of metabolites [70]. The major group produced is short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, which can differ from one to six carbons (Figure 1.4.) [71].

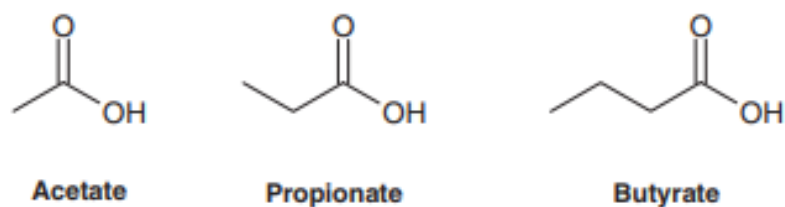


Figure 1.4. Chemical structures of acetate, propionate, and butyrate

SCFAs play a role in the prevention and treatment of various clinical studies due to their roles in energy homeostasis, cell proliferation, metabolic homeostasis, and regulation of inflammatory processes [66, 67]. Butyrate is found in dairy milk, parmesan

cheese, and butter but can also be produced by bacteria from resistant starches and dietary fiber [69, 72]. Butyrate is the primary energy source for colonocytes, and the remaining fraction goes through the hepatic portal vein to the liver [69]. Butyrate maintains cellular homeostasis in the intestine, stimulates cell proliferation in normal colonocytes, and inhibits cell proliferation in cancer cell lines *in vitro* [73]. Propionate is considered a substrate for hepatic gluconeogenesis [74]. In the liver, 90% of propionate is absorbed, and the rest sent into the peripheral blood system [75]. Propionate has beneficial anti-inflammatory properties, antihypertensive effects, cardioprotective effects, and influences T helper cell homeostasis [76].

1.4.2.2. Biosynthesis and Metabolism of Short-Chain Fatty Acids

The microbial transformation of dietary fiber to monosaccharides inside the gut involves multiple reactions facilitated by the enzymatic supply of specific gut microbiota (Figure 1.5.) [75]. The result of these fermentations leads to the production of SCFAs. Acetate comes from pyruvate via the acetyl-CoA route or the Wood-Ljungdahl pathway [75]. Next, acetate can form through two different branches, the Eastern branch via reduction of CO₂ to formate or the Western branch via reduction of CO₂ to CO, which is united with a methyl group to yield acetyl-CoA [75]. Propionate starts as phosphoenolpyruvate (PEP) via the succinate pathway, where succinate turns into methylmalonyl-CoA, and lastly, propionyl-CoA into propionate [75]. Other pathways include the acrylate pathway where the reduction of lactate produces propionate or the propanediol pathway, where deoxyhexose sugars such as fucose and rhamnose are substrates [75]. The third SCFA, butyrate, forms from two molecules of acetyl-CoA,

yielding acetoacetyl-CoA, which converts to butyryl-CoA via β -hydroxybutyryl-CoA and crotonyl-CoA [75].

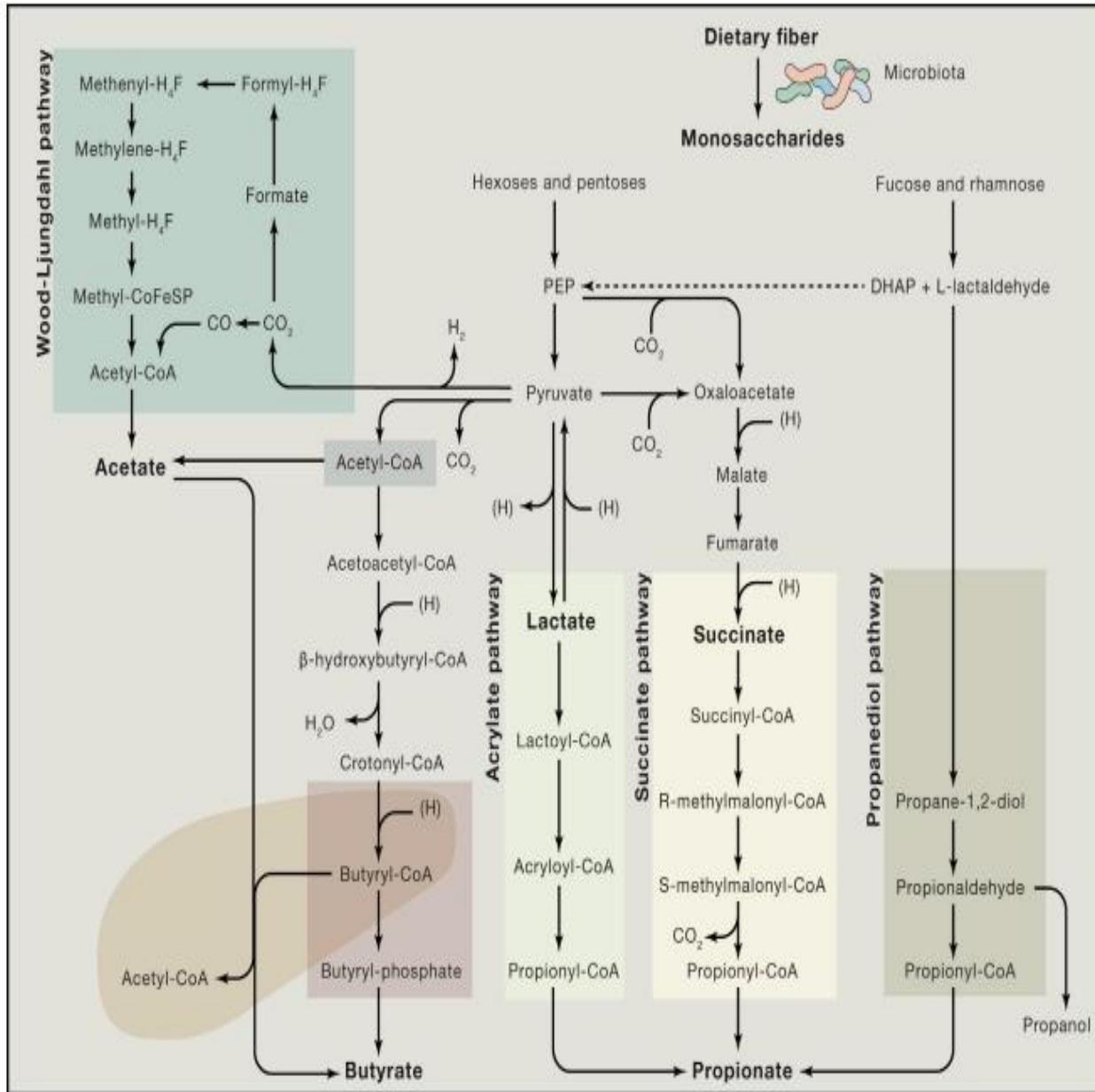


Figure 1.5. Pathways for the biosynthesis of short-chain fatty acids from dietary fiber

Once the generation of SCFAs occurs, they can enter the cells via monocarboxylate transporters (MCTs), passive diffusion, or exchange with bicarbonate (HCO_3^-) via partially oxidized CO_2 [77]. A list of all known SCFA transporters is shown in

Table 1.1. [77]. After transportation, the colonocytes absorb the SCFAs and enter the citric acid cycle inside the mitochondria to generate ATP and energy for the cells [78]. All three SCFAs via oxidation enter hepatocytes inside the liver as energy substrates or for the biosynthesis of glucose, cholesterol, and fatty acids [77].

Table 1.1. Short-Chain Fatty Acid Transporters

Transporter	Localization in the Body	Localization in the CNS and/or Brain	SCFA Substrate
MCT1 (SLC16A1)	Ubiquitous; apical membrane and basolateral membrane of the colonic epithelium and small intestine	Ubiquitous; brain endothelial cells, astrocytes, ependymocytes, and some neurons in rats	Acetate, propionate, and butyrate; butyrate uptake involves this transporter
SMCT1 (SLC5A8)	Entire large intestine (apical membrane), kidney, and retina	Neurons	Acetate, propionate, and butyrate; butyrate faster than propionate and acetate
SMCT2 (SLC5A12)	The apical membrane of the colonic epithelium and small intestine, kidney, and retina	Astrocytes and glia	Low affinity for propionate and butyrate
SLC26A3	Apical site of colonocytes and basolateral site of colonocytes	ND	Acetate, propionate, and butyrate
MCT2 (SLC16A7)	Stomach and small intestine	-Ubiquitous but high expression in cortex, hippocampus, and cerebellum -Neurons and some astrocytes in rats	Low affinity for acetate, propionate, and butyrate
MCT4 (SLC16A3)	The basolateral membrane of colonic epithelium, small intestine skeletal muscle, brain, kidney, placenta, leukocytes, heart, lung, and chondrocytes	-Ubiquitous but high expression in cortex, hippocampus, and cerebellum -Astrocytes	High affinity for butyrate
OAT7 (SLC22A9)	Liver and sinusoidal membrane of hepatocytes	ND	Butyrate
OAT2 (SLC22A7)	Kidney and liver	ND	Propionate

CNS, central nervous system; MCT, monocarboxylate transporter; ND, not determined; OAT, organic anion transporter; SCFA, short-chain fatty acid; SMCT, sodium-dependent monocarboxylate transporter.

1.4.2.3. Mechanism of Short-Chain Fatty Acids

The role of SCFAs involves cellular signaling pathways and their interaction with gut-brain pathways, which include immune, endocrine, vagal, and other humoral pathways (Figure 1.6.) [77].

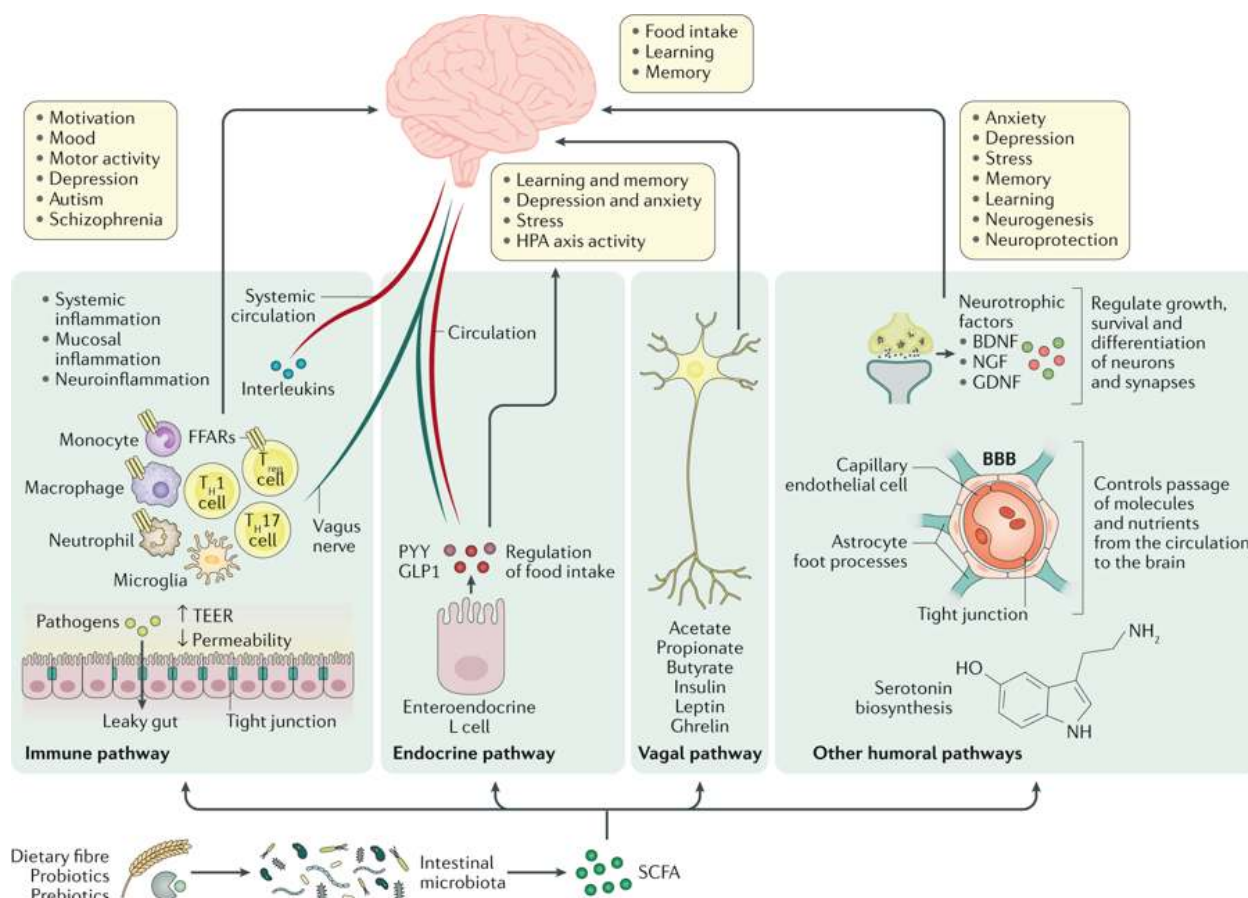


Figure 1.6. The gut-brain pathways where SCFAs may modify or control brain function. BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; HPA, hypothalamus-pituitary-adrenal; NGF, nerve growth factor; TH1, T helper 1; TH17, T helper 17; Treg cell, regulatory T cell.

Through the immune pathway, SCFAs can improve barrier integrity by upregulating the expression of tight junction proteins and increasing transepithelial electrical resistance (TEER) [77]. SCFAs can also activate free fatty acid receptors (FFARs) by interacting with intestinal epithelial cells and immune cells or by inhibiting

HDAC [77]. Histone acetylation begins as a fundamental switch that permits the interconversion between acetylation and deacetylation [77]. The removal of acetyl groups of histone tails occurs by HDAC inhibitors, while the additional acetyl groups of histone tails occur by histone acetyltransferases (HATs) [77]. Many studies report that HDAC inhibitors are involved in cancer therapy, brain development, and a range of neuropsychiatric diseases, including depression, schizophrenia, Alzheimer's disease, and addiction [79]. Butyrate and propionate are both known to act as HDAC inhibitors which possess antiproliferative effects, anti-inflammatory properties, reduce insulin resistance, and stimulate hepatic fatty acid β -oxidation [80, 81]. SCFAs via HDAC inhibition can also regulate cytokine expression in T cells and the production of Treg [82]. Butyrate is the most investigated SCFA due to its high presence in the gut lumen, and primary energy source for colonocytes [83]. Normal colonocytes oxidize butyrate, whereas nuclear extracts from cancer cells accumulate butyrate 3-fold [84]. Therefore, butyrate can act as an efficient HDAC inhibitor in cancerous cells and a HAT activator in healthy cells. Through HDAC inhibition, butyrate makes the immune system hyporesponsive to beneficial commensals by suppressing pro-inflammatory effectors in lamina propria macrophages [77]. The beneficial health outcomes of using SCFAs includes their HDAC-inhibiting activity, high abundance (mM range), and energetics of cells using fatty acids for energy and generation of ATP versus glycolysis.

The endocrine pathway is when SCFAs interact with their receptors on colonocytes, which induce indirect signaling to the brain by inducing the secretion of gut hormones such as glucagon-like peptide 1 (GLP1) and peptide YY (PYY) from enteroendocrine L cells via the systemic circulation or vagal pathways [77].

These hormones impact learning, memory, and mood [77]. SCFAs can also signal to the brain by directly activating vagal afferents via FFAR [85]. Propionate can activate GPCRs, FFAR-2 and FFAR-3, also known as GPR43 and GPR41, respectively [81]. Propionate has the highest rank order of potency compared to acetate and butyrate in both GPR41 and GPR43 [86]. The mechanism of propionate involves changing invasive phenotypes to non-invasive phenotypes [87]. GPR41 is in the gut, adipose tissue, and the peripheral nervous system (PNS), while GPR43 is in adipose tissue, immune cells, and the intestine [88]. GPR43 releases PYY and GLP-1, affecting satiety and intestinal transit [89]. GPR41 signaling involves propionate-induced intestinal gluconeogenesis by gut-brain neurocircuitry [90]. Both propionate and butyrate increase intestinal glucose production; however, butyrate directly upregulates intestinal gluconeogenesis genes (G6PC and PCK1), while propionate does indirectly [91]. Propionate receptor GPR41 is present in the nerve fibers of the portal vein wall and can send signals to the peripheral and central nervous system (CNS) areas to induce intestinal gluconeogenesis [77]. Butyrate can signal GPR109A, which exerts immune-suppressive mechanisms by increasing IL-18 secretion, generating Treg, and IL-10 producing T cells via signaling [92]. GPR109A is a receptor for nicotinate (niacin) and facilitates lipid-lowering mechanisms [92]. The signaling of GPR109A is present on adipocytes, immune cells, and colonocytes [92]. Through the binding of GPCRs, SCFAs may affect the CNS and PNS, which is a qualification for their assumed effects on psychological developments.

Lastly, SCFAs can cross the blood-brain barrier (BBB) via monocarboxylate transporters located on endothelial cells and influence BBB integrity by inhibiting pathways associated with inflammatory responses [77]. SCFAs also modulate levels of

neurotrophic factors centrally, via histone acetylation and can contribute to the biosynthesis of serotonin [83, 93]. Together, the interaction of SCFAs with these gut-brain pathways can directly or indirectly modulate processes associated with neural functioning, learning, memory, and mood.

1.5. Research Objective and Hypothesis

NAFLD is the most common cause of chronic liver disease [5]. Unfortunately, patients are asymptomatic in the early stages, which makes diagnosis difficult [5]. Currently, the only treatment is regular exercise and a healthy diet. The main objective of this doctoral dissertation research is to evaluate the mechanisms behind sodium butyrate (NaB) and sodium propionate (NaP), which could potentially serve as bioactive compounds that relieve oxidative stress and inflammation in NAFLD progression. We hypothesize that NaP alone, NaB alone, or in combination, will promote FAO and reduce pro-inflammatory cytokines in HepG2 cells.

1.6. Notes

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CHAPTER 2. SODIUM PROPIONATE AND SODIUM BUTYRATE PROMOTE FATTY ACID OXIDATION IN HEPG2 CELLS UNDER OXIDATIVE STRESS

2.1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a series of disturbances that involve various steps of liver damage including simple steatosis, which is fat accumulation in liver cells, to non-alcoholic steatohepatitis (NASH), fat build up plus inflammation in liver cells, that can eventually lead to hepatic fibrosis, cirrhosis, or liver cancer [1]. NAFLD affects individuals that consume the typical Western diet consisting of high levels of refined sugars, fats, and other refined carbohydrates such as starch. Consequently, the increase of circulating free fatty acids (FFAs) as a result of unhealthy white adipose tissue causes oxidative stress and liver inflammation. Palmitic acid, a saturated fatty acid, and oleic acid, a monounsaturated fatty acid, are the most abundant FFAs in the diet and fatty liver [2]. Palmitic acid is in palm oil, meat, dairy products, cocoa butter, olive oil, and breast milk [3, 4]. Oleic acid is mostly in olive and canola oil but also found in animal fats such as lard [5].

One important mechanism contributing to the development of NAFLD is gut dysbiosis, where the imbalance of gut bacteria affects liver homeostasis [6]. The liver is the first organ to drain the gut through the portal vein and plays a vital role in host-microbe interactions [6]. The portal blood contains water-soluble molecules and nutrients that cross from the gut to the blood, which makes the liver one of the most vulnerable organs to intestinal bacteria and bacterial-derived products [6]. A leaky and inflamed gut can lead to the translocation of lipopolysaccharide (LPS), which comes from the cell wall of gram-negative bacteria and worsens liver inflammation. The relationship between metabolic

disorders, inflammation, a Western diet with overconsumption of energy, oxidative stress, and the changes in gut microbiota might result from LPS exposure [7]. The liver, in response to the elevated levels of palmitic and oleic acid or LPS, activates the release of numerous pro-inflammatory cytokines, such as Interleukin (IL)-8, IL-6, and Tumor Necrosis Factor alpha (TNF- α), within the liver microenvironment [8, 9].

One of the major pathways disrupted in NALFD is fatty acid oxidation (FAO), which occurs primarily inside the mitochondria and generates adenosine triphosphate (ATP) [10]. Peroxisome proliferator-activated receptor alpha (PPAR α), a transcriptional activator of hepatic lipid metabolism genes such as carnitine palmitoyltransferase 1 alpha (CPT1 α), controls the FAO pathway [11]. CPT1 α is the rate-limiting enzyme of fatty acid beta (β)-oxidation because it is involved in getting palmitic acid across the outer mitochondrial membrane into the mitochondrial matrix [12, 13]. In NAFLD patients, both PPAR α and CPT1 α levels significantly decrease in comparison to a healthy liver [14]. Short-chain fatty acids (SCFAs) such as butyrate and propionate can activate free fatty acid receptors (FFARs) by inhibiting histone deacetylases (HDAC), leading to greater gene expression for FAO genes, which stimulates hepatic fatty acid β -oxidation [15]. Many studies report that HDAC inhibitors possess anti-proliferative effects, anti-inflammatory properties, and regulate cytokine expression [16-18]. Butyrate is found in butter, parmesan cheese, and the lipid component of dairy milk, but can also form from resistant starches and dietary fiber in the large intestine [19, 20]. After absorption of butyrate, colonocytes use it as their primary source of energy, and the remaining fraction goes through the hepatic portal vein to the liver [19]. Butyrate has beneficial effects that include anti-inflammatory properties, increases in mitochondrial activity, prevention of metabolic endotoxemia, increases in

intestinal barrier function, and helps reduce body weight [20, 21]. Butyrate also has low systemic toxicity, which makes it an excellent agent for clinical trials. Propionate can provide energy for epithelial cells and is considered a substrate for hepatic gluconeogenesis [22]. The liver absorbs about 90 % of propionate, and the rest is sent into the peripheral systemic blood [14]. Propionate has beneficial anti-inflammatory properties, anti-hypertensive effects, and cardioprotective effects [23]. Both butyrate and propionate reduce food intake, weight gain, and high blood sugar [24].

In this study, we are evaluating the mRNA expression of genes associated with fatty acid metabolism and the protein expression of pro-inflammatory cytokines related to NAFLD using the HepG2 human liver hepatocellular carcinoma cells exposed to palmitate and oleate or LPS. The mechanisms behind sodium butyrate (NaB) or sodium propionate (NaP) could potentially serve as bioactive compounds that relieve oxidative stress and inflammation in liver cells. We hypothesize that NaB or NaP or in combination will promote FAO and reduce pro-inflammatory cytokines in HepG2 cells.

2.2. Materials and Methods

2.2.1. Reagents

Radioimmunoprecipitation assay (RIPA) buffer (#89900), protease inhibitors (#5871S), phosphatase inhibitors (#5870S), Gibco™ fetal bovine serum (FBS) (#26140079), phosphate buffer solution (PBS) (#10010023), Gibco™ gentamicin/amphotericin solution (#R01510) and Gibco™ 1X minimum essential medium (MEM) (#11095072), and Ambion® RNAsecure™ (#AM7005) were obtained from ThermoFisher Scientific (Waltham, MA). TRI reagent (#T9424), fatty acid free-bovine serum albumin (BSA) endotoxin-free (#A8806), lipopolysaccharide from *Escherichia coli*

O111:B4 (#L4391), penicillin-streptomycin (#P0781), and 1-Bromo-3-chloropropane (BCP) (#B9673) were obtained from Sigma Aldrich (St. Louis, MO). Sodium palmitate (#P0007), sodium oleate (#O0057), sodium butyrate (#S0519), and sodium propionate (#P0512) were purchased from TCI America (Portland, OR).

2.2.2. Palmitate and Oleate Preparation

Stock solutions of 5mM sodium palmitate and 10mM sodium oleate were prepared in MEM with 10% defatted BSA. Briefly, sodium palmitate was dissolved in PBS and then heated at 70°C until completely clear, and quickly added to preheated MEM 37°C. Sodium oleate was directly added to preheated MEM. All preparations were filtered and stored at 4°C.

2.2.3. Sodium Butyrate and Sodium Propionate Preparation

The 100mM stock solutions were prepared for NaB and NaP in PBS. All preparations were filtered and stored at 4°C.

2.2.4. Cell Line and Culture Conditions

The HepG2 human hepatocellular carcinoma cells (ATCC HB-8065, Manassas, VA) were maintained in MEM with 10% FBS and 1% antibiotic (penicillin/streptomycin/amphotericin) at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. Just before reaching 80% confluence, cells were split (1:6) by trypsinization into 6, 24, or 96-well plates in MEM with 10% FBS and 1% antibiotic (penicillin/streptomycin/amphotericin) for 24h. All experimental treatments were conducted in MEM with 10% heat-inactivated FBS (inactivated by heating for 30 minutes at 56°C) and 1% antibiotic (penicillin/streptomycin/amphotericin) which was the vehicle control treatment. Cells were subjected to oxidative stress using LPS from *Escherichia*

coli O111:B4 (1ug/ml) in some experiments. In other experiments, cells were preloaded with a combination of palmitate (0.5mM) and oleate (0.5mM) for 24h. After 24h, cells were treated with various concentrations of NaB (2, 4mM), NaP (2, 4, and 8mM), or a combination of NaB (2mM) and NaP (4mM) for another 24h in the presence of LPS or the combination of palmitate and oleate.

2.2.5. Cell Viability using MTS Assay

The MTS assay (#G3582) was used to test cell viability from Promega (Madison, WI). The HepG2 cells were plated in a 96-well at 5×10^3 cells per well and treated with different concentrations of palmitate and oleate combined (0.5, 1, 2mM), LPS (400, 800, 1000 ng/ml), NaP (2, 4, 8mM), and NaB (2, 4, 8mM) for 24h. CellTiter 96® AQueous One Solution Reagent (100ul) was directly added to each cell culture well, incubated for 1h at 37°C in a humidified atmosphere containing 5% CO₂, and then absorbance was recorded at 490nm using the BioRad Model 680 microplate reader (Hercules, CA). The quantity of formazan product measured at 490nm absorbance is directly proportional to the number of living cells in culture. The relative cell viability equation was used to determine the percentage of living cells.

2.2.6. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

In a 24-well plate, cells were exposed to LPS from *Escherichia coli* O111:B4 (1µg/ml) or with a combination of palmitate (0.5mM) and oleate (0.5mM) for 24h. After 24h, cells were treated with various concentrations of NaB (2, 4mM), NaP (2, 4, and 8mM), or a combination of NaB (2mM) and NaP (4mM) for another 24h in the presence of LPS or the combination of palmitate and oleate. After 24h treatment, cell media was aspirated, and TRI Reagent (500µl) was added to each well. Total RNA was extracted

from cells using TRI Reagent and purified with the RNeasy Mini Kit (#74104) from Qiagen (Hilden, Germany). Briefly, cell homogenates were stored at room temperature for 5 minutes. Next, BCP (50 μ l) was added to each sample to facilitate the separation of organic (proteins) and aqueous phases (RNA). The samples were shaken by hand vigorously for 20 seconds, stored at room temperature for 5 minutes, and centrifuged at 12,000 rpm for 15 minutes at 4°C to clarify phases. The upper aqueous phase containing total RNA (200 μ l) was removed, added to ice-cold 100% RNA-free ethanol (220 μ l), and loaded onto RNeasy mini spin columns. The columns were washed with RW1 and RPE buffers to remove DNA and protein. Lastly, columns were transferred to new collection tubes with RNasecure (1 μ l). Total RNA was eluted in RNase-free water (50 μ l) and stored at -80°C until use.

The Qubit RNA BR Assay (#Q10210) and Qubit Fluorometer from ThermoFisher Scientific (Waltham, MA) were used to quantify RNA concentrations. Reverse transcriptase and qPCR were conducted sequentially in each reaction with the reverse PCR primer serving to prime cDNA synthesis using Superscript III Platinum One-Step Quantitative RT-PCR System (#11732020) from ThermoFisher Scientific (Waltham, MA). Primer and probe sets were purchased from Applied Biosystems (Pleasanton, CA) (Table 2.1.). Ribosomal Protein L13a (RPL13A) (Taqman® ID Hs04194366_g1, ThermoFisher Scientific, Waltham, MA.) was used as a housekeeping gene. The RT-PCR assay for each sample was performed each time in duplicate using the protocol for Applied Biosystems Instruments (7900 HT).

Table 2.1. Primer and Probe Sequences for qRT-PCR

Gene	Primer and Probe	Sequences	NCBI Reference
ATGL	Forward (5'-3')	CGTGTA CTGTGGGCTCATC	NM_020376.3
	Reverse (3'-5')	GGACACTGTGATGGTGTTCCTTA	
	Probe	ATGGTGGCATTTCAGACAACCTGC	
CPT1 α	Forward (5'-3')	GCGTTCTTTGTGACGTTAGATG	NM_001876.3
	Reverse (3'-5')	CGGCCGTGTTAGTAGAGATTTG	
	Probe	AGAAGGATACAGAAGTGAAGACCCGGA	
FGF21	Forward (5'-3')	GAGTCAAGACATCCAGGTTCC	NM_019113.4
	Reverse (3'-5')	GTATCCGTCTCAAGAAGCAG	
	Probe	CCTCAGGGTCAAAGTGGAGCGATC	
PGC-1 α	Forward (5'-3')	CACCAAACCCACAGAGAACA	NM_00145134.1
	Reverse (3'-5')	GGGTCAGAGGAAGAGATAAAGTTG	
	Probe	AAAGAAGTCCCACACACAGTCGCA	
PPAR α	Forward (5'-3')	GTCGATTTCAACAAGTGCCTTTC	XM_011530239.2
	Reverse (3'-5')	CAGGTAAGAATTTCTGCTTTCAGTT	
	Probe	AACGAATCGCGTTGTGGTGACATCC	
UCP2	Forward (5'-3')	CTACAAGACCATTGCACGAGAGG	NM_003355.2
	Reverse (3'-5')	AGCTGCTCATAGGTGACAAACAT	
	Probe	CCTCAGGGTCAAAGTGGAGCGATC	

ATGL, adipose triglyceride lipase; CPT1 α , carnitine palmitoyltransferase I alpha; FGF21, fibroblast growth factor 21; NCBI, National Center for Biotechnology Information; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator I alpha; PPAR α , peroxisome proliferator activated receptor alpha; qPCR, quantitative polymerase chain reaction; UCP2, Uncoupling Protein 2.

2.2.7. ELISA Analysis of TNF- α and IL-8

The cells were plated in 24-well plates and stimulated with LPS from *Escherichia coli* O111:B4 (1 μ g/ml) or with a combination of palmitate (0.5mM) and oleate (0.5mM). After 24h, cells were treated with various concentrations of NaB (2, 4mM), NaP (2, 4, and 8mM), or a combination of NaB (2mM) and NaP (4mM) for 24h in the presence of LPS or the combination of palmitate and oleate. After 24h treatment, culture plates were put on ice, each well was washed with ice-cold PBS, and cells were scraped into ice-cold RIPA buffer (300 μ l) containing protease and phosphatase inhibitors. Samples were pushed through an ice-cold 20G syringe needle four times to disrupt organelles and centrifuged at 12,000 rpm for 5 minutes. Cell supernatants were collected and immediately stored at -80°C until further use.

The levels of TNF- α (15.6pg/ml-1000pg/ml) (#EK0525) and IL-8 (15.6pg/ml-1000pg/ml) (#EK0413) were analyzed using ELISA kits from Boster Bio (Pleasanton, CA) following manufacturer's instructions. All samples were diluted using sample diluent provided in each ELISA kit at 1:25 for the LPS model and 1:50 for the palmitate and oleate model. The absorbance of each cytokine (pg/ml) was read at 450nm using a BioRad Model 680 microplate reader (Hercules, CA). Each sample was done in triplicate, and the protein concentration (pg/ml) of each sample is determined based on the standard curve.

2.2.8. Western Blot Analysis of CPT1 α

Cells were plated in 6-well plates and loaded with fat by exposure to a combination of palmitate (0.5mM) and oleate (0.5mM). After 24h, cells were treated with NaP (8mM) and the combination of NaB (2mM) and NaP (4mM) for 24h. Total cellular protein was harvested. Culture plates were put on ice, each well was washed with ice-cold PBS, and

cells were scraped into ice-cold RIPA buffer (300 μ l) containing protease and phosphatase inhibitors. Samples were pushed through an ice-cold 20G syringe needle four times to disrupt organelles and spun at 12,000 rpm for 5 minutes in a prechilled centrifuge to remove the insoluble fraction. Cell supernatants were collected and immediately stored at -80°C until further use. The protein concentration in samples was quantified using the bicinchoninic acid assay (BCA) (#23227) from ThermoFisher Scientific (Waltham, MA).

After BCA, to obtain equal sample volumes for loading on gels, RIPA buffer was added to each 50 μ g sample. Next, 4X Laemmli sample buffer (#161-0747, Bio-Rad) mixed with 5% 2-mercaptoethanol (#M6250, Sigma-Aldrich) was added to each sample and boiled at 100°C for 5 minutes to denature proteins. Samples were centrifuged and loaded onto TGX sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (7.5% TGX, Bio-Rad). Gels were run at 100V until the dye line was near the bottom. Proteins of interest were transferred to nitrocellulose membranes (#1620145, Bio-Rad), then blocked in 5% bovine serum albumin (BSA) in tris buffered saline with 0.1% tween-20 (TBST) for 1h. The primary antibody for β -actin (#A5316, Sigma) and CPT1 α (#sc-393070, Santa Cruz Biotechnology, Inc.) were prepared in TBS with 4% BSA and 0.5% tween-20 and incubated at room temperature for 3h. The membranes were washed three times for 5 minutes using TBST, then incubated for 1h with anti-mouse (#AP130P, Sigma-Aldrich) secondary antibody and washed three times for 5 minutes using TBST.

Visualization of proteins was performed in a dark room using chemiluminescence (Western Lightning Plus-ECL, PerkinElmer, Waltham, Massachusetts). Densitometric analyses were done using ImageJ software (National Institutes of Health), and the relative expression of the target protein versus β -actin was calculated.

2.2.9. Statistical Analysis

All experiments were performed at least three times. The differences between samples were analyzed using a one-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) test. Significance was set at $P < 0.05$ level. The results are expressed as mean \pm SE. Means with the asterisk (*) symbol indicate significantly different from the unstimulated control. Means with the pound (#) symbol indicate significantly different from LPS or palmitate/oleate. All analyses were performed using SAS 9.4 (SAS Institute, Cary, NC).

2.3. Results

2.3.1. Effect of Palmitate/Oleate, Lipopolysaccharide (LPS), and SCFAs on Cell Viability

To investigate the impact of palmitate/oleate, LPS, NaP, and NaB on cell survival in HepG2 cells, various concentrations were used for 24h. The results were consistent with other studies, showing palmitate/oleate at 1mM (80% live cells) and 2mM (70% live cells) were significantly different from the control (100% live cells). In the LPS model, all concentrations were not significantly different from the control, which suggests that up to 1ug/ml does not cause significant cell death in HepG2 cells. The concentrations of NaP and NaB were not significantly different from the control, which suggests that up to 8mM does not cause significant cell death in HepG2 cells.

2.3.2. FAO and Mitochondrial Related Genes in the LPS Model

The HepG2 cell line was used to investigate the response of NaP and NaB treatment in the LPS model. The dose of 1ug/ml for LPS has been used by others to induce inflammation and oxidative stress in HepG2 cells [25-28].

The treatment of NaB, NaP, or in combination increased the mRNA expression of peroxisome proliferator-activated receptor- γ coactivator (PGC-1 α), PPAR α , CPT1 α , and uncoupling protein 2 (UCP2).

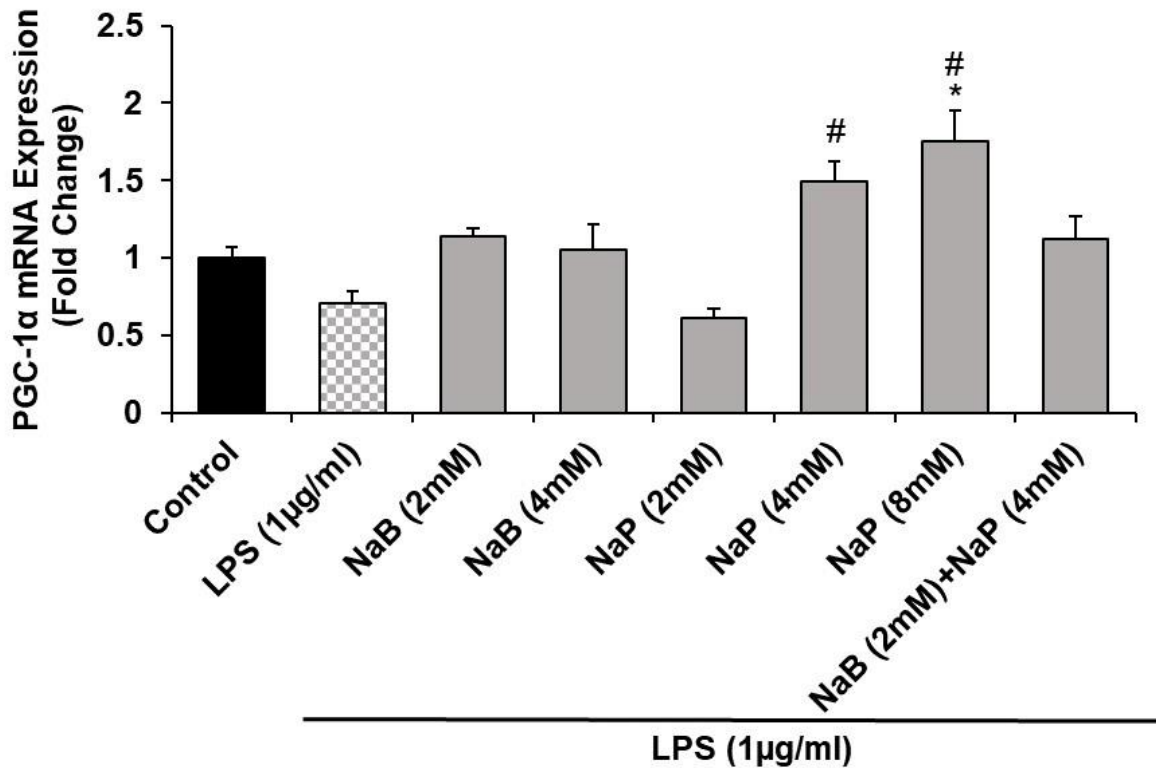


Figure 2.1. PGC-1 α mRNA expression in HepG2 cells alone (control), in the presence of LPS, or in the presence of LPS and various concentrations of NaB, NaP, or NaB and NaP mixture. All data were normalized using RPL13 α from three independent experiments. The results are presented as mean \pm SE (n=6). * p < 0.05 represents significantly different from the control; # p < 0.05 represents significantly different from LPS.

PGC-1 α is a potent activator of FAO and controls hepatic gluconeogenesis [29]. PGC-1 α expression was significantly increased in NaP 4mM by 1.49-fold in comparison to LPS and was significantly increased in NaP 8mM by 1.75-fold in comparison to the control and LPS (Figure 2.1.)

PGC-1 α is also known to control the transcriptional activity of PPAR α , an enzyme involved in lipid oxidation. PPAR α was significantly increased in NaB 2mM, NaB 4mM, NaP 4mM, NaP 8mM, and the combination of NaB 2mM and NaP 4mM by 1.28-fold, 1.33-fold, 1.46-fold, 1.68-fold, and 1.54-fold, respectively in comparison to LPS, while NaP 8mM and the combination of NaB 2mM and NaP 4mM was significantly different from the control (Figure 2.2.).

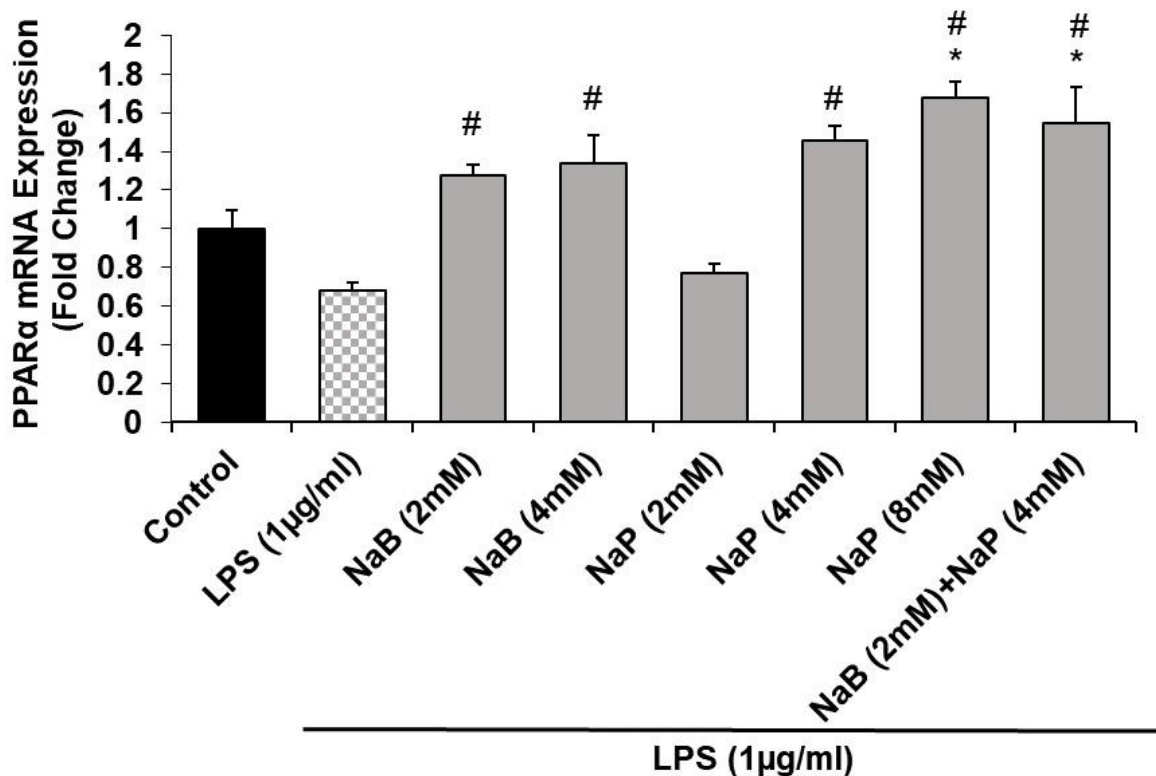


Figure 2.2. PPAR α mRNA expression in HepG2 cells alone (control), in the presence of LPS, or in the presence of LPS and various concentrations of NaB, NaP, or NaB and NaP mixture. All data were normalized using RPL13 α from three independent experiments. The results are presented as mean \pm SE (n=6). * p < 0.05 represents significantly different from the control; # p < 0.05 represents significantly different from LPS.

PPAR α is also a transcriptional activator of CPT1 α , which was significantly increased in NaB 2mM by 1.24-fold, NaB 4mM by 1.21-fold, and the combination of NaB 2mM and NaP 4mM by 1.37-fold in comparison to LPS (Figure 2.3.). However, there were no significant differences seen in CPT1 α among all treatments against the control.

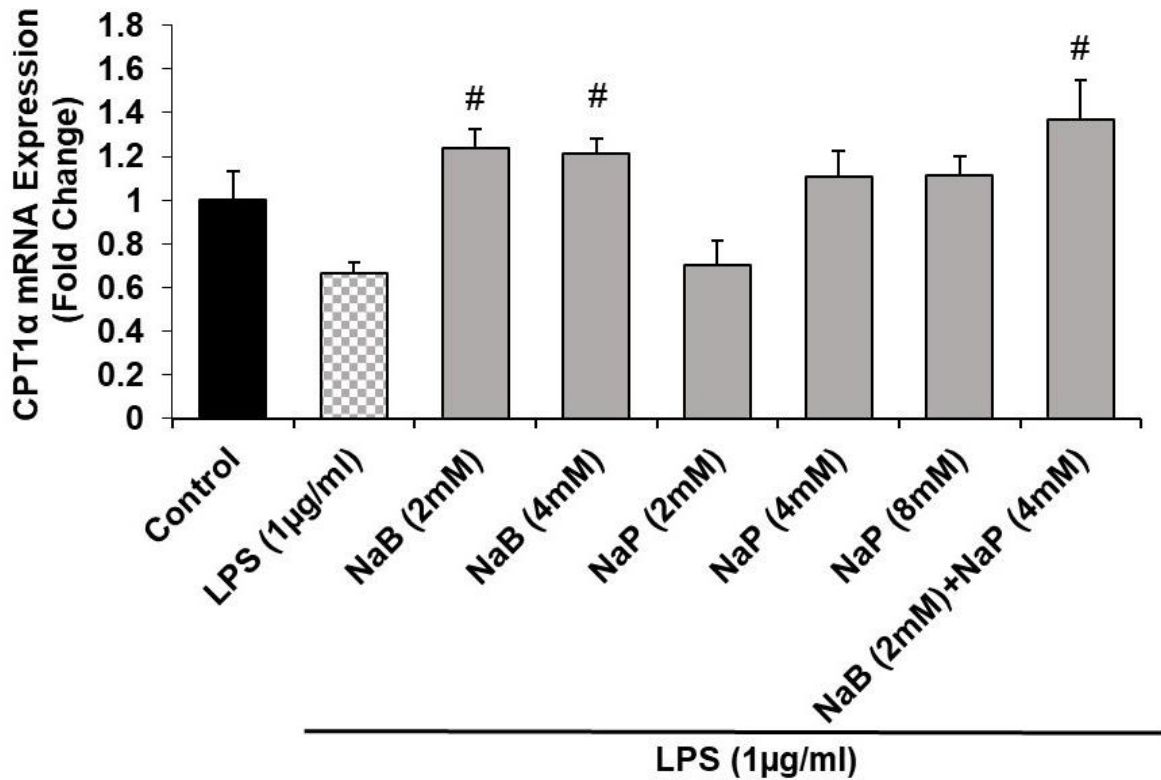


Figure 2.3. CPT1 α mRNA expression in HepG2 cells alone (control), in the presence of LPS, or in the presence of LPS and various concentrations of NaB, NaP, or NaB and NaP mixture. All data were normalized using RPL13 α from three independent experiments. The results are presented as mean \pm SE (n=6). * p < 0.05 represents significantly different from the control; # p < 0.05 represents significantly different from LPS.

UCP2, a mitochondrial protein, is a regulator of mitochondrial reactive oxygen species (ROS) production and plays a role in reducing ROS [12].

The upregulation of UCP2 was significantly increased in NaB 2mM, NaB 4mM, NaP 4mM, NaP 8mM, and a combination of NaB 2mM and NaP4mM by 2.15-fold, 2.51-fold, 1.88-fold, 2.18-fold, and 2.55-fold, respectively against the control and LPS; however, NaP 4mM was not significantly different from the control (Figure 2.4.).

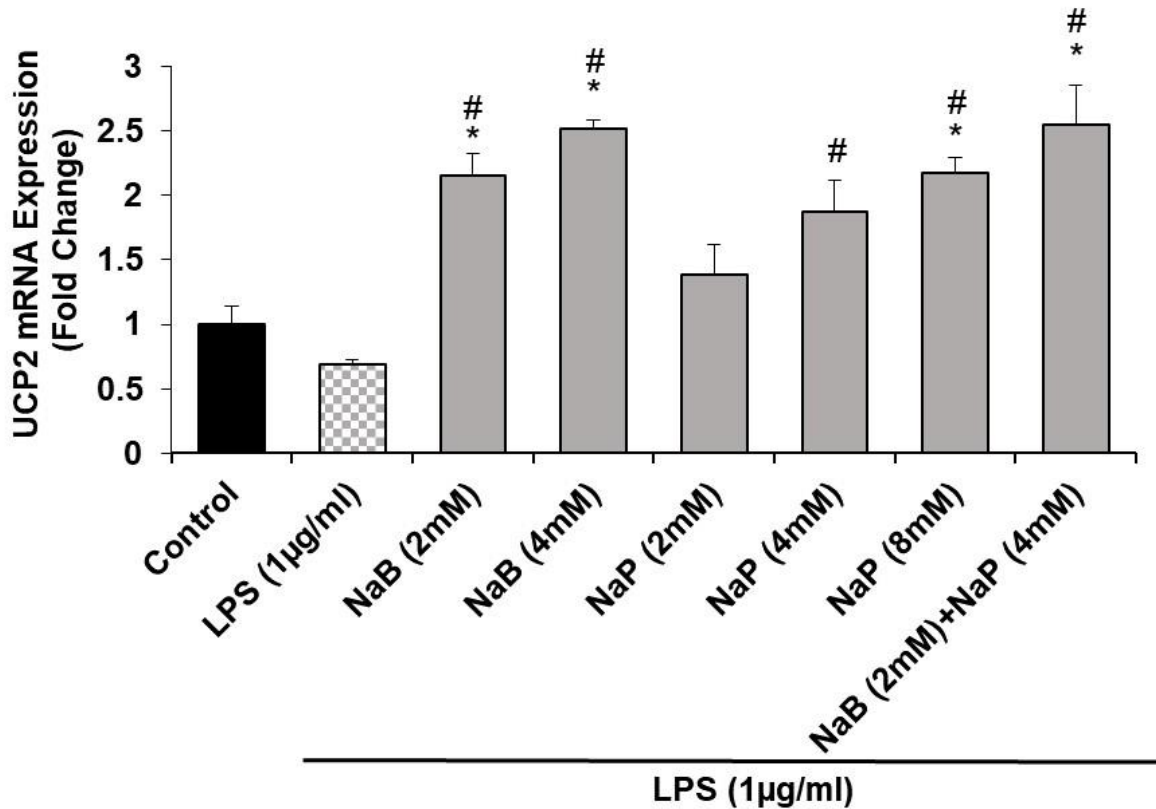


Figure 2.4. UCP2 mRNA expression in HepG2 cells alone (control), in the presence of LPS, or in the presence of LPS and various concentrations of NaB, NaP, or NaB and NaP mixture. All data were normalized using RPL13α from three independent experiments. The results are presented as mean ± SE (n=6). **p* < 0.05 represents significantly different from the control; #*p* < 0.05 represents significantly different from LPS.

2.3.3. FAO and Mitochondrial Related Genes in the Palmitate/Oleate Model

The oxidative stress and inflammation induced by palmitate/oleate was rescued by NaP and NaB treatment by upregulating mRNA expression of adipose triglyceride lipase (ATGL), PPARα, CPT1α, and fibroblast growth factor 21 (FGF21).

ATGL is a key lipase in the liver. The treatments of NaB 2mM, NaB 4mM, NaP 4mM, NaP 8mM, and the combination of NaB 2mM and NaP 4mM by 1.80-fold, 2.99-fold, 1.43-fold, 2.21-fold, and 2.55-fold, respectively, were significantly different in comparison to the control and palmitate/oleate (Figure 2.5.).

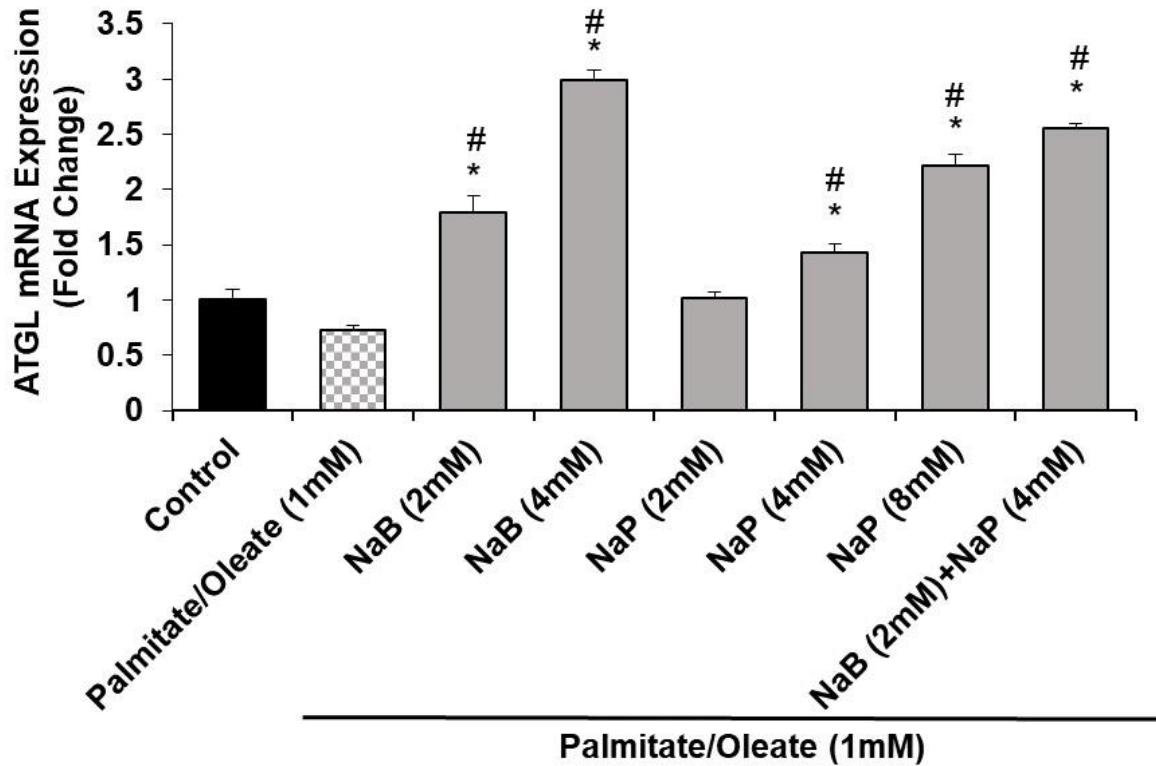


Figure 2.5. ATGL mRNA expression in HepG2 cells alone (control), in the presence of palmitate/oleate, or in the presence of palmitate/oleate and various concentrations of NaB, NaP, or NaB and NaP mixture. All data were normalized using RPL13 α from three independent experiments. The results are presented as mean \pm SE (n=6). * p < 0.05 represents significantly different from the control; # p < 0.05 represents significantly different from palmitate/oleate.

ATGL also plays a role in PPAR α signaling [30, 31]. The expression of PPAR α was significantly increased in NaB 2mM, NaP 4mM, and NaP 8mM by 1.24-fold, 1.53-fold, and 1.74-fold, respectively, in comparison to palmitate/oleate (Figure 2.6.).

The treatment of NaP 4mM and NaP 8mM was significantly different from the control. The rest of the treatments were not significantly different against the control or palmitate/oleate.

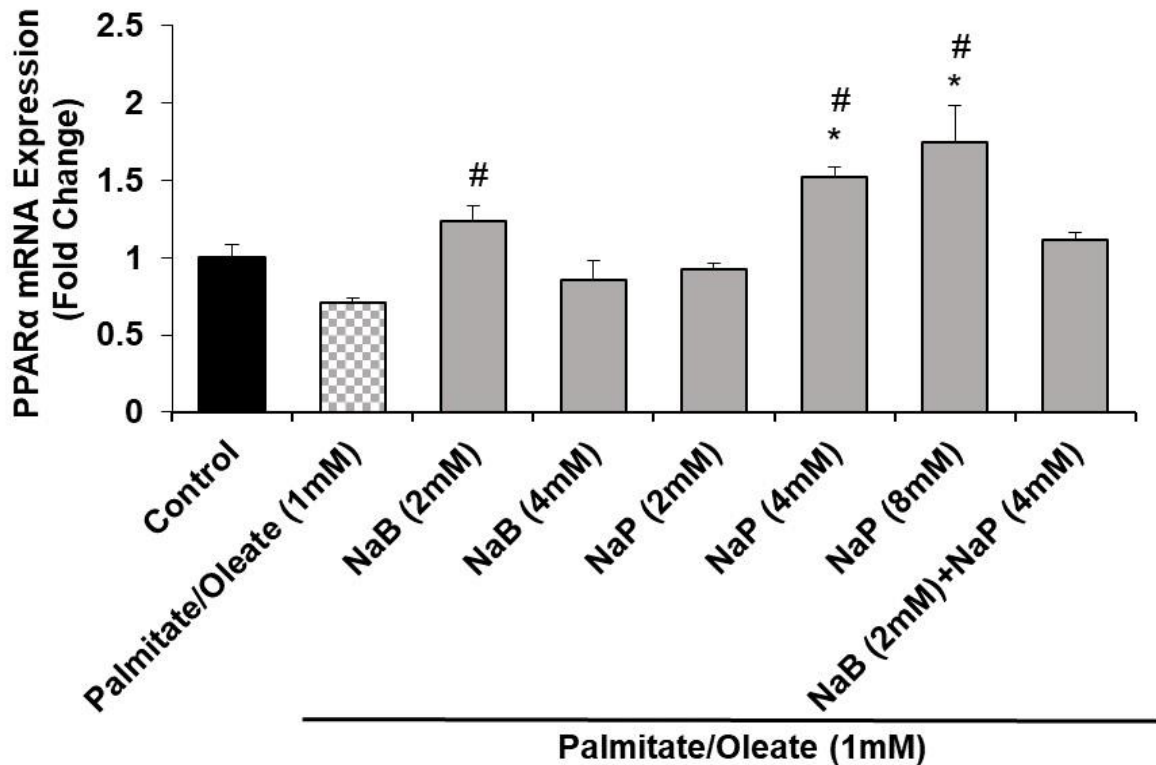


Figure 2.6. PPAR α mRNA expression in HepG2 cells alone (control), in the presence of palmitate/oleate, or in the presence of palmitate/oleate and various concentrations of NaB, NaP, or NaB and NaP mixture. All data were normalized using RPL13 α from three independent experiments. The results are presented as mean \pm SE (n=6). * p < 0.05 represents significantly different from the control; # p < 0.05 represents significantly different from palmitate/oleate.

The expression of CPT1 α was significantly increased by 2.25-fold in NaP 8mM and in the combination of NaB 2mM and NaP 4mM by 2.30-fold in comparison to the control and palmitate/oleate (Figure 2.7.).

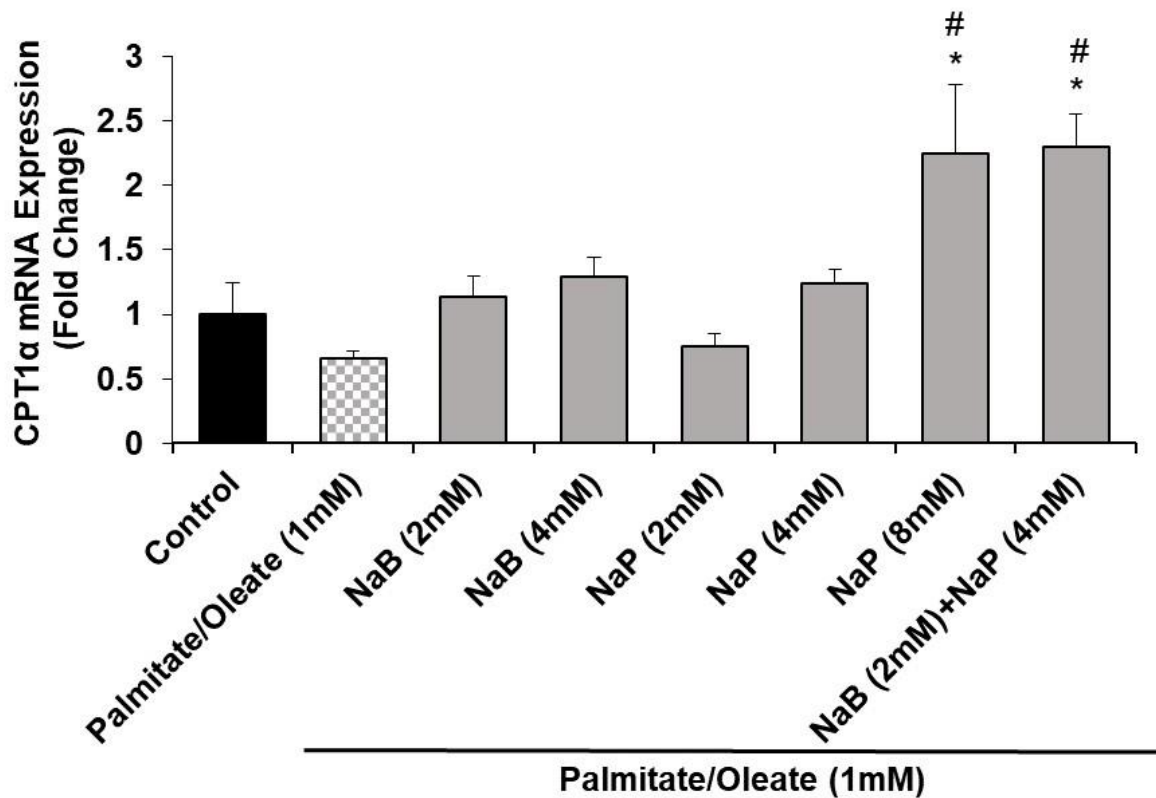


Figure 2.7. CPT1 α mRNA expression in HepG2 cells alone (control), in the presence of palmitate/oleate, or in the presence of palmitate/oleate and various concentrations of NaB, NaP, or NaB and NaP mixture. All data were normalized using RPL13 α from three independent experiments. The results are presented as mean \pm SE (n=6). * p < 0.05 represents significantly different from the control; # p < 0.05 represents significantly different from palmitate/oleate.

FGF21 expression, in comparison to the control and palmitate/oleate, were significantly upregulated in NaP 2mM and NaP 4mM by 1.97-fold and 2.21-fold, respectively (Figure 2.8.). However, NaP 8mM and the combination of NaB 2mM and NaP 4mM were undetermined and excluded from the results.

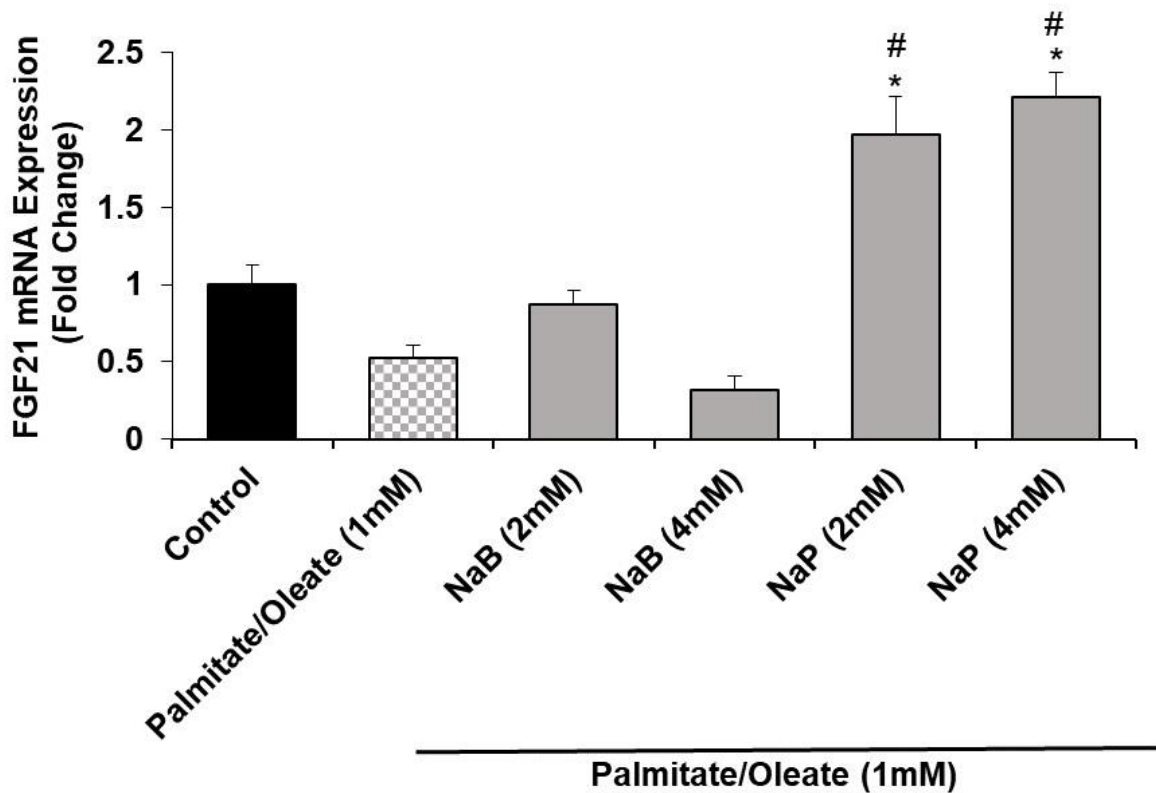


Figure 2.8. FGF21 mRNA expression in HepG2 cells alone (control), in the presence of palmitate/oleate, or in the presence of palmitate/oleate and various concentrations of NaB, NaP, or NaB and NaP mixture. All data were normalized using RPL13 α from three independent experiments. The results are presented as mean \pm SE (n=6). * p < 0.05 represents significantly different from the control; # p < 0.05 represents significantly different from palmitate/oleate.

2.3.4. Expression of TNF- α and IL-8 Levels

The protein levels of TNF- α and IL-8, released from LPS-stimulated or palmitate/oleate-stimulated HepG2 cells were determined using ELISA. LPS is a potent inducer of TNF- α and was significantly increased (2112 pg/ml) in comparison to the control (1644 pg/ml) (Figure 2.9.).

All the treatments of NaB and NaP were not significantly different from the control in the LPS model. However, in comparison to LPS (2112 pg/ml), the treatment of NaP 8mM (1400 pg/ml) and the combination of NaB 2mM and NaP 4mM (1285 pg/ml) had significantly decreased TNF- α expression.

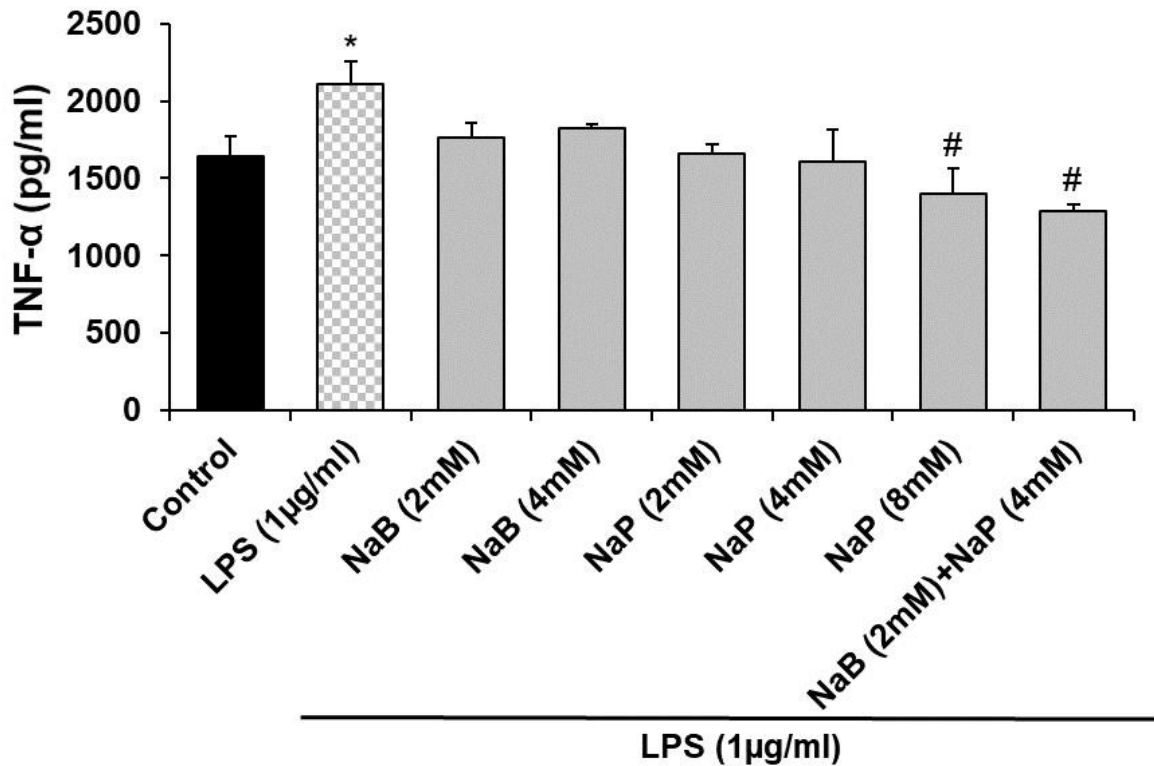


Figure 2.9. TNF- α levels in HepG2 cells alone (control), with LPS, or in combination with various concentrations of NaB, NaP, or NaB and NaP. TNF- α levels were measured by ELISA. The results are presented as mean \pm SE (n=3). *p < 0.05 represents significantly different from control; #p < 0.05 represents significantly different from LPS.

In the palmitate/oleate model, the levels of TNF- α in all treatments were not significantly different from the control (Figure 2.10.).

IL-8, also called neutrophil-activating peptide-1 or SCYB8, is a tissue-derived peptide secreted in response to stimulation by TNF- α .

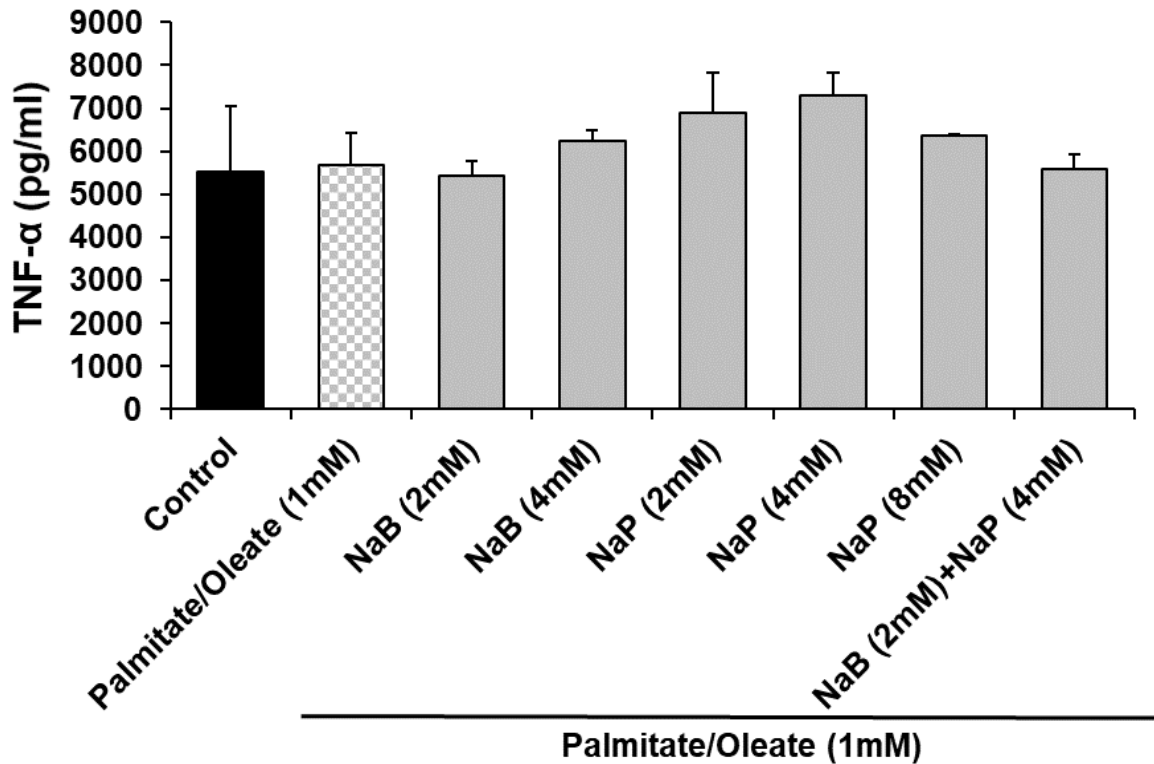


Figure 2.10. TNF- α levels in HepG2 cells alone (control), with palmitate/oleate, or in combination with various concentrations of NaB, NaP, or NaB and NaP. TNF- α levels were measured by ELISA. The results are presented as mean \pm SE (n=3). *p < 0.05 represents significantly different from control; #p < 0.05 represents significantly different from palmitate/oleate.

IL-8 levels in the LPS model showed no significant differences among all treatments. The two treatments, NaB 2mM (244 pg/ml) and NaB 4mM (262 pg/ml), were able to reduce IL-8 levels more effectively, however were not statically significant in comparison to the control (534 pg/ml) or LPS (654 pg/ml) (Figure 2.11.).

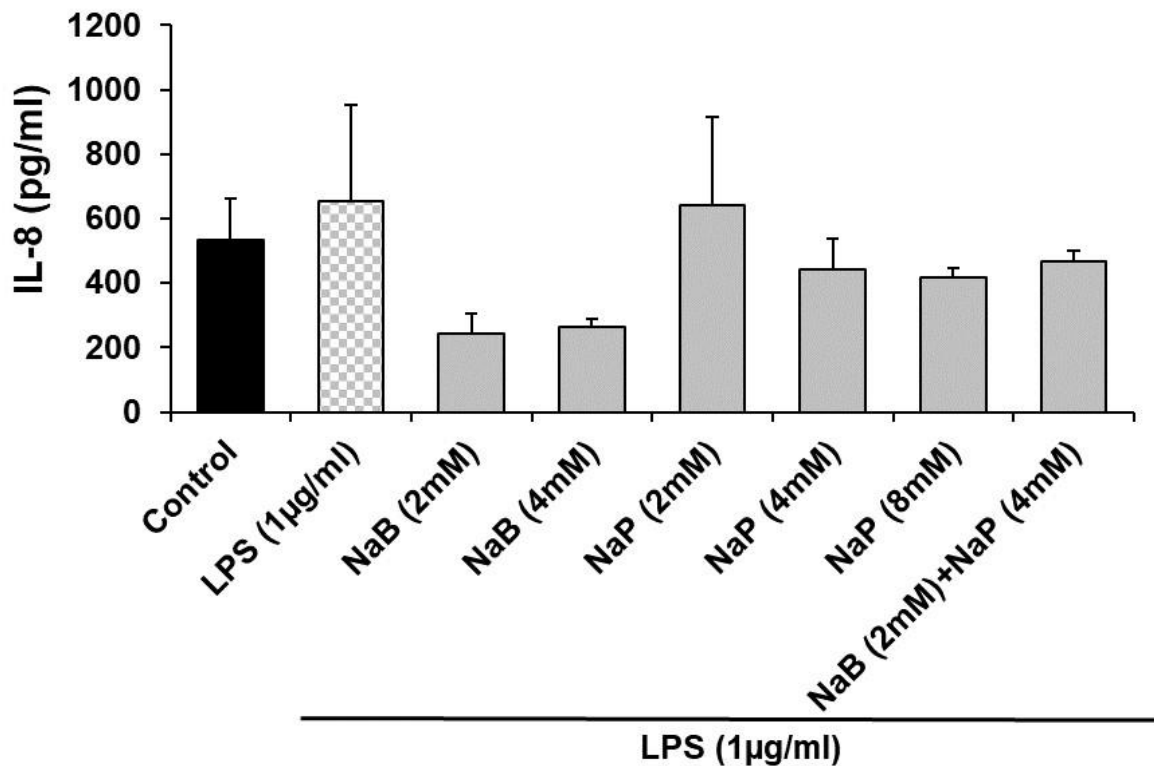


Figure 2.11. IL-8 levels in HepG2 cells alone (control), with LPS, or in combination with various concentrations of NaB, NaP, or NaB and NaP. IL-8 levels were measured by ELISA. The results are presented as mean \pm SE (n=3). *p < 0.05 represents significantly different from control; #p < 0.05 represents significantly different from LPS.

In the palmitate/oleate model, like the LPS model, IL-8 levels showed no significant differences among all treatments (Figure 2.12.). The two treatments, NaB 4mM (1074 pg/ml) and the combination of NaB 2mM and NaP 4mM (945 pg/ml), were able to lower IL-8 levels more successfully than all other treatments, however were not considered statically significant in comparison to the control (1461 pg/ml) or palmitate/oleate (1877 pg/ml).

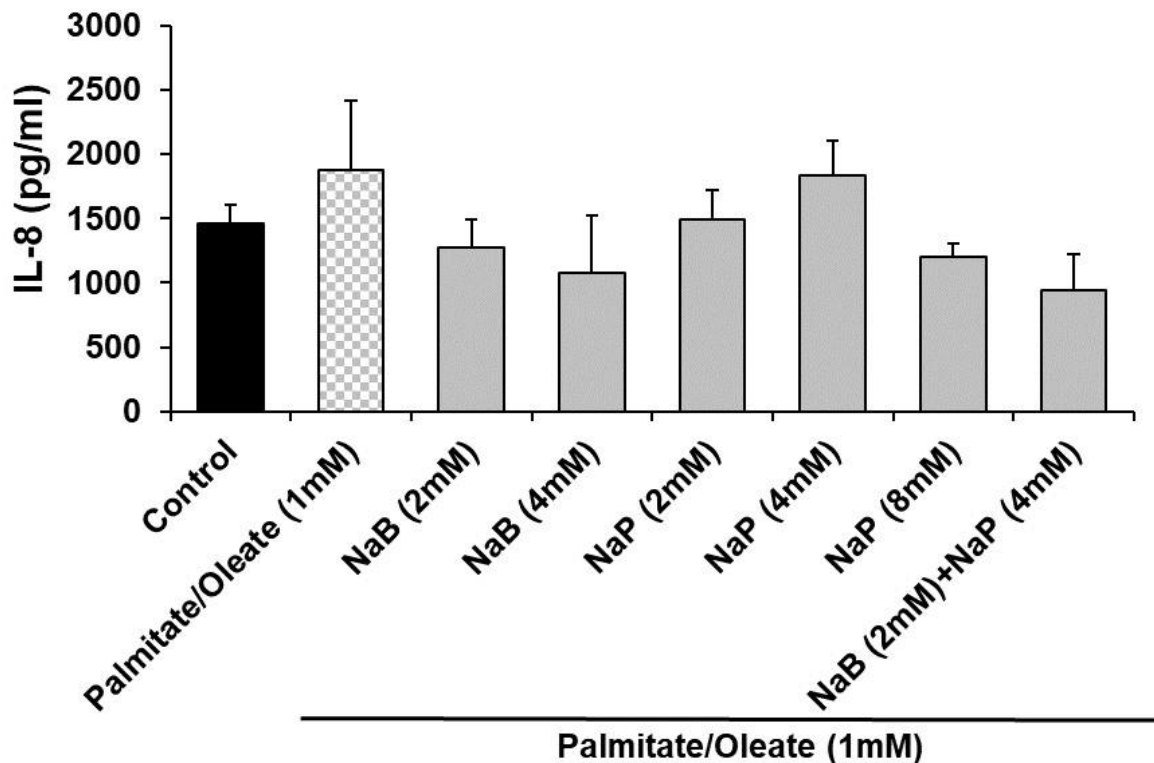


Figure 2.12. IL-8 levels in HepG2 cells alone (control), with palmitate/oleate, or in combination with various concentrations of NaB, NaP, or NaB and NaP. IL-8 levels were measured by ELISA. The results are presented as mean \pm SE (n=3). *p < 0.05 represents significantly different from control; #p < 0.05 represents significantly different from palmitate/oleate.

2.3.5. Effects of NaP and NaB on Carnitine Palmitoyltransferase 1 alpha (CPT1 α) Levels

One of the key proteins in FFA metabolism is CPT1 α . Therefore, protein levels of CPT1 α were studied in palmitate and oleate-stimulated HepG2 cells by Western blotting. The results indicated that CPT1 α , which is the rate-limiting enzyme of fatty acid β -oxidation, increased.

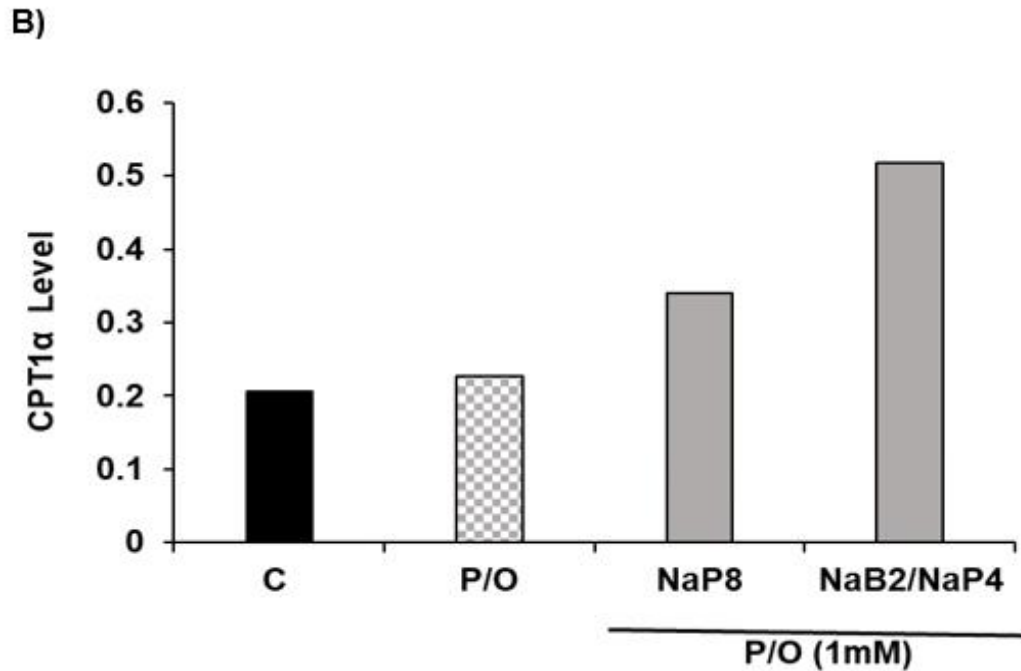
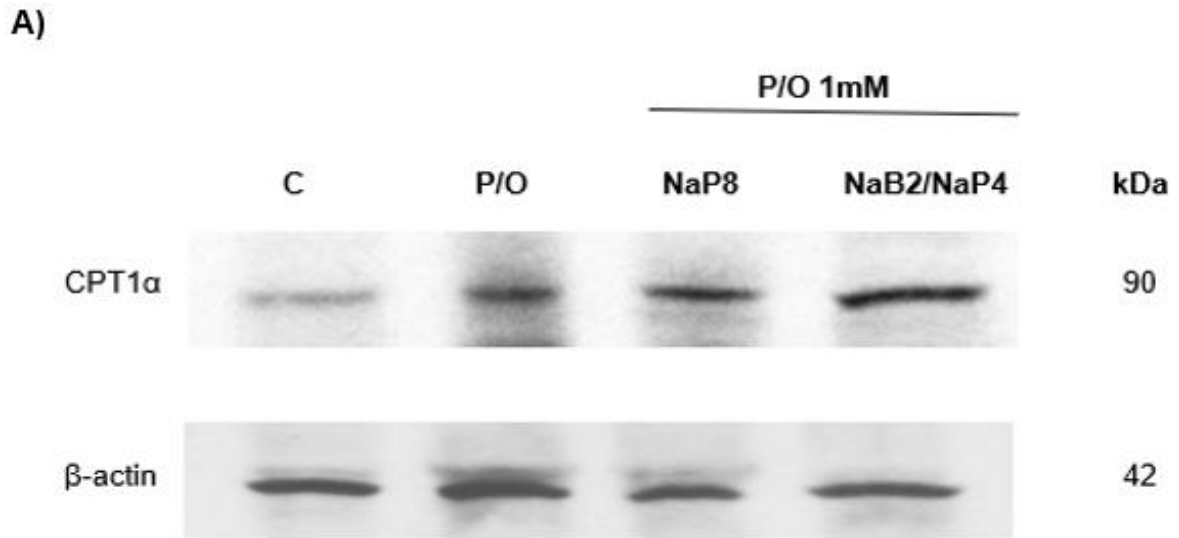


Figure 2.13. Western blot results of CPT1 α levels in HepG2 cells alone (control), with palmitate/oleate (P/O), NaP 8mM (NaP8) with P/O, or NaB 2mM and NaP 4mM (NaB2/NaP4) with P/O. A) Protein levels of CPT1 α and β -actin visualized by chemiluminescence. B) Densitometric analysis of the bands was performed using ImageJ software, and relative expression of CPT1 α versus β -actin was calculated.

The protein levels of CPT1 α in the palmitate/oleate model were increased by NaP 8mM and the combination of NaB 2mM and Nap 4mM (Figure 2.13.).

2.4. Discussion

In this study, we investigated two major risk factors that lead to the progression of NAFLD. The combination of palmitate and oleate or LPS, which are generated by the over consumption of the Western diet. Both risk factors are known to cause gut dysbiosis, oxidative stress, and inflammation in the liver. We found that mRNA expression levels of multiple genes involved in FAO were upregulated by NaP and NaB. Studies prove that propionate and butyrate, but not acetate, act as HDAC inhibitors, which possess antiproliferative effects, anti-inflammatory properties, and stimulate hepatic FAO [24, 32, 33]. Our results suggest that NaP and NaB can attenuate steatosis and liver injury by stimulating FAO and mitochondrial related genes, increasing protein expression of CPT1 α , and regulating inflammation by decreasing pro-inflammatory cytokines.

The LPS model, used to represent the consequence of gut dysbiosis, is significantly elevated in NAFLD human and animal studies [6]. The impact of LPS in HepG2 cells decreased PGC-1 α , UCP2, PPAR α , and CPT1 α mRNA expression but was not significantly different from the control. There is evidence that indicates hepatocytes uptake and detoxifies LPS and could explain why LPS was not able to significantly reduce FAO related genes [34, 35]. However, we can confirm that LPS caused inflammation in HepG2 cells through TNF- α expression, which was significantly increased. In the liver, PGC-1 α regulates energy homeostasis, controls gluconeogenesis, and interacts with genes such as PPAR α , which is involved in FAO [29, 36]. Studies show that the dietary intervention of PGC-1 α can prevent and treat metabolic syndrome [19]. Our study shows

that *in vitro*, NaP is a novel activator of PGC-1 α in the presence of LPS compared to cells treated with LPS only. The molecular mechanism of NaP could be the ability to stimulate hepatocyte mitochondrial content and function through PGC-1 α activation [29]. Furthermore, NaP, NaB, and in combination, may play protective roles against mitochondrial oxidative damage since they significantly upregulated UCP2, a transport protein in the inner mitochondrial membrane, that reduces ROS production [37]. In NAFLD, FFA overload in the mitochondria increases ROS production and oxidative stress [38]. The molecular mechanism of NaP and NaB may be the ability to prevent mitochondrial oxidative stress and hepatic fat accumulation in NAFLD through UCP2 promotion.

We used the combination of palmitate and oleate to represent the fatty acid composition of Western diets seen in NAFLD patients. The effect of combined palmitate and oleate only numerically decreased the mRNA expression of FGF21, ATGL, PPAR α , and CPT1 α in HepG2 cells but was not significantly different from the control. Palmitate at high concentrations (> 0.5 mM) causes cytotoxicity via endoplasmic reticulum stress in hepatocytes, while oleate does not [39, 40]. Studies have shown that when combined, oleate may protect cells from palmitate-induced cellular stress and apoptosis through efficient incorporation of fatty acids into triglyceride-rich lipoproteins for export, thus channeling excess lipids away from toxic pathways [39, 41-44]. Cell viability results confirmed that the combination of palmitate and oleate was significantly different from the control, which suggests that palmitate and oleate together can cause cell death but not significant reductions in FAO. In the liver, FGF21 is highly expressed and increases FAO by stimulating PGC-1 α expression [45]. PGC-1 α is a coactivator of PPAR α , which controls

FGF21 at a transcriptional level [45]. *In vivo* and *in vitro* studies show that PPAR α agonists can increase FGF21 expression [46-48]. Exogenous FGF21 administration has been shown to reduce plasma and hepatic triglycerides, reduce body weight, reverse hepatic steatosis, and increase whole-body energy expenditure in mice fed a high-fat diet [49]. We show for the first time that only NaP alone was able to significantly increase FGF21 expression in palmitate and oleate-induced HepG2 cells, which indicates its potential in stimulating FAO, improving lipid metabolism, and possibly reversing hepatic steatosis [50, 51]. We speculate that NaP uptake by the liver explains why it has a better impact than NaB in promoting FGF21 expression since it is highly expressed in the liver. ATGL is a major lipase in the liver and was significantly increased by NaP, NaB, and in combination, which suggests these treatments may play a significant role in regulating lipolysis in palmitate and oleate-stimulated HepG2 cells. ATGL is a rate-limiting enzyme for intracellular hydrolysis of stored triglycerides and a key regulator of lipid metabolism [31]. ATGL may serve as a protector of hepatic inflammation through increased PPAR α signaling, when levels of PPAR α are low, the risk of developing NAFLD rises [31].

In both models, NaP, NaB, or in combination, significantly increased PPAR α and CPT1 α mRNA expression in HepG2 cells. PPAR α is a transcription factor that plays a critical role in hepatic lipid metabolism and regulates fatty acid synthesis and oxidation [52]. PPAR α plays multiple roles in the liver involving FAO, anti-inflammatory, and anti-fibrotic effects [45, 52, 53]. PPAR α agonists have been considered effective treatments for NAFLD due to their lipid-lowering effects [54]. The treatment of NaP, NaB, or in combination stimulated FAO, and possibly produced anti-inflammatory and anti-fibrotic effects through the significant upregulation of PPAR α in both experiments. Additionally,

PPAR α activates the expression of CPT1 α , which leads to increased FAO [53, 55]. In the outer mitochondrial membrane, CPT1 α is the primary regulatory enzyme involved in mitochondrial β -oxidation. Therefore, NaP, NaB, or in combination may play a role in regulating mitochondrial β -oxidation through CPT1 α upregulation.

This study found that LPS or palmitate/oleate activates inflammation in HepG2 cells by stimulating pro-inflammatory cytokines TNF- α and IL-8. In a longitudinal analysis, high serum TNF- α levels in patients were associated with the development of NAFLD [56]. TNF- α is a major proinflammatory cytokine that causes triglyceride accumulation, oxidative stress, hepatocyte cell death, and hepatic steatosis [57-59]. In response to TNF- α stimulation, IL-8 is produced [60]. IL-8 is a chemokine that acts as a chemical signal attracting neutrophil migration to the site of inflammation [61]. These pro-inflammatory cytokines are known to stimulate further liver inflammation and damage. Therefore, decreasing TNF- α and IL-8 is an important step in preventing the development of NAFLD. Our findings suggest that LPS causes more inflammation than palmitate/oleate in HepG2 cells through the significant increase of TNF- α expression. Other studies confirm that LPS significantly increases TNF- α levels in HepG2 cells [25, 62, 63]. All treatments, including LPS and palmitate/oleate, showed no significant differences in IL-8 expression. However, other studies have shown that LPS and palmitate induce IL-8 in HepG2 cells [28, 59, 63, 64]. In both models, the treatments of NaP, NaB, and in combination may exert anti-inflammatory properties by regulating pro-inflammatory cytokines, TNF- α and IL-8, through HDAC inhibition since all treatments were not significantly different from the control. Propionate and butyrate are known to regulate cytokine expression through HDAC inhibition [32, 65].

The protein expression of CPT1 α increased in the palmitate/oleate model. The two treatments, NaP (8mM) and NaB (2mM) combined with NaP (4mM) were used based on the mRNA results. CPT1 α catalyzes the transfer of long-chain acyl group of the acyl-CoA ester to carnitine, which transports fatty acids into the mitochondrial matrix for β -oxidation. Therefore, NaP alone or, in combination with NaB, has the potential to increase FAO by permitting the mitochondrial entry of FFA through the increase of CPT1 α protein levels and mRNA expression.

2.5. Conclusion

NAFLD is one of the most common causes of liver dysfunction. The increase in circulating FFAs, palmitate and oleate, or gut-derived bacterial endotoxin, LPS, in the liver induces oxidative stress and pro-inflammatory cytokine production, which all contribute to NAFLD disease progression. In this study, we provide evidence that NaP, NaB, or in combination, have protective effects on palmitate/oleate- or LPS-induced cellular steatosis in HepG2 cells. LPS activates greater inflammation than palmitate/oleate by significantly increasing TNF- α expression in HepG2 cells. The treatment of NaP or NaB was able to promote FAO, regulate lipolysis, and reduce ROS production through the significant upregulation of PGC-1 α , PPAR α , ATGL, CPT1 α , FGF21, and UCP2 mRNA expression in HepG2 cells. Together, NaP and NaB may produce synergistic effects by significantly increasing CPT1 α , PPAR α , and UCP2 mRNA expression in LPS-induced HepG2 cells and by significantly increasing CPT1 α and ATGL mRNA expression in palmitate/oleate-induced HepG2 cells. Only NaP treatment may have the ability to reverse hepatic steatosis and increase whole-body energy expenditure by significantly increases FGF21 mRNA expression in palmitate/oleate-induced HepG2 cells.

The protein expression of CPT1 α was increased by NaP (8mM) and the combination of NaB (2mM) and NaP (4mM) in the palmitate/oleate model. The study shows promising results for the use of SCFAs, NaP and NaB, as a potential therapy in NAFLD. We suggest further investigation with NaP and NaB therapy in animal and human clinical trials be tested.

2.6. Notes

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CHAPTER 3. SUMMARY AND CONCLUSIONS

NAFLD is the most common chronic liver disease in the United States and continues to rise every year. The progression of NAFLD is caused by a combination of the Western diet with a sedentary lifestyle, which causes the increase of FFAs, palmitate and oleate, or gut-derived bacterial endotoxin, LPS, to circulate inside the liver. Consequently, the increase of palmitate, oleate, and LPS causes oxidative stress and inflammation in the liver.

Many studies have shown the benefits of SCFAs in the prevention and treatment of various metabolic diseases. The purpose of this dissertation was to evaluate the mechanisms behind NaP and NaB in FAO and explore their potential benefits in relieving oxidative stress and inflammation in liver cells. The mRNA expression of genes associated with fatty acid metabolism and the protein expression of CPT1 α and pro-inflammatory cytokines related to NAFLD using the HepG2 human liver cancer cell line were exposed to palmitate/oleate or LPS.

Our results suggest that NaP, NaB, or in combination, will promote FAO, regulate lipolysis, and reduce ROS production while regulating pro-inflammatory cytokines in HepG2 cells. Together, NaP and NaB may produce synergistic effects in both models by effectively increasing FAO. However, only NaP treatment may have the ability to reverse hepatic steatosis and increase whole-body energy expenditure. This study shows the beneficial uses of NaP and NaB in NAFLD treatment. Therefore, future studies in NaP and NaB therapy in animal and human models need to be investigated to validate our results.

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VITA

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