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FOR THE DEGREE OF MASTER OF SCIENCE

p-Coumaric acid prevents high fat and high sucrose-mediated hepatic inflammation and fibrosis

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CONTENT

List of Figure

List of tables

Abstract

Chronic intake of high fat and high sucrose (HFHS) cause hepatic inflammation and fibrosis. Activation of hepatic stellate cells (HSC), which is the dominant effector during liver fibrosis, is trigged by NLR family pyrin domain containing 3 (NLRP3) inflammasome. *p*-coumaric acid (4-hydroxycinnamic, PCA) found in edible plants and fruits has been known as a natural bioactive component to have powerful anti-oxidant and anti-inflammatory properties. However, it is not well understood. The impact of PCA on the induced hepatic fibrosis and it's underlying mechanism. To test this hypothesis, C57BL/6 male mice were randomized into three groups, low fat (LF) diets (11% calories from fat), high-fat diets (60% calories from fat) with high sucrose (20% sucrose, HFHS) drink, or HFHS plus PCA treatment (HFHS+PCA, 50 mg/kg b.w) by intraperitoneal injections. After feeding for 13 weeks, HFHS-fed mice increased body weight and lipid profiles with abnormal glucose metabolism compared to that of the LF diet group. Although HFHS+PCA did not make significant changes in most obesogenic parameters compared to the HFHS group, hypercholesterolemia and insulin resistance marker HOMA-IR were improved by PCA treatment. Interestingly, HFHS-induced hepatotoxicity and hepatic fibrosis which evidenced by endoplasmic reticulum (ER) stress, inflammation gene expression and collagen accumulation were significantly attenuated by PCA. We confirmed the anti-fibrogenic properties of PCA by using LX-2 hepatic stellate cells. NLR family pyrin domain containing 3 (NLRP3) inflammasome activation, which are main contributor of HSC activation, was significantly reduced by PCA in bone-marrow derived macrophages. These findings support the fact that PCA could reduce the HFHS diet-mediated hepatic fibrosis, and it is partly associated with inhibition of inflammasome.

Key words: p-coumaric acid; hepatic inflammation; hepatic fibrosis, inflammasome

Abbreviations:

ACC: AcetylCoA carboxylase

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

AMPK: AMP-activated protein kinase

ASC: Adaptor molecule apoptosis-associated speck-like protein containing

AUC: Area under curve

BMDM: Primary bone marrow cells

BW: Body weight

ChREBP: Carbohydrate response element binding protein

CLR: C lectin receptor

COX2: Cyclooxygenase-2

DAMP: Damage-associated molecular patterns

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinases

FAS: Fatty acid synthesis

GTT: Glucose tolerance test

HCC: Hepatocellular carcinoma

HDL: High-density lipoprotein

HFD: High-fat diet

HFHS: High fat and high sucrose

HSC: Hepatic stellate cell

FA: Fatty acid

IkBα: Inhibitory subunit of NF kappa B alpha

IL-1β: Interleukin 1β

IL6: Interleukin 6

ITT: Insulin tolerance test

IR: Insulin resistance

JNK: c-Jun N-terminal kinase

LDL: Low-density lipoprotein

LF: Low fat

LPL: Lipoprotein lipase

LXR: X-receptor

MCP1, Monocyte chemoattractant protein 1

mtROS: Mitochondrial reactive oxygen species

NAFL: Nonalcoholic fatty liver

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NEFA: Non-esterified fatty acids

NLR: Nucleotide binding domain leucine repeat

NLRP3: NLR family pyrin domain containing 3

LPS: Lipopolysaccharides

NF-kB: nuclear factor-kB

Ng: Nigericin

NOD: Nucleotide binding domains

PA: Palmitic acid

PAMP: Pathogen-associated molecular patterns

PCA: *p*-coumaric acid

PGE2: Prostaglandin E2

PPARγ: Peroxisome proliferator- activated receptor gamma

PRR: Pattern recognition receptors

RLH: RIGI-like RNA helicase

RLR: RIGI-like receptors

ROS: Reactive oxygen species

SCD: StearoylCoA desaturase

SREBP1c: Sterol regulatory element-binding protein 1c

STZ: Streptozotocin

T2DM: Type 2 diabetes

TC: Total cholesterol,

TG: Triglycerides

TGFβ: Transforming growth factor-beta

TIMP1: Tissue inhibitor of metalloproteinase 1

TLR: Toll-like receptor

TLR4: Toll-like receptor 4

TNFα: Tumor necrosis factor-alpha

ULK1: Unc-51-like kinase 1

VLDL: Very low-density lipoprotein

αSMA: α-smooth muscle actin

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide that effect patients from all ages, races, and ethnicities across the world. It encompasses a wide range of hepatic diseases, from basic steatosis, in which fat accumulates in hepatocytes, to nonalcoholic steatohepatitis and cirrhosis. NAFLD is defined as an accumulation of fat in the cytoplasm of hepatocytes, mainly in the form of triglycerides, that is more than 5% to 10% by weight, as determined by histology or imaging. Other causes of steatosis, such as excessive alcohol use, drugs, or genetic diseases, must be ruled out[1, 2]. It is estimated at 20%-30% of the general population gets NAFLD that varies considerably based on definitions, populations studied, and the diagnostic techniques (liver enzymes, imaging, histology, or their combinations)[3]. About 20% to 30% of adults with NAFLD will develop nonalcoholic steatohepatitis (NASH), which is the progressive form of a chronic hepatic inflammatory condition, which is the more severe form of the disease[4]. Increased fatty acid oxidation in the liver can generate reactive oxygen radicals (ROS), which can contribute to mitochondrial dysfunction, lipid peroxidation, and/or cytokine secretion[5]. A two-hit model has been proposed (Day and James, 1998; Garcia-Monzon, 2001) in hepatic steatosis, which involves a series of cellular events that contribute to inflammation, fibrosis, apoptosis, and cirrhosis. Fat accumulation in the liver is thought to be associated with insulin resistance (IR)[6] associated with NAFLD, but the exact mechanism is unclear[7]. Obesity, insulin resistance and hyperinsulinemia are the most common underlying abnormalities in people with NAFLD. According to overwhelming evidence, in most patients, identifying NAFLD is a sensitive surrogate diagnostic for the existence of underlying insulin resistance[8-11]. However, hepatic steatosis (i.e., triglyceride (TG) accumulation) is isolated from IR in patients with familial hypobetalipoproteinemia, providing further evidence that hepatic TG elevation may be a marker rather than a cause of IR[12]. Hepatic insulin sensitivity worsens with increased fat accumulation in the liver, which increases gluconeogenesis in the liver and increases

gluconeogenesis in the liver. Insulin resistance in NAFLD is characterized by decreased insulin sensitivity of the whole body, liver, and adipose tissue. The mechanism(s) by which fat accumulates in the liver may include excess dietary fat, increased free fatty acid transport to the liver, inadequate oxidation of fatty acids, and increased new adipogenesis[13] (Fig. 1).

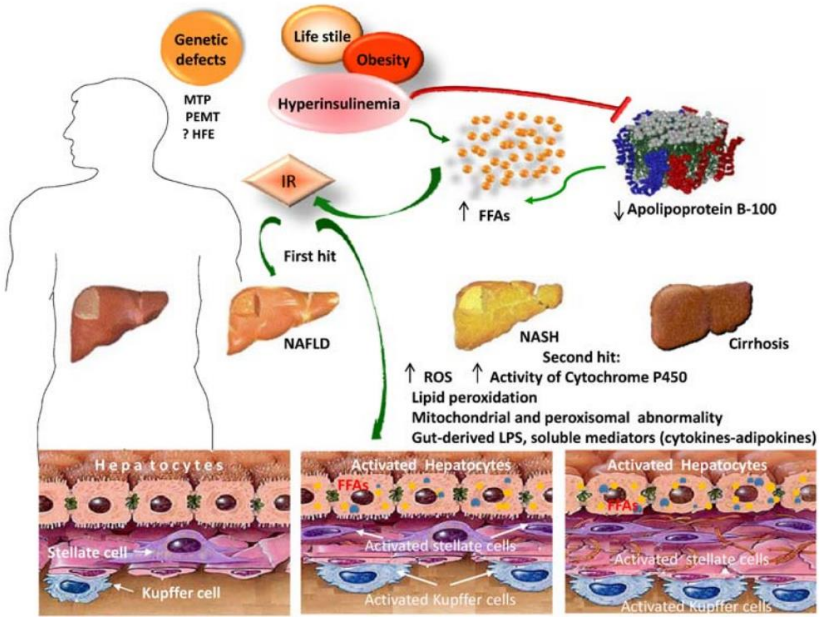

Figure 1. Sketch showing the developmental mechanism of NAFLD (From Malaguarnera, M. et al., J Mol Med (Berl). 2009, 87, 679–695).

Along with inflammation and varying degrees of sustained liver injury can lead to progressive fibrosis and cirrhosis, is a potentially serious condition, and ~10–25% of patients with NASH might progress to liver-related morbidity, and hepatocellular carcinoma (HCC)[14]. Much is known about how fat accumulates in the liver, but not much is known about how fat causes permanent hepatocellular damage and the consequences of trauma recognized as NASH and fibrosis. Insulin resistance and hyperinsulinemia may contribute to these pathological changes[15]. A chronic increase in the supply of free fatty acids to the liver from lipolytically active adipose tissue may also contribute to the pathogenesis of NASH. The prevalence and severity of NAFLD progressively increases with the number and severity of metabolic syndrome signs[16]. However, when fat accumulates in hepatocytes, the sensitivity of the liver to damage caused by various causes increase, impairing the ability of the liver to

regenerate[17, 18]. The obese rats excreted less hepatic endotoxin than non-obese control animals[18]. This additional stressor is also referred to as the "second hit" in the paradigm, which defines fat accumulation as the "first hit"[19].

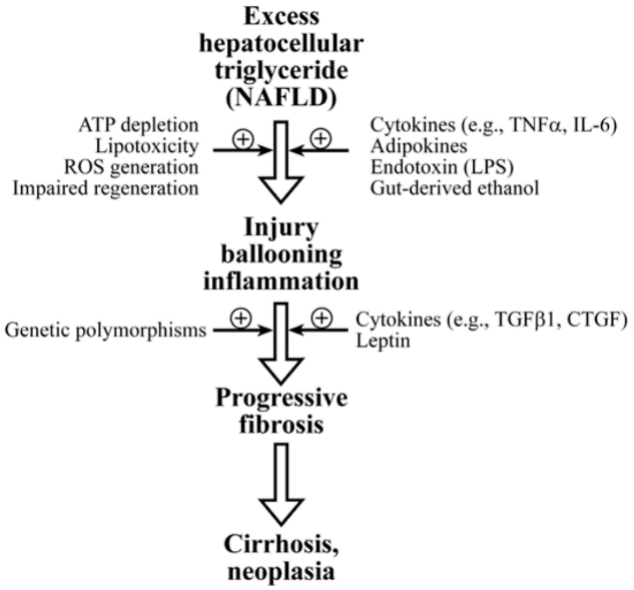

Figure 2. Possible pathways from NAFLD to NASH, cirrhosis, and hepatocellular carcinoma. Several factors, both intrahepatocellular (left) and extracellular (right), may contribute to fatty hepatocellular injury, leading to processes leading to fibrosis, cirrhosis.

Obesity, type 2 diabetes, and metabolic syndrome are prevalent worldwide[20, 21]. Nonalcoholic fatty liver disease (NAFLD) is a liver manifestation of metabolic syndrome that affects approximately 1 billion people worldwide[22]. While host genetics, Western diet, and gut microbiome components contribute to obesity and NAFLD, a positive energy balance from increased calorie intake and decreased activity is essential. Overnutrition, which leads to fat overload in non-adipose tissues, is a major cause of fat accumulation in the liver. In fact, fatty liver disease is widely considered to be a hepatic manifestation of obesity-associated metabolic syndrome, rather than an organ-specific pathology. Fatty liver disease has been demonstrated to be impacted by both hereditary and behavioral factors. Nutrition impacts have been broadly considered as supporters of nonalcoholic fatty liver disease (NAFLD), and discoveries of various studies demonstrate that dietary admission is critical in the rise and advancement of this problem. The strong association between increased food intake and fatty liver prompted the investigation of the contribution of certain major nutrients to liver fat accumulation. Increased prevalence of NAFLD and NASH in Western societies[23] indicates the contribution

of typical food ingredients to determine prevalence and disease progression. A key feature of the Western diet is its high-fat content, which directly supports steatosis by promoting excessive absorption of dietary lipids by hepatocytes[24]. Therefore, increased energy intake and obesity are major contributors to the pathophysiology of NAFLD (Fig. 3).

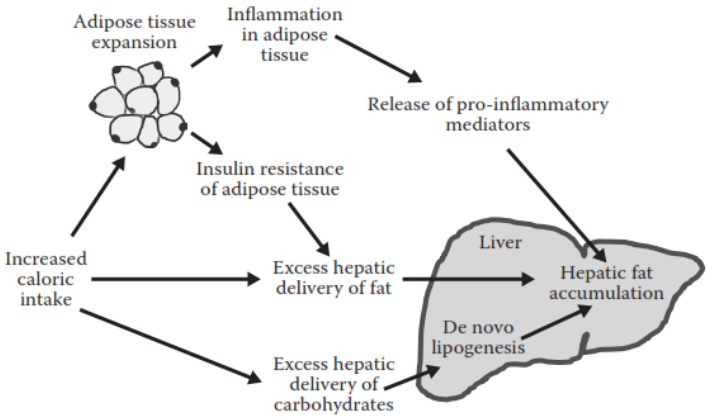

Figure 3. Prostatitis effects of overeating. Pathways of intrahepatic and extrahepatic steatosis due to overnutrition.

Consistent with this, the proportion of dietary fat was found to be significantly correlated with hepatic fat[25], indicating that NASH patients had increased fat consumption compared to healthy controls[26]. Moreover, interventional studies show the effect of dietary fat on hepatic steatosis, which is increased by consumption of a high-fat diet (HFD) and decreased by dietary intake low-fat diet[27]. Increased energy intake directly supports liver fat storage by transporting excess fat and carbohydrates to the liver, triggering an adipogenic pathway. In addition, the indirect effect of increased energy expenditure is mediated by the expansion of adipose tissue. When adipose tissue becomes enlarged due to obesity, it tends to cause inflammation[28-30], which makes it resistant to insulin-mediated inhibition of lipolysis[31-33]. Therefore, increased release of fatty acids (FA) from adipose tissue increases the serum pool of non-esterified fatty acids (NEFA), further enhancing hepatic uptake of free FA by the liver. Moreover, secretion of proinflammatory cytokines from adipose tissue induces fat accumulation in the liver by regulating the release of adipokines[34, 35]. Increased macrophage numbers in white adipose tissue have been shown to be significantly associated with measures of insulin resistance and fibro-inflammatory[36] liver injury, indicating an association between

adipose tissue inflammation and steatohepatitis in obesity. An additional mechanism by which HFD can contribute to NAFLD is by inducing hepatic endoplasmic reticulum (ER) stress. A high-fat diet induces markers of ER stress in the liver[37], which has been suggested as a mechanism for developing insulin resistance. The hepatic ER stress response stimulates the progression and exacerbation of NAFLD, leading to adipogenesis, decreased hepatic fat excretion, and inflammatory and fibrotic processes[38].

Dietary FA are either delivered to the liver by lipoproteins or "transfused" into serum NEFA pools (supplied by adipose tissue-derived FA) from chylomicrons and then taken up by hepatocytes as NEFA[39]. Inside hepatocytes, FA can be oxidized in the mitochondria[40] due to the cyclic breakdown of β-oxidation to acetyl-CoA units. However, when hepatocytes are overloaded with FA due to increased fat consumption, the rate of import exceeds the rate of catabolism, and the throughput of the β-oxidation pathway is superior. Increased availability of fatty acids in hepatocytes increases fatty acid esterification to triglycerides (TG), which accumulate and causes steatosis. Accumulation of TG can promote liver inflammation by increasing the activation of Kupffer cells[41], and the inclusion of TG in the composition is generally postulated as a protective mechanism against FA-mediated hepatotoxicity[42]. However, because the liver is not adipose tissue, its ability to store lipids is limited. Consequently, the accumulation of FA in the form of TG above a certain limit carried by hepatocytes converts these FA into the cytotoxic pathway. The lipotoxicity of fatty acids in the liver is evidenced by their ability to interfere with cellular function by acting on multiple pathways[43], which creates difficulties in studying the unique effects of each specific signaling pathway. In hepatocytes, FA can maintain the progression of NAFLD by inducing the expression of pro-inflammatory genes[44-46] and apoptotic responses[47-49], as well as promoting metabolic abnormalities by interfering with insulin signaling[50-52]. FA can also activate inflammatory signals dependent on toll-like receptor 4 (TLR4)[53] and promote inflammatory responses in macrophages[54]. Consequently, high levels of free fatty acids in the liver may

support the conversion of steatosis to NASH, promoting activation of the Kupffer cells, macrophages found in the liver.

Sucrose is composed of one molecule of glucose and one molecule of fructose, which are bound to each other. It is a disaccharide, a molecule consisting of two monosaccharides: glucose and fructose. Fructose is a monosaccharide that is widely used as a sweetener. In industry, it is often found in soft drinks and packaged foods. There is a correlation between dietary fructose intake and the prevalence of metabolic syndrome and hepatic obesity[55]. An exploratory observation pilot study has shown that excessive fructose consumption in NAFLD patients may increase the risk of fatty liver disease[56], modulating circulating adropine concentrations[57] and levels of lipocalin 2[58, 59], increasing liver fibrosis[60], and eventually leads to cirrhosis and liver failure. Fructose is metabolized primarily by fructokinase to produce fructose-1-phosphate, which irregularly releases glycerol phosphate and acyl-coenzyme A to form triglyceride, leading to the formation of triglycerides that are both secreted and stored in hepatocytes[61, 62]. The initial metabolism of fructose before convergence into the glycolytic pathway involves phosphorylation of fructose-to-fructose phosphate by fructokinase (ketohexokinase), which uses the substrate ATP. In contrast to glucokinase, phosphorylation of fructose by fructokinase is fructose-specific and not rate-determining. The high activity of fructokinase during the phosphorylation of fructose to fructose-phosphate in the liver can deplete ATP in the liver[63]. In fact, fructose has been shown to induce ATP depletion in humans[64-66], and a study using phosphorus 1 magnetic resonance spectroscopy to evaluate liver metabolism delayed recovery from fructose-induced ATP depletion in NALBD subjects[65, 67]. Excessive intake of fructose negatively affects hepatic lipid metabolism and insulin sensitivity. Fructose overload increases the metabolic load in the liver, stimulating the overproduction of acetyl-CoA in the mitochondria[68]. Through the tricarboxylate transport system, acetyl-CoA enters the cytoplasm and is used for fatty acid and cholesterol synthesis through the regulatory mechanism of the hepatic X-receptor (LXR) α, a protein that binds to regulatory factors sterol (SREBP) 1 / 1c, acetyl-CoA carboxylase (ACC),

fatty acid synthesis (FAS) and stearoyl-CoA desaturase (SCD). In contrast to glucose, which is widely used in tissues throughout the body, fructose is mainly metabolized in the liver, promoting oxidative damage and lipid peroxidation[69]. The process by which unsaturated lipids are oxidatively degraded due to inflammation into many products. Chronic moderate intake of fructose in mice has been shown to be associated with an increased translocation of lipopolysaccharides (LPS, endotoxins) from the intestine to the portal vein because of bacterial overgrowth and increased intestinal permeability. This may lead to further activation of hepatic Kupffer cells and the formation of reactive oxygen species (ROS) in the liver and induction of hepatic TNFα expression through the nuclear transcription factor κB (NFκB)[69].

The role of the innate immune system is to recognize invading pathogens and initiate a wide range of defenses until the adaptive immune system is activated. The three main classes of innate sensors are crowd-like receptors (TLR), RIGI-like receptors (RLR), and oligomerization and nucleotide binding domains (NOD)-like receptors (NLR)[70, 71]. These classes of proteins are known as pattern recognition receptors (PRR) because of their ability to recognize pathogen-associated molecular patterns (PAMP). Pattern recognition receptors (PRR) are important cellular sensors that coordinate early responses to a wide range of pathogens, including fungi, viruses, parasites, and bacteria. Pattern recognition receptors include TLR, RIGI-like RNA helicase (RLH), type C lectin receptor (CLR), and nucleotide binding domain leucine repeat (NLR) receptors[72]. These risk signals or risk-associated molecular patterns (Damage-associated molecular patterns, DAMP) are released in conditions of stress or cellular damage caused by pathogenic or sterile inflammatory strokes. The simultaneous presence of PAMP and DAMP can provide the immune system with the information it needs to differentiate between invasive pathogens and benign commensal organisms. NLRP3 is relatively conserved within species, indicating a role in the recognition of conserved DAMP and PAMP. NLRP3 is expressed by myeloid cells, keratinocytes, epithelial cells, and osteoblasts. Its expression is strongly induced by TLR agonists[73, 74].

Inflammation is a physiological reaction caused by a molecule derived from an invading microorganism or a host molecule released during tissue is damaged. "Self" molecules are recognized after chemical modification or when they appear in atypical locations where these molecules are usually absent[75]. In addition, the phase transition of exogenous crystalline or soluble substances to a solid state can induce an immune response[76]. The inflammatory response is mediated by the activation of immune signal receptors expressed in immune cells and certain non-immune cells. Therefore, both classical and non-immune cells help form an immune response. The last two families of signal receptors mentioned form a large multimolecular complex, the so-called "inflammasome," that acts to activate caspase-1 and IL-1β cytokines[77]. The term "inflammasome" was chosen to highlight the structural and functional similarities of these signaling complexes. It is like caspase, which serves as a molecular platform to trigger apoptosis, another complex to activate the apoptosome[78]. Although most inflammasomes are believed to be formed in response to single-molecule triggers, the NLRP3 inflammasome can be activated by a variety of chemically and structurally different substances[76]. The data confirm that aberrant activation of the NLRP3 inflammasome is associated with the pathogenesis of a variety of autoinflammatory, autoimmune, chronic inflammatory and metabolic diseases, including gout, atherosclerosis, and type 2 diabetes[79-81]. Therefore, NLRP3 inflammasome activation must be tightly regulated to prevent unwanted host damage and excessive inflammation.

Activation of immune signal receptors usually results in the production and secretion of various cytokines and other effector molecules. This molecule stimulates and modulates inflammation-promoting and antibacterial effects. Among immune effect molecules, the IL-1β family of cytokines, including IL-1β and IL-18, are the most studied cytokines and are important components of the inflammatory response to infection or damage of host tissues[82]. In addition to its many pro-inflammatory effects, IL-1β cytokines can induce T cell differentiation and participate in the maintenance of the T helper phenotype of Th1, Th2 and Th17 cells[83]. Activation of IL-1β cytokines results in severe local and systemic changes.

Immune cells such as neutrophils and macrophages are attracted to the IL-1β maturation site and cause changes in body temperature, feeding behavior, and sleep patterns due to the systemic action of IL-1[82]. Dysregulation of IL-1β production can lead to inflammatory diseases[84], and it is not surprising that the production and activation of IL-1β cytokines are tightly regulated (Fig. 4).

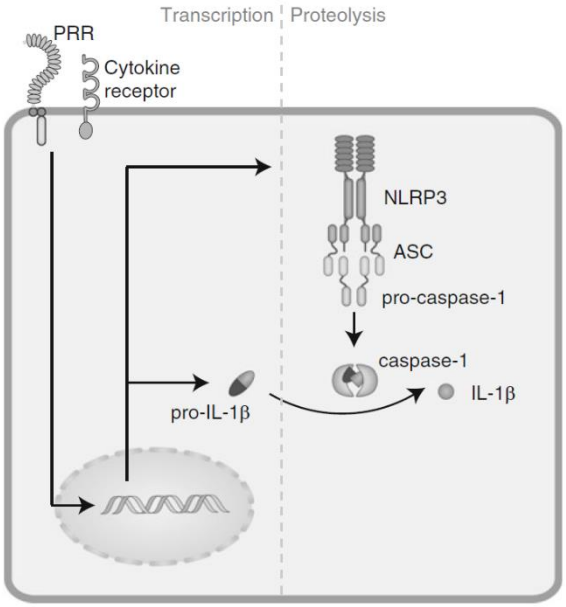

Figure 4. Activation of IL-1β by the NLRP3 inflammasome. Signals from IL-1β-activated immune pattern recognition receptors (PRR) or cell-surface cytokine receptors by the NLRP3 inflammasome increase the expression of genes encoding NLRP3 and IL-1β forms.

While most cytokines are proteins with a leader sequence that is released during progression along the secretory pathway, IL-1β cytokines are unique in that they are expressed in a biologically inactive form in the cytoplasm because of the lack of a leader sequence[85]. These performs must be catalyzed by the cysteine protease caspase-1 to become "mature" effector cytokines. Caspase-1 itself is produced in the cytoplasm in the form of an inactive pro form and requires an autocatalytic activation step[86]. This activation of caspase-1 relies on the assembly of the inflammasome to recruit adapter proteins involved in apoptosis, in which NLRP3 is a spotted protein containing a caspase recruitment domain (adaptor molecule apoptosis-associated speck-like protein containing, ASC). ASC is a bidirectional adapter protein that induces the proximity of recruited caspase-1 molecules when interacting with proinflammatory NLR or PYHIN family proteins. This proximity leads to self-cleavage of caspase-1 and the formation of two subunits (p10 and p20) that assemble into active caspase-

1 composed of two heterodimers[86]. Caspase-1-mediated cleavage is the major mode of activation of the IL-1 beta cytokine, but cleavage by neutrophil proteinase-3, elastase, matrix metalloproteinase-9 and granzyme-A has also been documented[82].

Despite the development made in expertise the law of IL-1β processing, controversy surrounded the capability of TLR ligands inclusive of LPS or lipopeptides to set off caspase-1 ensuing with inside the launch of lively IL-1β. Based on faulty responses of the monocyte-like leukemia mobileular line THP-1 to LPS stimulation, it's been recommended LPS through itself is useless as a stimulator of IL-1β launch[87]. It turned into consequently proposed that a second sign inclusive of MDP or ATP is needed to procedure and launch IL-1β. This second sign turns on the inflammasome and finally induces the activation of caspase-1[88]. However, this version is derived from research that investigated THP-1 cells[87] and number one mouse macrophages[89], and is in contradiction with research demonstrating that IL-1β is launched from monocytes after unmarried stimulation with a TLR ligand, inclusive of purified LPS, lipopeptides, or lipoteichoic acid[90, 91]. In macrophages want wonderful indicators: one sign that induces transcription and translation and a second sign that turns on caspase 1. These indicators will sooner or later bring about IL-1β processing and secretion[92] (Fig. 5). Macrophages are restrained to an environment (e.g., alveolar area and mucosal surfaces) wherein they're continuously uncovered to microbial stimuli and risk indicators. A touchy reaction of macrophages to launch IL-1β for every come across with such stimuli might bring about persistent deleterious inflammatory reactions.

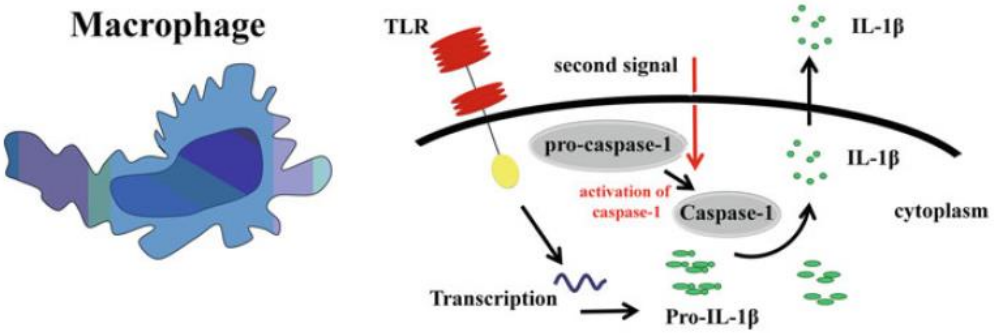

Figure 5. Macrophages require two signals: one, such as TLR ligand, which induces transcription, and a second that induces inflammatory activation as well as IL-1β processing and secretion. (From F.L. van de Veerdonk and M.G. Netea).

Long before the discovery of the inflammasome, the stimulatory effect of cytosol K+ depletion on proteolytic processing and IL-1β secretion from LPS prime macrophages and monocytes in response to compounds such as ATP and nigericin was observed[93-95]. Currently, this effect is known to be mediated by NLRP3[96], and K+ efflux remains the most characteristic minimal stimulus for NLRP3 inflammasome activation[97]. Conversely, incubation in media containing hyperphysiological [K+] may block NLRP3 inflammasome composition in response to most of the identified NLRP3 triggers[97]. The main classes of NLRP3 activators include millimolar extracellular ATP, K+ ionophore[96], and crystalline / particulate matter or other factors that cause lysosomal destabilization[98, 99]. All these stimuli are known to reduce cytoplasmic sol levels of K+ ions[97]. Transporting ions in the opposite direction to the electrical gradient (i.e., positive ions out of the cell or negative ions into the cell) requires significant energy consumption. In light of this, a sharp decrease in $[K+]_i$ required for NLRP3 activation (estimated to be at least ~20–30% decrease) [97] can be expected to be accompanied by either a counter-flux of cations or a "co-flux" of anions, which should also be provided by NLRP3 stimuli. Of the anions, this should also be provided by NLRP3 stimulation. It has been previously shown that a decrease in intracellular K+ concentration, the common activators NLRP3 ATP and nigericin, induce non-selective conduction of K+ across the cell membrane and changes in the intracellular ion content to initiate pro-IL-1β processing[94]. In addition, a decrease in intracellular K+ levels are important for NLRP3 inflammasome activation in monocytes/macrophages when triggered by a number of known NLRP3 activators, including the pore-forming toxin nigericin, ATP stimulation, or bacterial infection with live as *Escherichia coli* [100]. A carboxyl ionophore that can exist in nigericin in the form of a free membrane-impermeable anion or as a neutral membrane-permeable complex when attached to a K+ cation or proton (H+). In the electrically neutral K+ efflux cycle mediated by nigericin, ionophore anions bind to H+ outside the cell, pass through the plasma membrane as nigericin H+, and release protons on the cell side. There, the nigericin anion binds to K+, which is then transported across the plasma membrane as nigericin K and released

outside the cell[101]. This mechanism promotes the outflow of K+ by allowing the influx of H+, which leads to acidification of the cytosol. Activation of the NLRP3 nucleosome leads to the formation of mitochondrial ROS, which then induces lysosomal membrane permeation[102].

High plasma levels of fatty acids, mainly caused by consumption of high-fat diet, contribute to the development of several diseases in humans, including type 2 diabetes (T2DM)[103, 104]. Although there is evidence that chronic low-grade inflammation underlies insulin resistance, the hallmark of T2DM, the mechanistic details and the involvement of mediators of inflammation remains unresolved. Previous studies implicated high glucose levels and β-cell dysfunction in IL-1β production in T2DM[105, 106]. However, these studies could not fully explain the role of IL-1β secretion and inflammation in the pathogenesis of T2DM. Fatty acid palmitate (a saturated fatty acid), one of the most abundant fatty acids in human plasma[103], has been demonstrated, is capable of activating the inflammatory disease NLRP3[107]. BSA-conjugated palate exposure after priming LPS by macrophages and DCs resulted in increased secretion of IL-1β and IL-18[107]. Mechanistically, palmitate inhibit the phosphorylation and activation of AMP-activated protein kinase (AMPK), which is known to control autophagy directly through phosphorylation-dependent activation of unc-51-like kinase 1 (ULK1, the mammalian homolog of yeast ATG1)[108, 109]. Palmitate-exposed cells had higher amounts of mtROS, a known trigger for NLRP3 inflammasome activation, in the presence of abnormal autophagy. Consequently, exposing cells to the AMPK activator AICAR or transfecting them with constitutively active AMPK-1 restored autophagy via activating ULK1 phosphorylation, resulting in lower mtROS levels and IL-1 production[107]. Saturated fatty acid levels that are higher stimulate the formation of lipid species such as ceramides and DAG[110]. During obesity-induced diabetes, ceramide synthesis is linked to an inflammatory response[111-113]. Ceramide activation of LPS-primed BMDMs leads in caspase-1 cleavage and IL-1β release[114], which is consistent. Increased NLRP3 activity in adipose tissue macrophages, in turn, causes T cell activation and, as a result, reduces insulin sensitivity[115].

p-Coumaric acid (PCA) belongs to the family of hydroxycinnamic acid, a phenolic derivative. PCA is abundant in plants and mushrooms and is the most powerful antioxidant in nature. PCA is widespread in fruits and vegetables (apples, berries, pears, grapes, oranges, tomatoes, beans, potatoes, onions, etc.)[116]. Extensive studies have shown that PCA exhibits a variety of biological functions such as antioxidant[117], anti-inflammatory[118], anti-cancer activities[119], and etc.. Like other polyphenols, PCA reduces intestinal absorption of dietary carbohydrates, regulates enzymes involved in glucose metabolism, improves β-cell function and insulin action, stimulates insulin secretion, and has antioxidant and improves antioxidant and anti-inflammatory properties. Diabetes can be reduced by enhancing the inflammatory effect[120]. PCA and its conjugates inhibit the intestinal absorption of carbohydrates by inhibiting the enzyme glucosidase. α-Glucosidase inhibitors are potentially useful in the treatment of diabetes. Inhibition of α-glucosidase delays the digestion of starch or sucrose into glucose, which reduces glucose absorption and increases postprandial hyperglycemia[121]. PCA has a moderate inhibitory effect on α amylase and glucosidase or βglucosidase. In addition, PCA and its conjugates hinder aldose reductase, a protein that catalyzes the diminishment of glucose to sorbitol. The actuation and/or overexpression of aldose reductase are related to diabetic disorder. In this way, restraint of this protein is significant to avoid or treat persistent diabetic complications, counting neuropathy, nephropathy, cataracts, retinopathy, quickened atherosclerosis and expanded cardiovascular hazard[122]. Besides, PCA and its conjugates actuate AMP-activated protein kinase, an chemical that directs vitality homeostasis by expanding glucose take-up, β-oxidation fatty acid oxidation, and triacylglycerol synthesis; hence, the activators of AMP-activated protein kinase (AMPK) are regularly utilized as anti-diabetic specialists[123]. Furthermore, PCA and its conjugates inhibit gluconeogenesis. Gluconeogenesis, which plays a role in preventing hypoglycemia, is a metabolic pathway that forms glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol, and glucogenic amino acids. However, increased gluconeogenesis has been reported to

contribute to insulin resistance in the liver of type 2 diabetes; in this way, restraint of gluconeogenesis could be a target for the therapeutic target for type 2 diabetes[124, 125]. Interestingly, PCA increases insulin sensitivity. Insulin resistance develops during the period from prediabetes to the advanced stage of type 2 diabetes. Adiponectin, produced and secreted only by adipocytes, is involved in energy homeostasis and regulates insulin sensitivity[126]. Thus, upregulation of adiponectin increases insulin sensitivity. Coumaric acid enhances adiponectin expression in 3T3-L1 adipocyte. PCA can inhibit adipogenesis. Obesity has gotten to be the driving metabolic disease in the world and is related with type 2 diabetes. In mice and rats, PCA inhibits lipid accumulation and reduces the low-density lipoprotein cholesterol and its oxidation[117]. In vitro studies have shown that PCA inhibits adipogenesis in 3T3-L1 cells and triglyceride accumulation in HepG2 cells[127, 128]. PCA also inhibited tumor necrosis factor (TNF) α-induced changes in the levels of monocyte chemoattractant protein 1 (MCP1), interleukin 6 (IL6), plasminogen activity inhibitor 1 and reactive oxygen species in 3T3-L1 adipocytes[129]. These data suggest that the anti-inflammatory and antioxidant effects of PCA may help alleviate complications associated with obesity. Consistent with in vitro findings, an in vivo study showed that PCA (100 mg/kg bw) ameliorated HF diet-induced hyperlipidemia and steatosis in mice by reducing oxidative stress[130, 131]. Finally, PCA can alleviate diabetes syndrome due to its antioxidant, anti-inflammatory and immunomodulatory activities. Oxidative stress, inflammation, and immunological changes are common pathogens of type 2 diabetes[131]. Due to its antioxidant, anti-inflammatory and immunomodulatory activities[132], PCA may alleviate diabetes by protecting against oxidative stress and inflammation and increasing its immunomodulatory effects.

Our previous report showed that PCA-enriched peanut sprout inhibits HF diet-induced obesity and protects against inflammatory-induced inhibition of adipocyte browning through mitochondrial activation[57]. We found that PCA was identified as the most abundant polyphenol in peanut sprouts mimicking the action of peanut sprouts. Base on the evidence presented, we aimed to evaluate the role of PCA in the prevent hepatic inflammation, fibrosis,

and NAFLD in HFHS diet-induced obese mice. With the intention of revealing the potential we set up HFHS fed C57BL/6 mice as an animal model; induced macrophage inflammasome by lipopolysachride (LPS), nigericin (Ng), and palmitate (PA) stimulation in bone marrow-derived macrophages (BMDM) differentiated or TGFβ was stimulated in LX-2 human hepatic stellate cell line (LX-2) as a cell model. In this study, treatment with PCA (50 mg / kg body weight) is effective in reversing HFHS diet-mediated metabolic disorders such as insulin resistance (IR), adipocyte inflammation, and hepatic stress, along with the characteristics of fibrosis.

2. Materials and Methods

2.1. Experimental chemicals and reagents preparation

All cell culture dishes and were purchased from SPL (Seoul, Korea) unless otherwise stated. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco (Grand Island, NY, USA). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

p-Coumaric acid (PCA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). We freshly prepared 400 mg/mL PCA stock was diluted with phosphate-buffered saline (PBS) and diluted 200 mM of PCA stock with dimethyl sulfoxide (DMSO) for animal and cell culture, respectively.

2.2. Animals and diets

All animal procedures complied and approved by the Institutional Animal Care and Use Committee at the Jeju National University (Approval ID # 2020-0015). C57BL/6J 6-week-old mice (ORIENT BIO Animal Center, Seongnam-si, Korea) were used to build the animal experiment and housed in a dark/light cycle at Jeju National University. After one week of acclimation, the C57BL/6 mice were divided into three (n=6): low-fat diet (LF, 11% kcal from fat), or high-fat diet (HF, 60% kcal from fat) with high sucrose drink (HFSH, 20% sucrose), or HFHS diet with PCA intraperitoneal (i.p.) injection (HFHS+PCA, 50 mg/kg BW) (Fig. 4). The experimental period was 13 weeks. AIN-93G diet was used as the LF control diet, and the HF diet is modified from AIN-93G and the typical HF diet (60% kcal from fat)

(**Supplementary Table 1**) as previously described[133]. Food intake and drink intake were freely accessed and measured 3 times a week. Body weight was recorded every weekly.

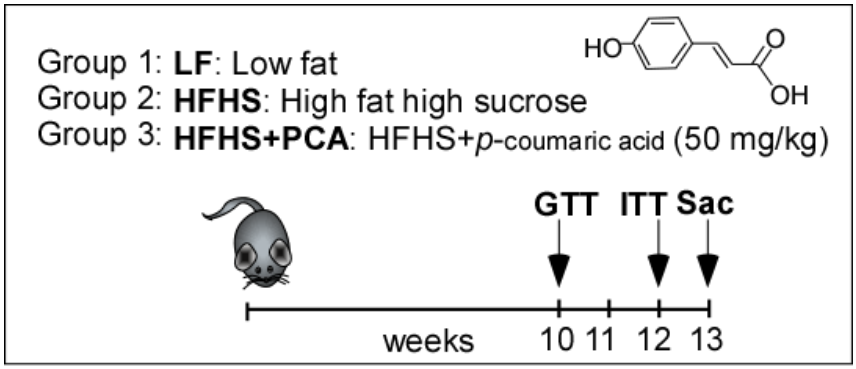

Figure 6. Animal experiment design. 7-week-old male mice were divided to three group low-fat diet (LF, 11% kcal from fat), or high-fat diet (HF, 60% kcal from fat) with high sucrose drink (HFSH, 20% sucrose), or HFHS diet with PCA intraperitoneal (i.p.).

2.3. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

The experimental animals underwent (GTT) and (ITT) at the 11th and 12th weeks of the experiment, respectively. For GTT, after 12 hours fasting, 10 % D-glucose solution (0.5 g/kg BW) was administered intraperitoneally and blood glucose levels were measured at 15, 30, 60, and 120 min by using glucose meter (Midium Blood Glucose Analyzer, Kia Ace Co., Ltd., Gyeonggi, Korea). For ITT, mice were fasted 12 hours before intraperitoneal injection 0.75 mU/kg BW insulin and blood glucose levels were measured at set time points by using glucose meter (Midium Blood Glucose Analyzer, Kia Ace Co., Ltd., Gyeonggi, Korea).

2.4. Serum biochemistry parameters measurement

After the experiment ended, C57BL/6J were fasted 12 hours and sacrificed by carbon dioxide narcosis. Blood was collected from a cardiac puncture to determine serum biochemistry. The concentration of serum triglyceride (TG, mg/dl) and total cholesterol (TC, mg/dl) were measured spectrophotometer using enzyme assay kit (Asan Pharmaceutical Co., Seoul, Korea) followed by manufacture's protocol with absorbance at 500 nm, 550 nm, respectively.

The serum glutamic oxaloacetic transaminase (GOT, IU/L) and serum glutamic pyruvic transaminase (GPT, IU/L) were analyzed using (Asan Pharmaceutical Co., Seoul, Korea) with either absorbance at 505 nm.

The serum high-density lipoprotein (HDL, mg/dL) cholesterol and low-density lipoprotein (LDL)/ very low density lipoprotein (VLDL) cholesterol (LDL/VLDL, mg/dL) were performed using EZ-HDL, LDL/VLDL Assay Kit, respectively (Dozen Bio Co., Ltd., Sudogwon, Korea) with absorbance at 570 nm.

The quantitative insulin in mouse serum was measured by following ultra-sensitive mouse insulin ELISA kit (Crystal Chem, Elk Grove Village, IL 60007, USA) protocol with absorbance at absorbance 450 nm and subtract absorbance 630 nm.

The homeostatic model assessment for insulin resistance (HOMA-IR) was measured as $\frac{\text{Fasting plasma glucose} \times \text{Fasting serum insulin}}{22.5}$ (mmol/L).

The plasma was used to measure the IL-1 beta/IL-1F2 by following the protocol of Mouse IL-1 beta/IL-1F2 quantikine ELISA Kit (MLB00C, R&D Systems, Inc., Minneapolis, MN 55413, USA) with absorbance 450 nm colorimetric or subtract readings at 540 nm from the readings at 570 nm.

2.5. Tissue preparation and histological examination

After autopsy, epididymal adipose and liver tissue was removed from the mice and flash-fixed with 10% formalin buffer. Adipose tissue embedded in paraffin was cut into 5-7 μm sections and treated for hematoxylin and eosin (H&E) staining as previously described[134]. Bright field images were taken at 10x magnification using an Invitrogen microscope (Invitrogen ™ EVOS ™ FL Digital Inverted Fluorescence Microscope, Invitrogen, CA, USA). H&E stained sections of epididymal adipose tissue was used for size determination by following the published protocol by Seo et al[134]. Briefly, we used ImageJ software (NIH, USA) to analyze adipocyte size by analyzing digital images of H&E stained paraffin sections.

Liver tissues were harvested from mice and immediately fixed in 10% buffered formalin. Tissues were embedded in paraffin, sectioned to a thickness of 4 μm, and stained with Masson's trichrome to detect collagen fibers. All slide images were taken using a LEICA DM 2500 microscope (Tucsen Photonics Co., Ltd.). The percentage of blue collagen

deposition over the total tissue area reflecting liver fibrosis was calculated using MIPAR software (MIPARTM, Worthington, OH, USA).

Liver tissues were harvested from mice and immediately fixed in 10% buffered formalin, processed, and embedded in paraffin for Sirius red staining. The images were taken at 40x magnification using an Invitrogen microscope (Invitrogen™ EVOS™ FL Digital Inverted Fluorescence Microscope, Invitrogen, CA, USA) and liver collagen was quantified by image analysis of Sirius red stained sections.

2.6. Accumulation of lipids in the liver

Total liver lipids were extracted from 200 mg of liver tissue in methanol/chloroform (1: 2 volume ratios [v/v]). After samples were incubated at 60^{O}C for 3 hours, samples were incubated at room temperature overnight. The next day, the resulting extract was filtered through Whatman® filter paper. The sample was then washed several times with chloroform and the mixture was dried at room temperature. The resulting sample was then resuspended in deionized water.

Measurement of TG and TC content in lipids at 550 nm and 500 nm, respectively, using an enzyme assay kit (Asan Pharmaceutical Co., Seoul, South Korea). The liver lipid nonesterified fatty acid was measured by LabAssayTM NEFA (FUJIFILM Wako Pure Chemical Co.,Osaka, Japan**)** flowing protocol attachment at 550 nm.

The liver free cholesterol was performed by following protocol of cholesterol assay kit-HDL and LDL/VLDL (ab65390, Abcam, Cambridge, MA, USA) with fluorescence at excitation/emission = 535/587 nm (fluorometric).

2.7. Malondiadehyde (MDA) assay

During oxidative stress, malondialdehyde (MDA) is one of the most common consequences of lipid peroxidation. Briefly, total liver protein was isolate from 25 mg of liver tissue in RIPA Buffer containing protease inhibitor and phosphatase inhibitor. The procedure was followed protocol of TBARS Asay Kit (Item No. 10009055, Cayman Chemical ,1180

East Ellsworth Road, Ann Arbor, Michigan 48108 USA) with the absorbance at an excitation wavelength of 530 nm and an emission wavelength of 550 nm

2.8. Cell culture and treatment

The LX-2 Human Hepatic Stellate Cell Line were purchased from Sigma (St. Louis, MO, USA). Briefly, LX-2 cells were cultured in a basal medium containing 2% FBS, DMEM containing 1000 mg/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate, 100 units/mL penicillin and 100 g/mL streptomycin.

For investigation of anti-inflammatory or anti-fibrogenic properties of PCA, LX-2 cells were stimulated TGFβ. LX-2 cells were seeded at 1.2 x 10^6 cells cm^{-2} in 35-mm culture plates and pre-incubated with 1000X DMSO (as a vehicle) or 200 μM PCA for 48 h. After 48 h, LX-2 cells were starved in DMEM with or without 200 μM PCA for 2 h. Then, LX-2 cells were stimulated with 5 ng/mL TGFβ + 200 μM PCA for 24 h. In the end of period, protein, RNA, and media were collected.

Primary bone marrow cells were isolated from the femurs of 20-week-old C57BL/6 mice and stimulated to differentiate for 7–10 days in L-cell conditioned medium (CM) as we described previously[135]. The resulting differentiated bone marrow-derived macrophages (BMDMs) were pretreated with 200 μM PCA or vehicle (1000X DMSO) for 24 h, then primed with lipopolysaccharide (LPS) (100 ng/ml) for 1 h and stimulated either with nigericin (Ng; 6.5 μM, a K+/H+ ionophore) for 1 h or palmitate (PA; 400 μM complexed with BSA) for 12 h.

2.9. Analysis of mRNA by Real-time Polymerase Chain Reaction

0.2 g of liver and adipose tissue and cells rapidly stored in the freezer were subjected to RNA extraction using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA). Extracted RNA concentrations were measured using nanodroplets (Nano200 MicroSpectrophotometer, Hangzhou, China) and then cDNA was synthesized with a high-performance cDNA reverse transcription kit (Applied Biosystem, USA). Gene expression was determined by real-time PCR (CFX96 ™ real-time PCR detection system, BioRad, USA) (Supplementary Table 2).

The relative gene expression was normalized ribosomal protein lateral stalk subunit P0 (RPLP0, *36B4*), *Gapdh*, *β-actin* and *Hprt* (Cosmo Genetech).

2.10. Protein Isolation and Western Blotting

Tissue samples were collected and homogenized with a homogenizer in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, MA, USA) with protease and phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA) and centrifuged to collect the supernatant. We separated 10-15 μg of protein using 10%-12.5% SDS-PAGE and transferred the protein to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, MA, USA) with tris-buffered saline/Tween 20 and blocked for 1 hour at room temperature. The membranes were washed several times with Tris-buffered saline with tween 20 (TBST) solution and reacted overnight at $4^{O}C$ with primary antibodies against phosphor or total of Caspase 1(Merck KGaA, Darmstadt, Germany) extracellular signal-regulated kinases (ERK), Kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα), phosphor or total of stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK), phosphor or total of p38 (p38), BiP, CHOP, β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell signaling Technology, Danvers, MA, USA, anti-alpha smooth muscle Actin (αSMA), anti-Collagen I (Collagen I) (Abcam, Cambridge, MA, USA), and human/mouse NLRP3/NALP3 antibody (NLRP3) (R&D Systems, Inc., Minneapolis, MN 55413, USA). The next day, the membrane was washed several times with 1X TBST and then incubated with the secondary antibody, goat anti-rabbit, or anti-rat IgG, HRP-linked antibody (Cell signaling Technology, Danvers, MA, USA), or goat anti-mouse IgG-HRP (Santa Cruz biotechnology, CA, USA) for 1 hour. The membranes were washed and reacted with enhanced chemiluminescence (ECL) reagent (PerkinElmer, Waltham, MA, USA). To identify the band, ChemiDoc (Bio-Rad, CA, USA, at Bio-Health Materials Core-Facility at Jeju National University) was used, and the expression level was calculated using Image Lab (Bio-Rad, CA, USA) or Image J (NIH, MD, USA).

2.11. Statistical Analysis

The experiment's results are expressed as the mean ± standard error of the mean (SEM). Statistical calculations were performed using ANOVA (one-way analysis of variance) with Bonferroni's multiple comparison test or student's t-test. P-value <0.05 was considered statistically significant. All analyses were performed with GraphPad Prism 8.02.263 (La Jolla, California., USA), and SPSS 16.0 (SPSS software, Inc).

3. Result

3.1. PCA supplementation ameliorated HFHS-mediated glucose and insulin tolerance

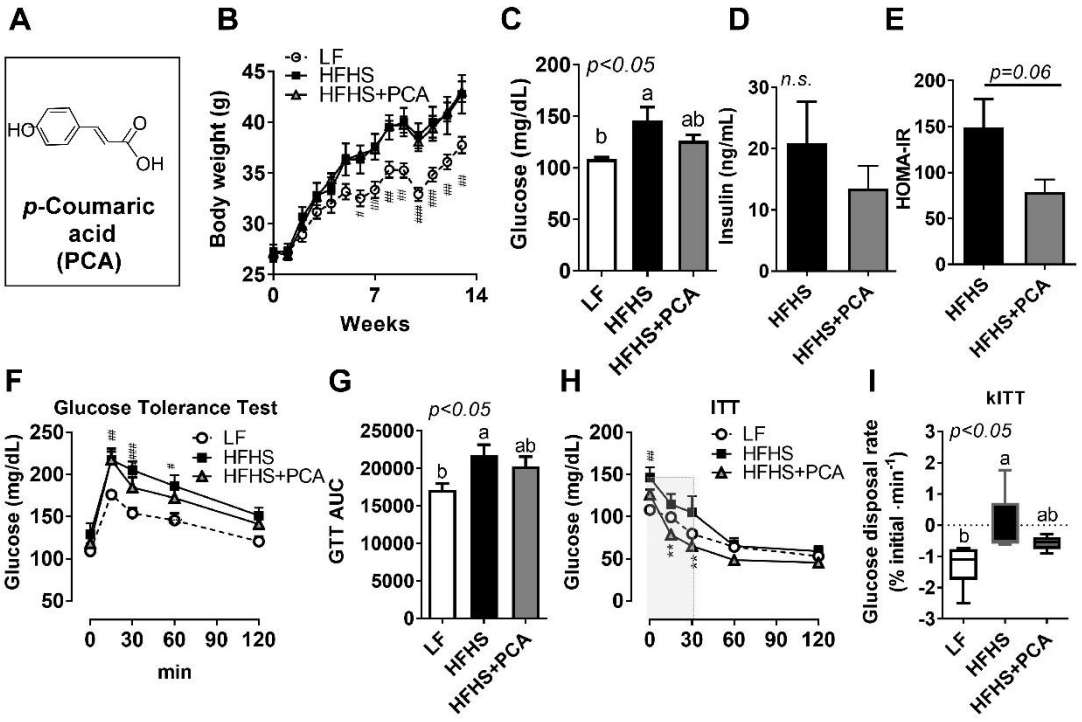

Figure 7. PCA supplementation ameliorated glucose and insulin tolerance in C57BL/6 mice against HFHS diet. Seven-week-old C57BL/6 male mice were random separated three group: LF group (black circle, black bar) or HFHS diet and 5% DMSO (grey square, grey bar) in PBS or 50 mg / kg PCA were supplemented by intraperitoneal (i.p.) injection (HFHS + PCA, white triangle, white bar) for 13 weeks (n = 6 per group). **A**. *p*-Coumaric acid structure. **B.** Body weight; **C**. Fasting plasma glucose levels (ng/ml); **D**. Quantify levels of plasma insulin (mg/dL); **E**. Homeostatic model assessment for insulin resistance (HOMA-IR); **F**. Glucose tolerance test (GTT, mg/dL); **G.** Area under curve (AUC); **H**. Insulin tolerance test; **I**. Glucose disposal rate (% initial.min-1). Data are expressed as the mean ± SEM (n=6). n.s. represents no significance. Bars with different letters are significantly different according to one-way ANOVA with Bonferroni's comparison test; **p<0.01 (HFHS vs. HFHS+PCA); #p<0.05, ##p<0.01, ###p<0.001 (LF vs. HFHS).

7-week-old C57BL / 6 mice were treated with PCA or vehicle and fed an LF or HFHS diet for 13 weeks. To investigated whether PCA supplementation affected glucose metabolism mediated -mediated obesity. Despite the reduction in food intake (g/day) (Supplementary table 2), HFHS fed mice no different daily caloric intake compared with LF group (LF = 22.08 kcal/day vs. HFHSD = 19.83 kcal/day). As expected, the HFHS fed mice were obese, significantly increased BW, body weight gain compared to LF (Fig. 7B, Supplementary table 2). In addition, the mass of liver, and epididymal fat were remarkable rise compared with the

LF mice (Supplementary table 2). Consistent with this result, a 13-week chronic intake of the HFHS diet significantly increased the fasting plasma glucose which was partly reduce in HFHS+PCA fed mice (Fig. 7C). Although there was not different fasting plasma insulin level between HFHS diet and HFHS+PCA group (Fig. 7D) but PCA supplementation reduce HOMA-IR content, an indicator for insulin resistance, by ≈50% compared to HFHS group alone (Fig. 7E). HFHS group experienced a slow decrease in the rate of glucose tolerance during GTT while PCA supplementation partially normalized GTT (Fig. 7F) which was confirmed by quantification of GTT AUC (Fig. 7G). Similarly, the HFHS group experienced a slow decrease in the rate of glucose clearance during hyperglycemia and GTT. The HFHS + PCA group showed a partial improvement of glucose intolerance confirmed by quantifying the area of the GTT under the curve (AUC) (Fig. 7G). During ITT, HFHS mice maintained higher glucose levels (Fig. 7H) and glucose tolerance (kITT) than the LF group (Fig. 7I). In contrast, HFHS diet-mediated glucose tolerance (Fig. 7H) and glucose disposal rate (Fig. 7I).

3.2. PCA protects against HFHS- mediated hepatic lipogenesis

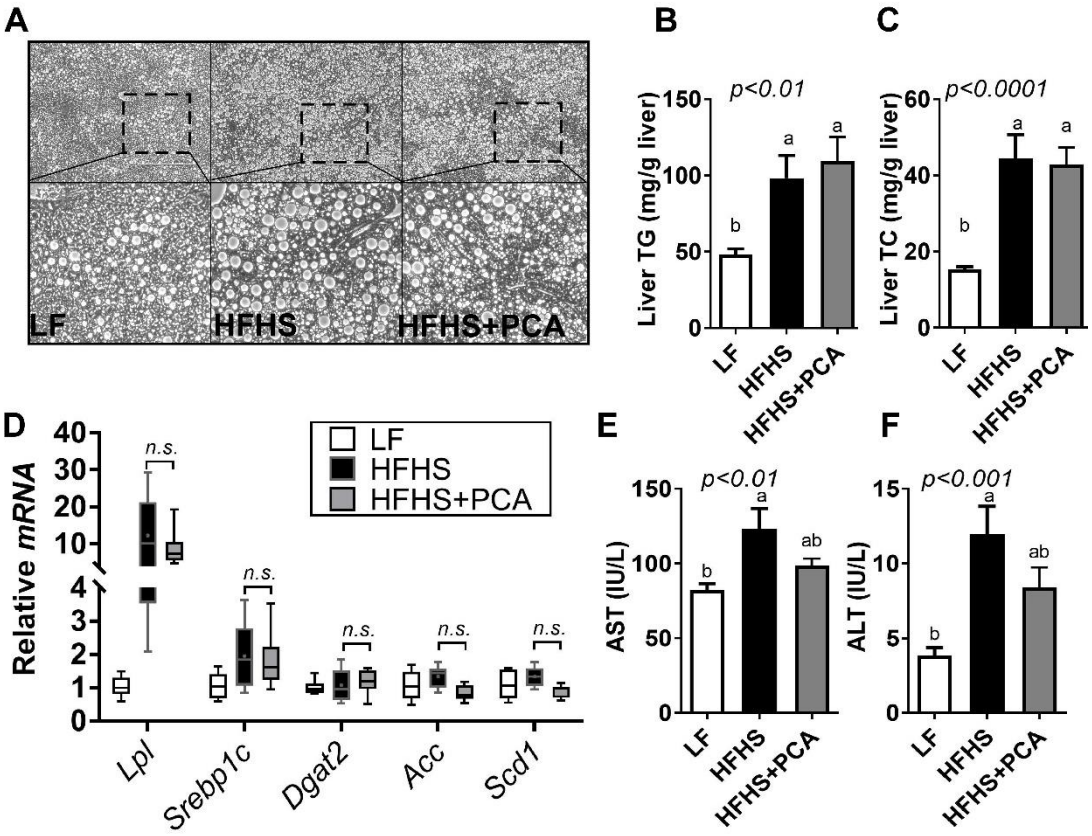

Figure 8. Effect of PCA treatment on HFHS-mediated hepatic inflammation and hepatic stress. **A.** Representative microscopic images of liver tissue (magnification 10X) by hematoxylin and eosin (H&E) staining; **B.** Hepatic triglyceride (TG) content (mg/g liver weight); **C.** Liver total cholesterol (TC) content (mg/g liver weight); **D.** Expression of adipogenic genes Lpl, Srebp1c, Dgat2, Acc, and Scd1 mRNA in isolated hepatocyte tissue using Real-time PCR; **E.** Aspartate transaminase levels (AST, IU/L); **F.** Alanine transaminase levels (ALT, IU/L). Data are expressed as the mean ± SEM. n.s. represents no significance. Bars with different letters are significantly different according to one-way ANOVA with Bonferroni's comparison test.

Chronic intake of HFHS is known to lead to hepatic lipogenic and NAFLD[136-138]. Therefore, we hypothesized that PCA supplements reduce HFHS-mediated lipogenesis. After 13 weeks, HFHS fed mice increased significantly liver mass (Supplementation table 2), lipid droplets in liver histology (Fig. 8A), and liver lipid content (TG and TC levels) (Fig. 8B-C) compared to the LF group. However, there was no difference in liver weight and hepatic lipid profile (TG and TC levels) between the HFHS group and the HFHS+PCA group (Supplementation table 2, Fig. 8B-C).

Next, we examined hepatic lipogenic-related genes such as *Lpl, Srebp1c, Dgat2, Acc, and Scd1*. The *Lpl, Srebp1c*, and *Dgat2* mRNA expression in the HFHS group were elevated relative to the LF group, which was trended decrease in PCA treatment (Fig. 9D). Despite no significant Acc, and Scd1 mRNA expression between the HFHS group and HFHS+PCA group but PCA treatment trended reduced (Fig. 8D).

Aspartate transaminase levels (AST test) and alanine transaminase levels (ALT test) are the severity of tissue damage. AST and ALT are a useful biomarker for indicator liver disease. Serum AST level tended to increase markedly in the HFHS group compared to the LF group which was partly reduced in PCA supplement (Fig. 9E). ALT level was also extreme growth in the HFHS group compared to the LF group, which showed HFHS-mediated liver damage. Elevated ALT levels were slightly normalized by the HFHS+PCA group (Fig. 9F).

3.3. PCA suppressed HFHS diet-mediated hepatic inflammation, and hepatic stress

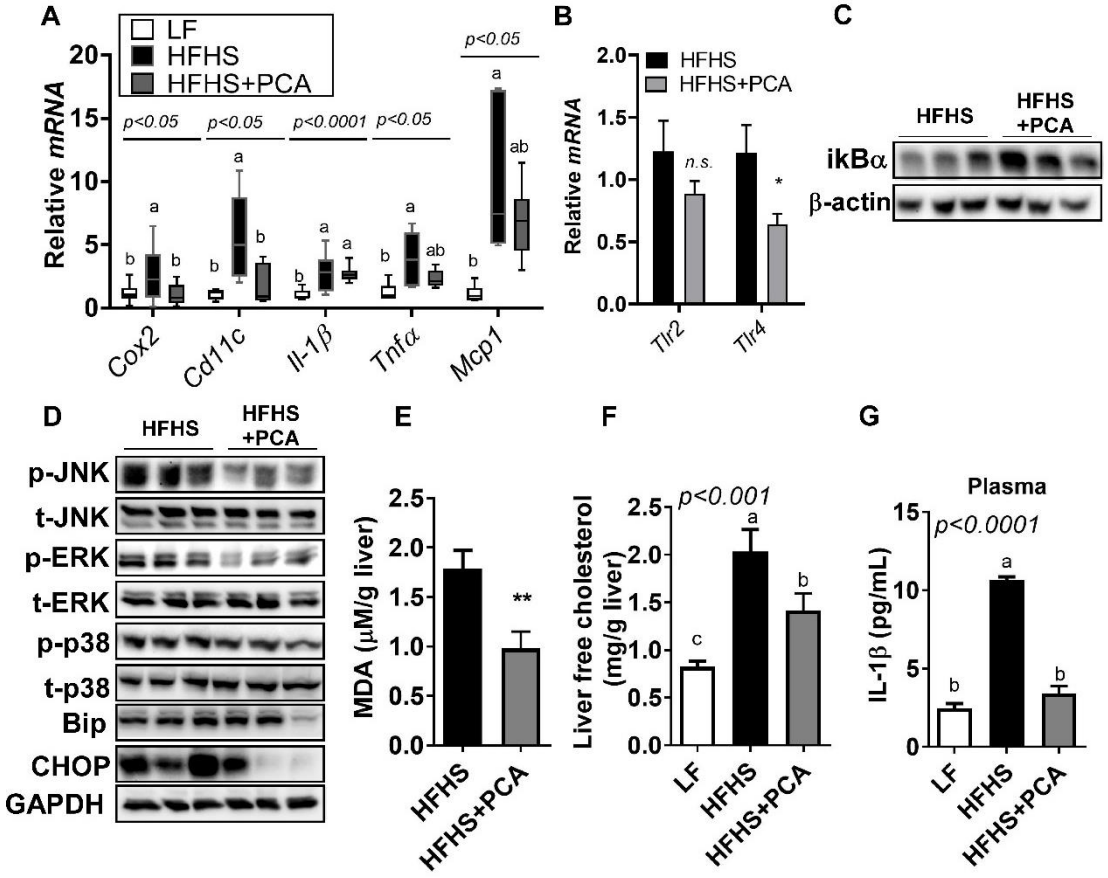

Figure 9. Effect of PCA treatment on HFHS-mediated hepatic inflammation and hepatic stress. **A**. Expression of inflammatory genes associated *Cox2, Cd11c, Il-1β, Tnfα* and *Mcp1* in isolated liver tissue using Real-Time PCR; **B**. Expression of the activation of innate immune cell as Tlr2 and Tlr4 in isolated liver tissue using Real-Time PCR; **C**. Protein expression of iκBα in liver tissue and β-actin served as a control (n=3); **D**. Representative western blot analysis of p-JNK, t-JNK, p-ERK, t-ERK, p-p38, t-t38, Bip, and CHOP protein levels, and GAPDH used for control (n=3); **E.** Malondiadehyde (MDA, μM/g liver); **F.** Liver free cholesterol in liver (mg/g liver); **G.** The plasma IL-1 beta/IL-1F2. Data are expressed as the mean ± SEM. n.s. represents no significance. Bars with different letters are significantly different according to one-way ANOVA with Bonferroni's comparison test.

Chronic intake of HFHS is known to lead to hepatic inflammation[139-141] and hepatic stress[142-144]. Therefore, we hypothesized that PCA supplements reduce HFHS-mediated steatohepatitis and endoplasmic reticulum (ER) stress. The effect of the HFHS diet on liver endoplasmic reticulum stress is associated with liver inflammation and steatosis[142]. We measured inflammatory gene expressions and ER stress markers in the liver. HFHS led to a significant increase in *Cox2, Cd11c* compared with LF diet, while decreased in HFHS+PCA diet compared with HFHS diet. The mRNA levels of *Tnfα* and *Mcp1* in HFHS group was dramatically elevated compared with that of the control and partly reduced in HFHS+PCA fed

mice. *IL-1β* mRNA expression in the HFHS group were elevated relative to the LF group, which was unaffected by PCA treatment (Fig. 9A). TLR4 and TLR2 are evolutionarily conserved pathogen recognition receptors that mediate the NF-B pathway, which leads to the development of an immune response[145]. Although *Tlr2* mRNA expression was no significant between the HFHS group and HFHS+PCA group but PCA treatment trended reduce. The PCA supplementation was markable diminution *Tlr4* mRNA expression compared to HFHS fed mice (Fig. 9B). Moreover, the iκBα, which inhibits the NFkB transcription factor that plays an important role in inflammation and immune response, was significantly increased in the PCA treatment (Fig. 9C).

Increasing activation JNK, ERK, and p38 via HFHS feeding was downregulated in the HFHS+PCA group. Similarly, the alteration of BiP, CHOP, and Caspase 1 activity were observed. Compared with the HFHS group, there was a decrease in the BiP (is a major ER stress) and CHOP (induced by ER stress and mediates apoptosis) by PCA treatment in HFHS+PCA groups (Fig. 9D).

MDA is a naturally occurring lipid peroxidation product. Lipid peroxidation is a well-known cause of cellular damage in animals and is utilized as a marker of tissue oxidative stress[146, 147]. The HFHS+PCA fed mice significantly fell MDA compared with the HFHS (Fig. 9E). Increasing cholesterol uptake speed up liver fibrosis. Free cholesterol accumulation is the critical key which accelerate liver fibrosis in the hepatocyte. Free cholesterol increases the level of TLR4 by inhibiting the endosome-lysosomal degradation pathway of TLR4, thereby down-regulating the expression of bone morphogenetic protein to make cells sensitive to transforming growth factor TGFβ-induced activation and activin membrane binding inhibitors[148]. Consolidating result of the reduction of *Tlr4* mRNA expression (Fig. 9B), the free cholesterol grown dramatically in the HFHS group (more than doubled compared to LF group which was considerable decline in PCA treatment (Fig. 9F). Similarly, the plasma IL-1β-related to the activation of NFκB signaling, the up-regulation of pro-inflammatory cytokines, liver damage and apoptosis[149]-increased dramatically in HFHS group compared with the LF group. Meanwhile, the HFHS+PCA group was tremendous reduction compared

to the HFHS group and became normal compare with LF group (Fig. 9G). Taken together, these data suggest that PCA reduces liver inflammation, AMPK activation and ER caused by the HFHS diet.

3.4. PCA supplementation improved HFHS-mediated fibrosis in the liver and LX-2 hepatic stellate cells

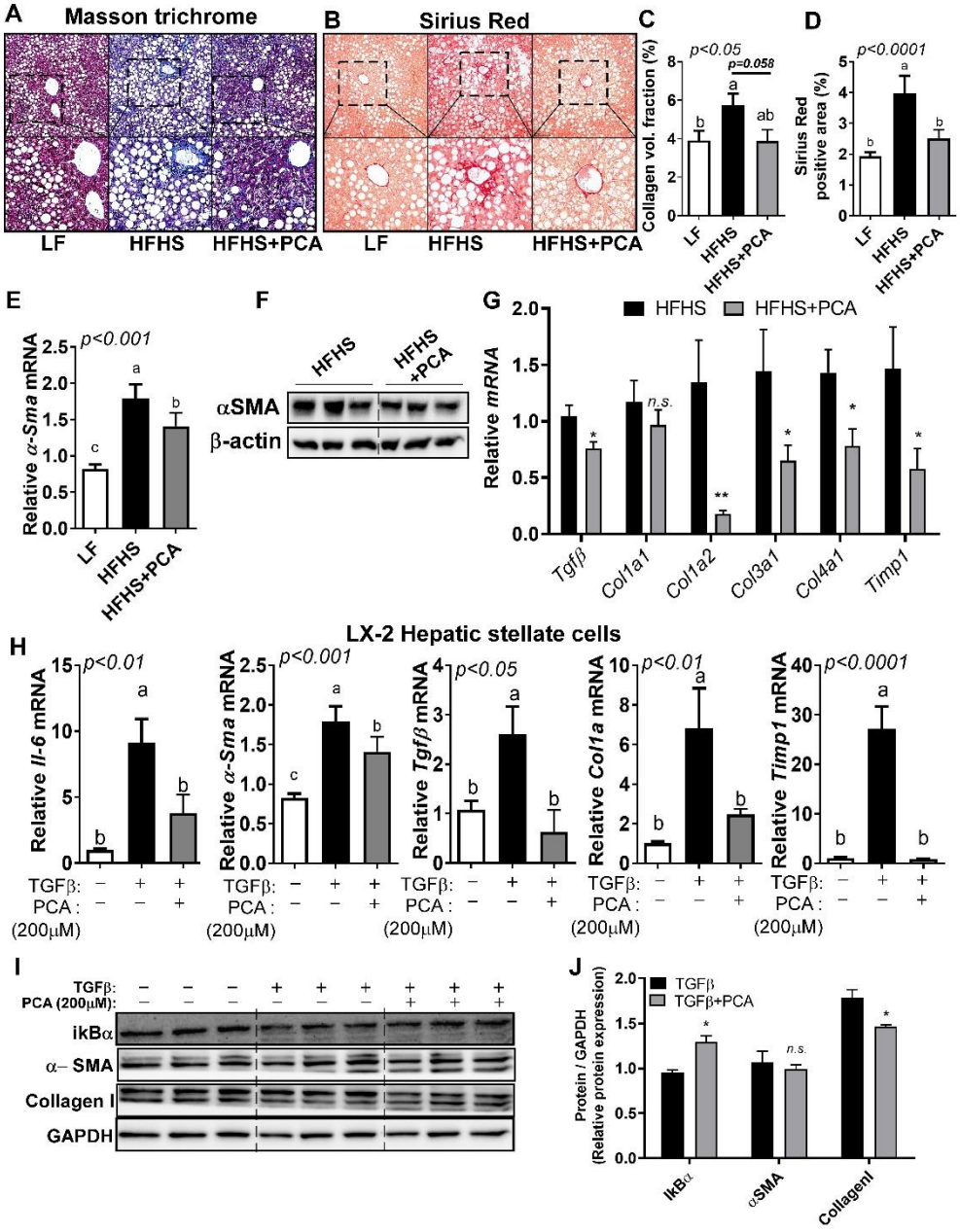

Figure 10. PCA improved HFHS-mediated liver fibrosis in the mice livers and LX-2 cells. **A**. Histopathological examination of Masson's trichrome staining (above, 40-fold magnification); **B.** Sirius red staining (below, 40-fold magnification) in liver tissue; **C**. Quantification of fibrosis in the Masson's trichrome staining (volume fraction of collagen, %); **D.** Quantification of collagen area in the Sirius red staining; **E**. mRNA expression levels of αSMA in liver tissue were performed by real-time PCR; **F**. Protein expression of α-SMA in liver tissue and β-actin served as a controls (n=3). **G.** mRNA expression levels of fibrosis-related gene expression including *Tgfb, Col1a1, Col1a2, Col3a1, Col4a1,* and *Timp1* in liver tissue were performed by real-time PCR. **H.** The mRNA expression levels of inflammatory marker *Il-6,* and fibrosis-related gene expression of *α-Sma, Tgfβ, Col1a,* and *Timp1* in the LX-2 hepatic stellate cells. **I.** Protein expression of ικBα, α-Sma, and Collagen I in the LX-2 hepatic stellate cells and GAPDH served as a control (n=3). **J.** The protein quantification of ικBα, α-Sma, and Collagen and GAPDH served as a control. Data are expressed as the mean ± SEM. Bars with different letters are significantly different according to one-way ANOVA with Bonferroni's comparison test.

Long-term intake of HF and HS diets causes mild hepatic fiborsis[12, 150, 151]. We asked if PCA would reduce HFHS diet-mediated liver fibrosis. By Masson's trichrome staining, identify an increase in collagen in diseases such as cirrhosis, the hepatocytes were generally increase in liver fibrosis in the HFHS diet compared with LF fed mice while there was significantly reduced in HFHS+PCA fed mice. In addition, hepatocyte lipid droplet was high present in the HFHS groups, especially reduced in HFHS+PCA mice (Fig. 10A). The software was used to quantify the histopathological fibrosis score (collagen volume fraction) in Masson's trichrome staining liver sections. As a result, the HFHS group had the highest percentage of collagen that could lead to liver scarring, whereas PCA treatment significantly reduced the amount of collagen comparable to the LF group (Fig. 10C).

Collagen accumulation was determined on Sirius-red stained sections. Histological examination of collagen accumulation by Sirius-red staining showed excessive deposition of collagen fibers in HFHS group with liver fibrosis, which was visualized as a red fibrous tissue band around the portal and central veins. On the other hand, treatment with PCA prevented the deposition of collagen fibers (Fig. 10B). These results were confirmed by collagen quantitative fraction (Fig. 10D). The result illustrated that the HFHS fed mice dramatically grown compared to LF group which showed a downward trend in the PCA treatment. In addition, the HFHS diet up-regulated the expression of the αSMA gene and protein, reflecting the activation of myofibroblast-like cells abolished by PCA (Fig. 10C-D). Furthermore, PCA supplementation normalized the HFHS diet-induced fibrosis-related gene expression of transforming growth factor beta (*Tgfβ*), procollagen type I, α2 (*Col1a2*) procollagen type III, α1 (*Col3a1*), procollagen type IV, α1 (Col4a1) tissue inhibitor of metalloproteinase-1 (*Timp1*), but not fibrosis-related procollagen type I, α1 (*Col1a1*) (Fig. 10G).

To mimic the fibrosis stage in the liver, we stimulated 5 ng/mL TGFβ in the LX-2 hepatic stellate cells. We examined that PCA significantly reduced LX-2 hepatic stellate cells inflammation and fibrosis evidenced by dampened levels of inflammatory marker *Il-6*, and fibrosis-related gene expression of *α-Sma, Tgfβ, Col1a,* and *Timp1* while dramatically

increased in the HFHS group (Fig. 10H). To further test whether PCA improves fibrosis, we examined the fibrosis markers in in the LX-2 hepatic stellate cells. The supplementation of PCA blunted the HFHS diet- induced IκBα degradation-a sign for reduced NFκB activation, reduced Collagen I-was increased during the progression from simple steatosis to NASH[152], but not α-SMA protein expressions (Fig. 10I). These results were confirmed by protein quantitative in the Fig. 10J.

3.5. PCA inhibited NLPR3 inflammasome activation in macrophages

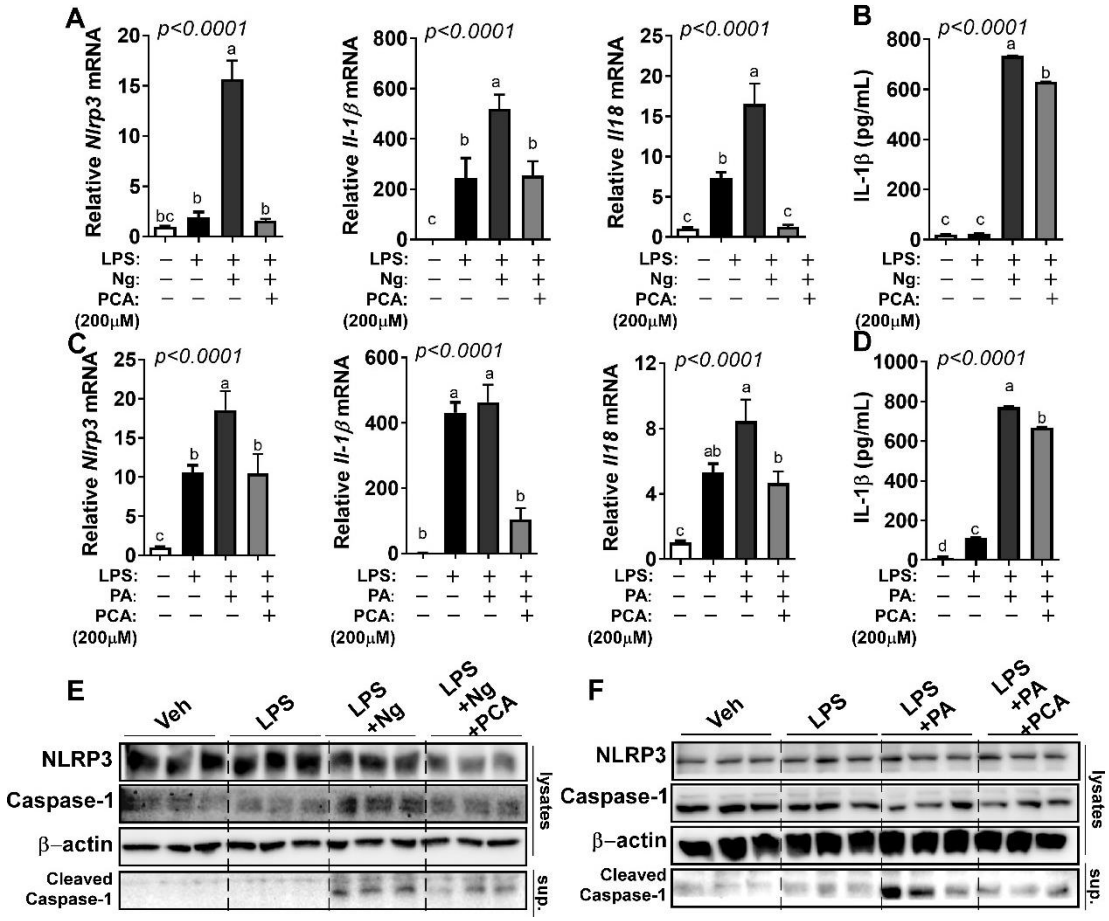

Figure 11. PCA inhibited NLPR3 inflammasome activation in macrophages. **A-B**. The mRNA expression levels of *Nlrp3, Il-1β,* and *Il18* were measured and normalized against that of *36B4* (using real-time PCR); the IL-1 beta/IL-1F2 from cell supernatant (n=3) after 1h primed LPS and then stimulated Ng with or without PCA for 1 h, respectively. **C-D.** The mRNA expression levels of *Nlrp3, Il-1β,* and *Il18* were measured and normalized against that of *36B4* (using real-time PCR); the IL-1 beta/IL-1F2 from cell supernatant (n=3) after 1h primed LPS and then stimulated PA with or without PCA for 12 h, respectively. **E.** Protein expression of NLRP3, caspase 1, and β-actin served as a control (n=3) in cells extract; cleaved caspase 1 in supernatant (n=3) after 1h primed LPS and then stimulated Ng with or without PCA for 1 h. **F.** Protein expression of NLRP3, caspase 1, and β-actin served as a control (n=3) in cells extract; cleaved caspase 1 in supernatant (n=3) after 1h primed LPS and then stimulated PA with or without PCA for 12 h. Data are expressed as the mean ± SEM. Bars with different letters are significantly different according to one-way ANOVA with Bonferroni's comparison test.

Fatty liver disease caused by being overweight with diabetes and a high risk of heart attack, termed non-alcoholic steatohepatitis (NASH), is the most common serious liver disease with no current treatment. There could be several causes of inflammation in NASH, but activation of a protein scaffold within cells termed the inflammasome (NLRP3) has been suggested to play a role. When LPS induce in macrophages can cause inflammasome activation - responsible for the activation of inflammatory responses of the innate immune

system[153]. We asked if the observed improvement diabetes by PCA was due to the suppression of inflammasome activation and associated inflammatory processes. PA may indirectly lead to insulin resistance by causing inflammatory reactions in macrophages[154]. To determine whether PCA can suppress PAMP- triggered inflammasome activation, LPS-primed BMDMs were stimulated with 6.5 μM of Ng for 1 h. The mRNA level of *Nlrp3, Il-1β,* and *Il18* significantly reduced by PCA treatment (Fig. 11A) which showed an upward trend in HFHS diet, suggesting that PCA decreased the priming NLRP3 inflammasome. In addition, PCA supplementation significantly reduced the secretion of IL-1β compared to Ng stimulation (Fig. 11B). Moreover, the expression of NLRP3 and caspase-1 extracted from cells and cleaved caspase-1 in the supernatant were decrease in PCA supplementation (Fig. 11E).

To determine whether PCA is able to suppress DAMP-triggered inflammasome activation, LPS-primed in primary BMDM cells were stimulated with 0.4 mM PA for 12 h. The mRNA levels of NFkB target genes (including *Nlrp3, Il-1β,* and *Il18*) were significant downregulated in the macrophages of PCA treatment (Fig. 11C). Beside that PCA supplementation significantly reduced the secretion of IL-1β compared to PA stimulation (Fig. 11D). The western blot analysis in cells extraction showed that the macrophages of PCA treatment has less p-AMPK, IL-1β (Fig. 12B). Furthermore, the western blot analysis the expression of NLRP3 and caspase-1 extracted from cells and cleaved caspase-1 in the supernatant were decrease in PCA treatment (Fig. 11F).

4. Discussion

Intake overnutrition is common in Western countries can induce NAFLD (Taylor and Francis group, supplementation table 4)- associated with obesity, insulin resistance, and other features of the metabolic syndrome such as hyperlipidemia. NAFLD is a group of common liver diseases that an umbrella term describing two-stage: NAFL (nonalcoholic fatty liver) and NASH (nonalcoholic steatohepatitis), which result in the accumulation of fat and can lead to other health problems. NAFL is characterized by hepatic steatosis and begins when fat accumulates in the liver, but with little or no inflammation or liver damage, which is reversed by reduced caloric intake and exercise. Progressed stage NASH is featured by steatosis with hepatic inflammation and liver injure[155]. Even though NASH is conceivably reversible with diet and exercise[156], NASH often progresses to cirrhosis, liver failure, and hepatocellular carcinoma (HCC)[155]. Apart from increasing caloric intake, the involvement of food ingredients in the Western diet is evaluated. The impact of dietary high fat-high sucrose content and composition are discussed, with a focus on the effect and metabolic pathways of trans-fat and carbohydrate-induced steatosis are presented and the contribution of increased carbohydrates consumption to NAFLD. Thus, we assessed the impact of PCA in improving diet enriched high-fat high sucrose-induced to NAFLD/NASH. This study in HFHS fed C57BL/6 male mice demonstrated that diet interventions, like chronic intake of high fat and high sucrose, constitute powerful for already manifest and severe obesity with increase body weight (Supplementation table 2, Fig. 7B), glucose tolerance, and reduced insulin sensitivity (Fig. 7C-I). Contrast with that, PCA supplementation against HFHS diet-mediated glucose and insulin tolerance (Fig. 7C-I). Consistent with this, PCA treatment changed the effect of the HFHS diet and further reduced hepatic injure which was evidenced by attenuating free cholesterol, inflammation, ER stress, and fibrosis without altering lipogenesis upon the HFHS diet (Fig. 8 and Fig. 9).

Many reports suggested the effects of PCA on systemic glucose homeostasis. PCA (200 mg/kg) supplementation for 8 weeks via oral gavage was maintained glucose homeostasis in high fat-fed mice[157]. After treating 2 mg/ml PCA (dissolved in drinking) for 14 weeks, the glucose homeostasis in high fat high fructose-fed mice was improved (during GTT and ITT)[158]. C57BL/6J mice treated with 10 mg/kg body weight/day for 16 weeks showed lower fasting blood glucose and significantly decreased HOMA-IR in high-fat diet-fed mice[159]. Moneim et al. reported that 40 mg/kg body weight PCA via oral gavage for 6 weeks was demonstrated increased glucose tolerance and decreased hemoglobin A1c (HbA1c) levels in a streptozotocin (STZ)-induced T2DM model. Different doses of PCA (25, 50, and 100 mg/kg body weight) were treated in rats by oral gavage for a period of 45 days that[160] were showed a significantly decreased level of plasma glucose in STZ-induced diabetic rats[161]. Consistent with these results, our result indicated that PCA improved abnormal glucose homeostasis, and HOMA-IR in HFHS diet-fed mice (Fig. 7). Insulin resistance impairs glucose excretion, leading to a compensatory increase in beta cell insulin production and hyperinsulinemia. Insulin resistance metabolic results can lead to hyperglycemia, hypertension, dyslipidemia, visceral obesity, hyperuricemia, elevated inflammatory markers, endothelial dysfunction, and thrombosis-promoting conditions[161].

The PCA has previously reported that it has about 20% higher radical scavenging activity than ascorbic acid, a potent antioxidant used as a positive control[133]. Antioxidants protect damaged tissue and prevent unwanted inflammatory reactions[162]. Consistent with this concept, several studies support that PCA has anti-inflammatory effects in some disease models. Oral administration of PCA (40 mg / kg body weight) for 6 weeks significantly reduced TNFα levels in STZ-induced T2DM rats. In vitro studies as well as animal models showed that PCA reduced *Cox2*, PGE2 and NF-kB protein expression in PA-induced fatty liver models[163]. In our study also showed a decrease in hepatic inflammation/ER stress as ALT levels decreased (Fig. 8 and Fig. 9). This anti-inflammatory effect of PCA was accompanied by an improvement in mild fibrosis, as evidenced by a decrease in collagen

volume fraction (Masson's trichrome staining), quantification of collagen area in the Sirius red staining, and hepatic αSMA levels (Figure 10). The anti-fibrogenic function of PCA was confirmed in LX-2 hepatic stellate cells stimulated with TGF-β1 (Figure 10) and in BMDM cells stimulated with either LPS/Ng or LPS/PA (Figure 11). The mechanism by which the effects of PCA contribute to liver anti-inflammatory and fibrosis remains unclear. Based on the above reports and our results, it is reasonable to assume that the high antioxidant capacity due to the structural location of the hydroxyl groups in PCA protects and prevents liver damage, inflammation, and fibrosis.

Recent evidence reports that PCA prevents obesity by promoting the development of heat in brown adipose tissue[158]. As a result, previous data showed the potential for PCA heat generation by maintaining high temperatures when exposed to the cold. In addition, PCA significantly reduced adipocyte lipid accumulation by activating mitochondria. The mice were given PCA via i.p. injection for 13 weeks in our investigation, although most other studies used oral gavage or drinking water to deliver the drug[157, 158, 160]. We additionally didn't see whether PCA was adequately conveyed to the liver or potentially fat tissue, our review's objective organs. In this manner, it is still difficult to explain whether the wellbeing advancing impacts of PCA were in reality because of PCA or not. Future review is important to get the ideal portion time frame scope of PCA which is all around conveyed to the objective organ.

In summary, this study shows the potentially beneficial effect of PCA on liver damage and distension in HFHS-fed C57BL/6 mice. High susceptibility to insulting and anti-inflammatory / fibrotic highlights of PCA was also confirmed by LX-2 cells and BMDM cells. With remain restrictions, we will investigate the optimal concentration and duration of PCA in the liver and/or adipose tissue; specific mechanisms effect of PCA; and if PCA PCA are physiologically achievable in our future study. However, our study found that PCA was impaired glucose tolerance mediated by the HFHS diet, as evidenced by fasting blood glucose levels and glucose clearance during GTT and ITT. In addition, stresses including HFHS-mediated hepatic stress and ER stress, inflammatory responses, and fibrosis with inflammation of adipose tissue were improvement by PCA treatment. Taken together, our study provides

insight into vegetables and nuts containing PCA which may be a potentially promising nutritional strategy for the prevention of fatty liver and fibrosis-induce by HFHS diet.

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Supplement Table

Table S 1. Dietary composition of low-fat (LF) diet, high-fat (HF) diet

Ingredients	**LF**	**HF**
	g/kg	g/kg
Casein	200	200
L-Cysteine	3	3
Sucrose	0	69
Corn starch	600	0
Maltodextrin 10	50	125
Lard	10	245
Cholesterol	0	2
Soybean oil	39	39
Cellulose	50	50
Mineral mix	35	35
Calcium phosphate	4	4
Vitamin mix	10	10
Choline bitartrate	2	2
Total	1003	784
	kcal (%)	kcal (%)
Carbohydrate	67.8	19.8
Protein	20.9	19.3
Fat	11.3	60.8

The AIN-93G diet was modified

Table S 2. Characteristic of diet intake, metabolic parameters and blood lipid profiles*

Group	LF	HFHS	HFHS+PCA	p-value
Diet intake				
Food intake (g/mouse/day)	5.69±1.19[b]	2.38±0.57[a]	2.26±0.43[a]	*<0.01*
Drink intake (g/mouse/day)		9.13±1.89	8.95±2.70	*n.s.*
Total kcal intake (kcal/mouse/day)	22.08±4.60	19.83±4.17	18.57±3.78	*n.s.*
Phenotypes				
BW (g)	37.75±0.82[a]	42.75±1.01[b]	43.00±1.03[b]	*<0.05*
ΔBW gain (g)	11.00±0.58[a]	15.50±0.89[b]	15.83±1.58[b]	*<0.0001*
Liver (g)	1.22±0.06[a]	1.65±0.11[b]	1.59±0.06[b]	*<0.01*
Liver/BW (%)	100±3.65[a]	119.13±4.63[b]	114.23±2.72[b]	*0.007*
Epididymal fat (g)	1.70±0.08[a]	2.29±0.27[ab]	2.36±0.20[b]	*0.066*
Epididymal fat/BW (%)	100±3.52	117.65±10.24	121.10±9.29	*n.s.*
Blood Chemistry				
Triglyceride (mg/dL)	95.72±5.76[b]	64.89±11.15[a]	63.31±10.43[a]	*<0.05*
Total Cholesterol (mg/dL)	91.28±4.70	130.64±18.28	99.14±12.58	*n.s.*
HDL (mg/dL)	9.00±0.98	11.08±0.50	10.89±1.99	*n.s.*
LDL/VLDL (mg/dL)	0.71±0.23	0.67±0.14	0.64±0.17	*n.s.*

* Values are mean ± S.E.M. All groups n= 6.

Table S 3. Primer sequences for real-time PCR

Gene	Forward	Reverse
mTnfα	GGCTGCCCCGACTACGT	ACTTTCTCCTGGTATGAGATAGCAAAT
mMcp1	AGGTCCCTGTCATGCTTCTG	GCTGCTGGTGATCCTCTTGT
mCox2	AAAGGTTCTTCTACGGAGAGAGTTCA	TGGGCAAAGAATGCAAACATC
mCd11c	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTC
mIL-1β	AAATACCTGTGGCCTTGGGC	CTTGGGATCCACACTCTCCAG
mIL-18	GACAGCCTGTGTTCGAGGAT	TGGATCCATTTCCTCAAAGG
mNlrp3	ATGCTGCTTCGACATCTCCT	AACCAATGCGAGATCCTGAC
mLpl	CATCTCATTCCTGGATTAGCAGAC	CCGATACAACCAGTCTACTACAATG
mSrebp1c	GTGAGCCTGACAAGCAATCA	GGTGCCTACAGAGCAAGAGG
mDgat2	CCGCAAAGGCTTTGTGAAG	GGAATAAGTGGGAACCAGATCA
mAcc	TGGACAGACTGATCGCAGAGAAAG	TGGAGAGCCCCACACACA
mScd1	GGGACAGATATGGTGTGAAACTATG	TTACAGACACTGCCCCTCAAC
mTlr2	CAAACTGGAGACTCTGGAAGC	GCACCTACGAGCAAGATCAACA
mTlr4	AGTGGGTCAAGGAACAGAAGCA	CTTACCAGCTCATTTCTCACC
mTgfβ	TTGCCCTCTACAACCAACACAA	GGCTTGCGACCCACGTAGTA
mCol1a1	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
mCol1a2	GTAACTTCGTGCCTAGCAACA	CCTTTGTCAGAATACTGAGCAGC
mCol3a1	CAGGTCCTAGAGGAAACAGA	TCACCTCCAACTCCAGCAATG
mCol4a1	CTGGCACAAAAGGGACGAG	ACGTGGCCGAGAATTTCACC
mTimp1	GATATGCCCACAAGTCCCAGAACC	GCACACCCCACAGCCAGCACTAT
m36B4	GGATCTGCTGCATCTGCTTG	GGCGACCTGGAAGTCCAACT
mGapdh	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA
mHprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAGC
hIl-6	CTTCTCCACAAGCGCCTTC	CAGGCAACACCAGGAGCA
hαSma	TGGCCGAGATCTCACTGACTA	CTTCTCAAGGGAGGATGAGGA
hTgfβ	CAACAATTCCTGGCGATACC	GAACCCGTTGATGTCCACTT
hCol1a	CCCTGGAAAGAATGGAGATGAT	ACTGAAACCTCTGTGTCCCTTCA
hTipm1	GGGGACACCAGAAGTCAACC	GGGTGTAGACGAACCGGATG
h36B4	GAAGGCTGTGGTGCTGATG	GTGAGGTCCTCCTTGGTGAA
hGapdh	GGCCTCCAAGGAGTAAGACC	AGGGGAGATTCAGTGTGGTG

Table S 4. Characteristic Components of the Western Diet Associated with Obesity-Related NAFLD

Nutrient Overconsumed	Involvement in NAFLD Pathology
Saturated fat	Steatogenic,[a] highly lipotoxic, proinflammatory
Trans-fat	Steatogenic,[b] proinflammatory, fibrogenic(?)
Cholesterol	Pro-lipogenic,[b] proinflammatory, fibrogenicc
Simple sugars	Pro-lipogenic, proinflammatory[b,d]

[a] Particularly when consumed together with simple carbohydrates

[b] Enhanced when consumed in setting of HFD

[c] Mainly induced by cholate

[d] In particular fructose