

INVESTIGATING HYPERLIPIDEMIA-DRIVEN
ORGANELLE STRESS AND NEUROINFLAMMATION
ON THE MOUSE CEREBRAL CORTEX: INSIGHTS
INTO THE INTERVENTION OF PERK PATHWAY

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We certify that we have read this thesis and that in our opinion it is fully adequate, in
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ABSTRACT

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Deficits in the metabolism of lipids called hyperlipidemia have been linked to a higher risk of developing neurodegenerative diseases. Protein Kinase RNA-like Endoplasmic Reticulum Kinase (PERK) signaling is crucial in cellular homeostasis. Abnormalities in the PERK have been associated with neurodegeneration. Mitophagy and the PERK pathway emphasize how cellular stress responses are regulated to preserve cellular homeostasis and mitochondrial quality control. The activity of main mitophagy regulators, such as Parkin and PINK1 (PTEN-induced kinase 1), is regulated by the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2 α) by PERK. If lipid metabolism is at a high level, abnormalities in the mitochondria and endoplasmic stress (ER) emerge. During the ER stress activation, the PERK pathway is induced, and mitophagy is blocked, causing an enhancement in the neuroinflammation. The underlying molecular mechanism by which hyperlipidemia impacts the PERK pathway and mitophagy in the cerebral cortex, as well as the relationship between mitophagy and neuroinflammation, is not fully understood. In this study, Apoe^{-/-} and C57BL/6 mice were given a chow or western diet to stimulate hyperlipidemia. Moreover, western diet-fed Apoe^{-/-} mice were injected with PERK inhibitors, GSK2606414 and Trans-ISRIB, intraperitoneally for six weeks to suppress the PERK

pathway. This study explores the effects of hyperlipidemia on the PERK pathway, inflammatory and mitophagy markers in the cerebral cortex of chow and western diet-fed C57BL/6 and Apoe^{-/-} mice and investigates whether the inhibition of the PERK pathway can change the levels of inflammatory and mitochondrial markers in the cerebral cortex of hyperlipidemic mice subjects. mRNA and protein expression levels of mitophagy and inflammatory markers were assessed using the RT-qPCR and western blot, respectively.

PERK pathway activation under hyperlipidemia conditions was not determined. Nevertheless, significant alterations in mitophagy markers and inflammation were detected in Apoe^{-/-} mice apart from the diet. Furthermore, significant alterations were not seen in the PERK pathway markers; however, mitophagy was stimulated, and some inflammation markers were significantly decreased mildly at the cortical tissue of WD-fed Apoe^{-/-} mice administrated with PERK pathway inhibitors, GSK2606414 and Trans-ISRIB. Besides, no statistically significant changes were observed in the transcript levels of the inflammatory markers. Taken together, hyperlipidemia did not cause the PERK pathway to be activated in the cerebral cortex of mice; nevertheless, it mildly altered inflammation and caused mild effects of the dysregulation of the mitochondria by hyperlipidemia independent from the PERK pathway. Furthermore, although the PERK pathway was not inhibited by the administration of PERK pathway inhibitors, mitophagy was induced, and inflammation was decreased mildly. Targeting the PERK pathway with GSK2606414 and Trans-ISRIB inhibitors from the cerebral cortex would not be a therapeutic approach for neurodegenerative diseases.

Keywords: Hyperlipidemia, ER Stress, PERK Pathway, Mitochondria Stress, Mitophagy, Neuroinflammation, Western Diet, Cerebral Cortex

ÖZ

FARE SEREBRAL KORTEKSİNDE HİPERLİPİDEMİ KAYNAKLI ORGANEL STRESİNİ VE NÖROİNFLAMASYONUNUN İNCELENMESİ: PERK YOLAĞINA MÜDAHALEYE İLİŞKİN ANLAYIŞLAR

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Hiperlipidemi olarak adlandırılan lipid metabolizmasındaki bir problem, nörodejeneratif hastalıkların gelişme riskinin daha yüksek olmasıyla ilişkilendirilmiştir. Protein Kinaz RNA benzeri Endoplazmik Retikulum Kinaz (PERK) sinyali hücrel homeostazda çok önemlidir. PERK'teki anormallikler nörodejenerasyona neden olur. Mitofaji ve PERK yolağı, hücrel homeostazı ve mitokondriyal kalite kontrolünü korumak için hücrel stres yanıtlarının nasıl düzenlendiğini vurgular. Örneğin Parkin ve PINK1 (PTEN kaynaklı kinaz 1) gibi ana mitofaji düzenleyicilerinin aktivitesi, ökaryotik başlatma faktörü 2 alfanın (eIF2a) PERK tarafından fosforilasyonu ile düzenlenir. Lipid metabolizması yüksek düzeyde ise mitokondri ve endoplazmik stres (ER) anormallikleri ortaya çıkar. ER stres durumu sırasında PERK yolağı indüklenir ve mitofaji bloke edilir, bu da nöroinflamasyonun artmasına neden olur. Hiperlipideminin serebral kortekste PERK yolunu ve mitofajiyi etkilemesinin altında yatan moleküler mekanizma ve mitofaji ile nöroinflamasyon arasındaki ilişki henüz tam olarak anlaşılamamıştır. Bu çalışmada Apoe^{-/-} ve C57BL/6 farelerine hiperlipidemi uyarmak için yemek veya batı diyeti verildi. Ayrıca batı diyetiyle beslenen Apoe^{-/-} farelerine, PERK yolağını baskılamak

iin altı hafta boyunca PERK inhibitörleri GSK2606414 ve Trans-ISRIB intraperitoneal olarak enjekte edildi. Bu alıřma, hiperlipideminin PERK yolaęı üzerindeki etkilerini, yiyecek ve batı diyetiyle beslenen C57BL/6 ve Apoe^{-/-} farelerinin serebral korteksindeki inflamatuvar ve mitofaji belirtelerini arařtırıyor ve PERK yolaęı inhibisyonunun PERK yolaęının seviyelerini deęiřtirip deęiřtiremeyeceęini arařtırıyor. PERK yolaęı, hiperlipidemik farelerin serebral korteksindeki inflamatuvar ve mitokondriyal belirteler. Mitofaji ve inflamatuvar belirtelerin mRNA ve protein ekspresyon seviyeleri, sırasıyla RT-qPCR ve western blot kullanılarak deęerlendirildi.

Hiperlipidemi kořulları altında PERK yolaęı aktivasyonu belirlenmemiřtir. Bununla birlikte, Apoe^{-/-} farelerinde diyet dıřında mitofaji belirtelerinde ve inflamasyonda önemli deęiřiklikler tespit edildi. Ayrıca PERK yolaęı belirtelerinde önemli deęiřiklikler görölmedi; ancak mitofaji uyarıldı ve PERK yolaęı inhibitörleri GSK2606414 ve Trans-ISRIB uygulanan batı diyetiyle beslenen Apoe^{-/-} farelerin kortikal dokusunda bazı inflamasyon belirteleri önemli ölçüde hafif bir řekilde azaldı. Ayrıca inflamatuvar belirtelerin transkript düzeylerinde istatistiksel olarak anlamlı bir deęiřiklik gözlenmedi. Sonuç olarak, hiperlipidemi, farelerin serebral korteksinde PERK yolaęının aktive olmasına neden olmadı; yine de inflamasyonu hafife deęiřtirdi ve PERK yolaęından bağımsız olarak hiperlipidemi nedeniyle mitokondride düzensizlięine neden oldu. Ayrıca PERK yolu inhibitörlerinin uygulanmasıyla PERK yolu inhibe edilmese de mitofajiyi indüklendi ve inflamasyon hafif bir řekilde azaldı. PERK yolaęının GSK2606414 ve serebral korteksteiki Trans-ISRIB inhibitörleriyle hedeflenmesi, nörodejeneratif hastalıklar iin terapötik bir yaklaşım olmayacaktır.

Anahtar Kelimeler: Hiperlipidemi, ER Stresi, PERK Yolaęı, Mitokondri Stresi, Mitofaji, Nöroinflamasyon, Batı Diyeti, Serebral Korteks

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LIST OF ABBREVIATIONS

BSA: Bovine Serum Albumin

CHOP: CCAAT-Enhancer Binding Protein (C/EBP) Homologous protein

DAMP: Danger-associated Molecular Pattern

DAPI: Fluoroshield Mounting Medium with 4,6-Diamidino-2-Phenylindole

DNA: Deoxyribonucleic Acid

EDTA: Ethylenediaminetetraacetic

GADD34: Growth Arrest and DNA Damage-inducible Protein

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenaseacid

GSK 2606414: 7-Methyl-5-(1-{[3-(Trifluoromethyl) Phenyl] Acetyl}-2,3- Dihydro-1H-Indol-5-Yl)-7H-Pyrrolo[2,3-D] Pyrimidin-4- Amine

HDL: High-Density Lipoprotein

ISRIB: N, N'-Trans-1,4-Cyclohexanediylbis [2-(4-Chlorophenoxy) Acetamide]

IL-1 β : Interleukin 1 Beta

IL-6: Interleukin 6

IRE1: Inositol-requiring Enzyme Type 1

LDL: Low-Density Lipoprotein

NEUN: Neuronal nuclei

NLRP3: Nucleotide-binding oligomerization domain leucine-rich repeat and pyrin domain-containing protein 3

PAMP: Pathogen-associated Molecular Pattern Molecules

Parkin: RBR E3 Ubiquitin-protein Ligase

PBS: Phosphate-buffered saline

PERK: Protein Kinase RNA-Like ER Kinase

PINK1: PTEN-induced Kinase 1

P-PERK: Phospho-protein kinase RNA-like endoplasmic reticulum kinase

PVDF: Polyvinylidene Fluoride

PIC: Preinitiation Complex

PMSF: Phenyl-Methane Sulfonyl Fluoride

RIPA: Radioimmunoprecipitation assay

RNA: Ribonucleic Acid

RT-qPCR: Reverse Transcriptase Quantitative PCR

TBS: Tris Buffered Saline

TBS-T: Tris Buffered Saline-Tween 20

TLR: Toll-like Receptor

TNF: Tumor Necrosis Factor

TXNIP: Thioredoxin-interacting Protein

T-TBS: Tris-buffered saline-Triton X



CHAPTER 1

INTRODUCTION

1.1. Hyperlipidemia

1.1.1. Lipid Metabolism

Hyperlipidemia is a group of inherited and acquired illnesses characterized by increased amounts of lipids, such as triglycerides and cholesterol [1]. This increase causes an elevation in the levels of lipoproteins and plasma lipids. Low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), very-low-density lipoprotein (VLDL), chylomicrons (CM), and high-density lipoprotein (HDL) are among the lipoproteins seen in plasma [2]. Lipids and proteins are the constituents of the lipoproteins, enabling a structure that eases the combination of lipids and other aqueous body fluids [3]. Besides these, apolipoproteins (Apo), amphiphilic proteins, are categorized as specialized proteins, allowing the connection and transportation between lipids and plasma proteins [4].

Dietary triglycerides are converted into CMs in the small intestine, where the pathway of lipid transportation starts [2]. They pass through the lymphatic system with the help of the HDL, which provides ApoE and Apo C-II. Furthermore, mature VLDL, which delivers lipids from the liver to peripheral tissues, is generated after obtaining ApoE and Apo C-II from the HDL [5]. Afterward, Apo C-II induces lipoprotein lipase (LPL) in peripheral tissues, enabling triglycerides to release free fatty acids [6]. Thus, free fatty acids and monoglycerides are produced when the activated LPL breaks down the VLDL [7]. Furthermore, CM remnants, which are then taken up by the liver, are generated when triglycerides, transported in the chylomicrons, are broken down by LPL in adipose tissue, and free fatty acids come out [8]. At the same time, insulin

accelerates the breakdown of triglycerides. Once adequate triglycerides have been delivered to the tissues, HDL and LDL are reduced and altered into IDL, of which the liver absorbs half, and the rest is converted into LDL [9]. LDL particles have an additional layer of cholesterol enrichment compared to IDL and VLDL particles [2]. Next, phospholipids and cholesterol are carried by the LDL particles from the liver to promote the regeneration of peripheral tissues [10].

High dietary fat consumption, which leads to weight gain and fat accumulation, causes LDL synthesis to occur faster than the removal. Therefore, LDL accumulation and abundance come out in the blood. After that, pancreatic beta-cells fail, and insulin levels decrease in the circulatory system [11]. For this reason, elevated cholesterol levels are seen in the body's circulatory system, and hyperlipidemia occurs. Indeed, LDL abundance causes such diseases as cardiovascular diseases (CVD) [12], obesity [13], and type 2 diabetes [14]. Since the dysregulation of lipid metabolism influences the central nervous system (CNS) and insulin signaling of the CNS is disrupted, brain glucose metabolism declines [15]. Thus, neurodegenerative disorders such as Dementia and Alzheimer's Disease (AD) result from a decline in glucose metabolism and chronic inflammation in the brain [16],[17]. Therefore, it can be inferred from those that lipid metabolism is essential for optimal CNS tissue physiology and cell signaling pathways.

1.1.2. Apoe^{-/-} mice model

A ligand for lipoprotein receptors included in identifying and clearing lipoproteins is called Apolipoprotein E (Apoe) [18]. The most used research model of hyperlipidemia and atherosclerosis is the Apoe^{-/-} mice model, created in 1992 through embryonic stem cells' homologous recombination [19], [20]. Apoe intervenes in the absorption of

chylomicrons and VLDL and the transportation of cholesterol [21]. Following the loss of the Apoe gene, more cholesterol is gathered in VLDL, which is carried in high concentrations and incapable of being broken down by lipoprotein receptors on the cell surface. Therefore, this build-up of cholesterol causes atherosclerosis [22]. A study [23] shows that Apoe^{-/-} animals exhibit deferred lipoprotein clearance, leading to hyperlipidemia and atherosclerotic lesions. Moreover, high levels of neuronal cholesterol and other lipids can cause these molecules to deposit in the brain, where they lead to building neurofibrillary tangles and amyloid plaques, two pathological features of AD [24]. Thus, the lack of the Apoe gene is also associated with AD. On the other hand, instability of cholesterol loading in the macrophages occurs because of the lack of Apoe gene expression. Therefore, the release of cytokines is induced, leading to inflammation and deformation in the extracellular matrix [25]. Consequently, the Apoe^{-/-} mice model gives a chance to investigate specific diseases related to inflammation [26], AD [27], and obstructive pulmonary diseases [28].

Apoe is included in significant processes as a neurotrophic part of the central nervous system (CNS) [29]. In the nervous system, microglial cells and astrocytes produce the Apoe [30]. Apoe expression is enhanced throughout the development process, and an in vitro study shows that lipoproteins, including the Apoe, can stimulate neurotic outgrowth [31]. Esterified cholesterol is transported by the Apoe to the neurons, experiencing reinnervation in the CNS, where it is absorbed by the route linked to the LDL receptor-related protein (LRP) pathway and utilized as a precursor for synthesizing new synaptic terminals [32]. One study displayed an age-dependent decline in synaptophysin-immunoreactive nerve terminals in the Apoe^{-/-} mice [33]. Studies have been conducted in the literature on the relationship between hyperlipidemia and neurodegeneration in Apoe^{-/-} mice [34], [35]. Taken together,

Apoe has an essential function as a neurotrophic role and is included in the regeneration and development of the CNS.

1.1.3. Impacts of High-fat Diet on Hyperlipidemia

Fatty acids serve various functions and have diverse metabolic activities; dietary fat content may influence obesity development by changing deposition rates [36]. Insulin and nutritional intake modulate the synthesis of lipids, and the greater availability of insulin induces the formation of fat and glucose in the liver [37]. TGs and unsaturated fatty acids are generated at higher rates when insulin promotes the transcription of genes associated with lipogenesis [38]. The consumption of fats in excess causes metabolic illnesses such as obesity and obesity-related hyperlipidemia, which may be extremely serious for people [39]. For instance, a possible reason for the generation of fatty liver is that a high-fat diet leads to the formation of excess free fatty acids, which boosts the intake of glucose through the glycolytic pathway and develops insulin resistance in the peripheral cells, leading to hyperinsulinemia [40]. Researchers stimulated hyperinsulinemia at in vitro, and they displayed that the insulin resistance mice showed impairments in neuroinflammation, which was characterized by activated microglia and enhanced pro-inflammatory factors in distinct brain areas, as well as deficiencies in spatial learning and memory [41]. Therefore, there is a possible link between AD and type-2 diabetes.

One study shows that an intensification of neurological impairment, stroke, and neuroinflammation in the brain of a high-fat diet (HFD)-fed cerebral ischemia-reperfusion injury rats were associated with hyperlipidemia caused by HFD [42]. Serum TGs, cholesterol, and LDL increased significantly, while serum HDL decreased noticeably. Furthermore, researchers investigated that Apoe^{-/-} mice have delayed

lipoprotein clearance even on a regular diet, which results in hyperlipidemia and atherosclerotic plaques leading to deterioration in the blood-brain barrier (BBB) [23]. Additionally, it was shown that the Apoe^{-/-} mice model fed with HFD displayed renal and arterial autoimmune damage [43]. Furthermore, cognitive impairments in specific brain regions, including cortical tissues and the hippocampus, result from the Apoe^{-/-} mice fed with HFD [44], [45]. Nevertheless, there is still uncertainty regarding the molecular mechanism underlying the cerebral cortex of Apoe^{-/-} mice fed with HFD.

1.2. Neuroinflammation

Inflammation, which brings on an infection, including chemical and physical stressors, is mediated by the pattern recognition receptors (PRRs) generated on innate immune cells [46]. PRRs identify two essential branches, which are damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). While DAMPs detect endogenous distress molecules caused by damaged cells, PAMPs identify microbes' and pathogens' chemical motifs [47]. PRRs enable innate immune responses by adhering directly to the invasive bacteria and stimulating phagocytosis [48]. Studies have demonstrated that HFD and hyperlipidemia are caused by HFD-induced triggers of inflammation of peripheral tissues, including the liver and skeletal muscle tissue, and lead to CNS inflammation [49], [50].

Neuroinflammation characterizes an intricate response to brain damage associated with the activation of astrocytes and microglia [51]. Upon activation of microglia cells, they undergo morphological changes comprising an enlarged cell body and circular shape, enabling them to move to injury sites and gain phagocytic ability. Hence, microglial receptors such as toll-like receptors 2 and 4 (TLR2 and TLR4) and calcium-sensing receptors (CASR) are released thanks to the activated microglia [52]. Although

astrocytes are not so effective in recognizing pathogens, contrary to the activated microglia, they become active by the polarized microglia. As a result of these, chemokines (CCL5, CCL2) and pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) are secreted, and reactive oxygen species are produced [53]. In this context, toll-like receptors (TLRs) are crucial PRRs that detect DAMPs and PAMPs and release pro-inflammatory cytokines [54]. The TLRs that are mainly implicated in neurodegenerative disorders are TLR2 and TLR4 [55]. When PAMPs or DAMPs are activated, aggregates like alpha-synuclein bind to the TLRs [56].

1.2.1. NLRP3 Inflammasome

NLRP3 inflammasome is a complex that is prevalent in the CNS. Activation of TLRs also stimulates the NLRP3 inflammasome pathway [57]. Proinflammatory cytokines are released when the MyD88-dependent pathway is initiated, which is caused by TLR2 and TLR4 activation. Besides the MyD88-dependent pathway, activation of TLR4 also promotes the IRF3 transcription factor and generates type I interferons (IFNs). NF- κ B and mitogen-activated protein kinase (MAPK) are activated, resulting from the MyD88-dependent pathway [58]. Expression of NF- κ B dependent genes, including IL-1 β , IL-18, caspase-1, and the NLR family pyrin domain containing 3 (NLRP3), that are the essential parts of the inflammasome is stimulated by the NF- κ B in the nucleus [53]. Pro-IL-18, pro-IL-1 β transcription plays a boosting signal for the NLRP3 inflammasome pathway [59].

Once a variety of stimuli involving misfolded protein aggregates is sensed, NLRP3 is activated, which causes pro-caspase-1 to transform into caspase-1 [60]. As a result of the generation of activated caspase-1, cleavage of pro-IL-1 β and pro-IL-18 occurs, and pro-inflammatory cytokine IL-1 β , IL-18 is secreted [61]. The maturation of

Gasdermin D (GSDMD), caused by the activation of NLRP3 inflammasome, goes through the plasma membrane and builds a channel that enables IL-1 β and IL-18 to deliver to the extracellular space [62]. In the end, pyroptosis, inflammatory cell death, which is explained by the GSDMD-driven breakdown of the plasma membrane, also happens by the caspase-1 [58] (Figure 1.1). Throughout the various downstream signaling, inflammatory responses are triggered by the IL-1 β and IL-18 and lead to neuronal turnover [63]. Consequently, NLRP3 inflammasome is a critical factor in the emergence of neuroinflammation.

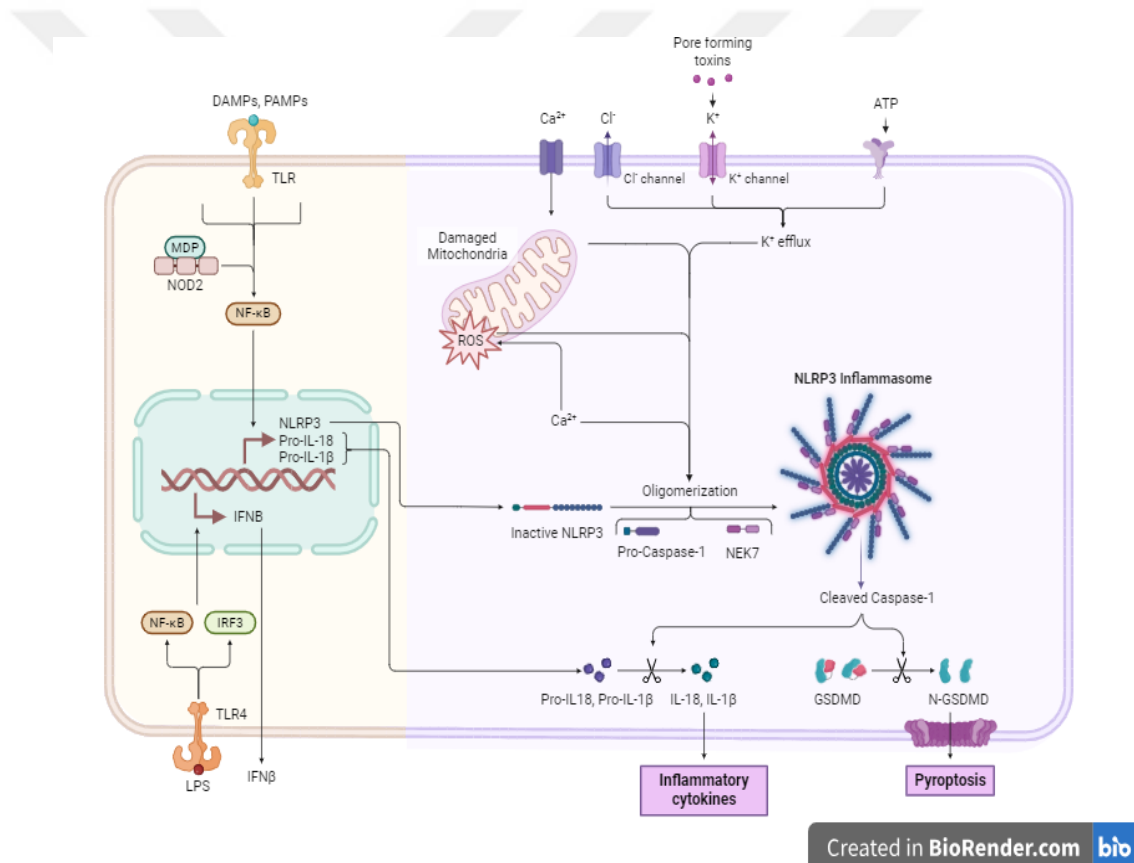


Figure 1.1. Schematic Description of NLRP3 Inflammasome. Adapted from ‘‘NLRP3 Inflammasome Activation’’, by BioRender.com (2019). Retrieved from <https://app.biorender.com/biorender-templates>. The part on the left, painted in yellow, indicates the priming step of the NLRP3 Inflammasome. It is activated by stressors such as an essential PRR, TLRs detect DAMPs and PAMPs, nucleotide-binding

oligomerization domain-containing protein 2 (NOD2), which cause the activation of NF- κ B and NLRP3, pro- IL-1 β , pro-IL18 transcription. Furthermore, TLR4 induces the NF- κ B and IRF3 transcription factors. Production of interferon beta (IFN β) is initiated, leading to MAPK activation. NLRP3 protein is activated by the distinct post-translational modifications. The other part, painted in purple, demonstrates the activation step of the NLRP3 inflammasome, which is stimulated by various factors comprising ion effluxes, mitochondrial dysfunction, and metabolic alterations. Generation of the NLRP3 inflammasome complex leads to pro-caspase-1 to cleaved caspase-1. As a result of this, pro-IL-1 β and pro-IL-18 are cleaved, and IL-1 β and IL-18 are produced. Moreover, cleavage of GSDMD takes place by caspase-1, leading to pyroptosis.

1.3. Organelle Stress

1.3.1.ER Stress and Unfolded Protein Response

The endoplasmic reticulum (ER) is the key contributor to the many structural processes such as protein folding, synthesis, carbohydrate metabolism, and Ca²⁺ homeostasis [64], [65]. Misfolded proteins are released from the folding system, conveyed from ER to the cytoplasm, and broken down in the ubiquitin-proteasome in a cascade of processes known as ER-associated degradation (ERAD) [66]. Nevertheless, some misfolded and unfolded proteins persistently accumulate. Because of such aggregation of misfolded and unfolded protein, severe impairment in the ER function results in ER Stress [67]. As a response to this situation, an adaptive mechanism, which is unfolded protein response (UPR), is activated. It plays an essential role in diminishing the accumulation of unfolded protein by upregulating the molecular chaperone expressions to boost the capacity of folding and enhance protein degradation [68].

Protein kinase RNA-like ER kinase (PERK), inositol-requiring transmembrane kinase/endoribonuclease 1 (IRE1), and activating transcription factor 6 (ATF6) are the three transmembrane proteins of the UPR and act as a stress sensor by identifying misfolded or unfolded proteins [69].

1.3.1.1. PERK Pathway

PERK is an ER transmembrane protein with three domains: transmembrane domain, luminal sensor domain, and cytosolic domain [70]. PERK is attached to ER membranes with the help of the transmembrane domain. A chaperone protein, BiP/Grp78, binds to PERK at the luminal sensor domain. When the ER luminal sensor domain is exposed to ER stress, PERK dissociates from the BiP/Grp78 to build an interaction with the misfolded and unfolded protein; it autophosphorylates and dimerizes at the cytoplasmic domain [71]. As a result, global protein synthesis is halted by phosphorylating the eukaryotic initiation factor 2 alpha (eIF2 α) expression by the phosphorylated PERK [72]. In other words, eIF2B, guanine nucleotide exchange factor, which converts inactive eIF2 α -GDP into active form eIF2-GTP, is hindered by the p-eIF2 α , and translation is blocked by the eIF2 phosphorylation [73]. Although global protein synthesis is prohibited, selective translation is processed [74]. Activating transcription factor 4 (ATF4), mRNA translation is initiated with the help of the p-eIF2 α and regulates the gene expression of GADD34 and ERO-1 α by interacting with C/EBP-homologous protein (CHOP) [75]. Cell apoptosis is the result of the PERK pathway [71].

AD, PD, Huntington's Diseases (HD), and dementia are the most common neurodegenerative diseases associated with the accumulation of misfolded proteins and malfunction of the genes, preserving the protein homeostasis and resulting in

neurodegeneration [69]. Upon the role of neurodegeneration of the PERK pathway, it is possible to develop therapeutic interventions by stabilizing ATF4 activity, inhibiting the PERK pathway [76]. Different inhibitors, such as GSK2606414 and ISRIB, suppress the PERK pathway from distinct locations [77]. On the one hand, GSK2606414, a PERK pathway inhibitor, prohibits the autophosphorylation of PERK, which in turn decreases the eIF2 α phosphorylation and ATF4 [78]. On the other hand, ISRIB (Integrated Stress Response Inhibitor) is an inhibitor downstream of the PERK pathway, increasing the expression of eIF2B, not suppressing the eIF2 α phosphorylation [79]. In the literature [80], it has been shown that suppressing the PERK pathway, which minimizes neurodegeneration, improves cognitive abilities in AD disease models.

1.3.1.2. PERK Pathway and Inflammation

As eIF2 α is phosphorylated by the PERK, it partially activates the NF- κ B pathway and downstream pathway of TXNIP during the ER Stress [81]. NF- κ B, a transcription factor, resides in the cytosol by attaching to its inhibitor, I κ B [82]. Upon eIF2 α phosphorylation, I κ B is translationally inhibited, resulting in dissociation from the NF- κ B. Next, NF- κ B is turned into an active state, and it is transmitted through the nucleus to commence the transcription of pro-inflammatory cytokines (Figure 1.2) [83]. Through the NF- κ B pathway, ER stress boosts the generation of IL-1 β and NLRP3, which in turn engages in the NLRP3 inflammasome [84]. Furthermore, as mentioned previously, the expression of CHOP is increased by the signaling pathway of PERK-eIF2 α -ATF4, and it triggers the TXNIP to transfer from the nucleus to mitochondria, where the mitochondrial ROS (mtROS) production starts [85]. As a result, TXNIP binds to NLRP3 by separating from the thioredoxin (TRX), and it brings about the

NLRP3 inflammasome activation and releases the mature form of IL-1 β [81]. In the literature, it has been shown that HFD can be linked to a rise in the expression level of the TXNIP, which is a protein controlled by glucose metabolism, and local generation of IL-1 β in many tissues [86]. To conclude, PERK is a complex pathway that is responsible for the regulation of cellular processes and the potential treatment of neurodegenerative diseases.

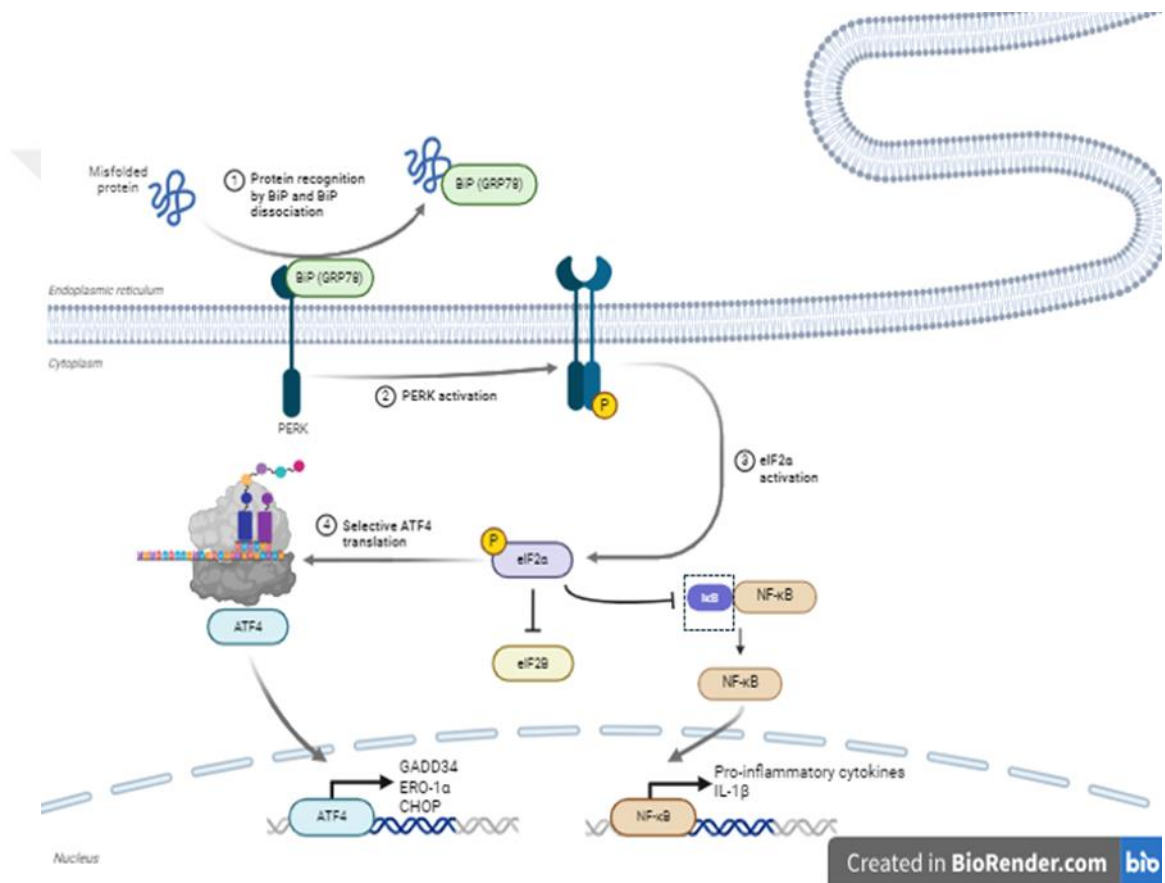


Figure 1.2. Relationship Between PERK Pathway and Inflammation. Adapted from ‘‘Activating the Unfolded Protein Response: PERK-ATF4 Signaling’’, by BioRender.com (2000). Retrieved from <https://app.biorender.com/biorender-templates>. (1) During the ER Stress, BiP/Grp78 detects misfolded protein accumulation. (2) PERK dissociation from the BiP/Grp78 happens and autophosphorylates itself. (3) Phosphorylated PERK leads to activation of p- eIF2 α ,

which halts the global protein translation and inhibits eIF2B translation. (4) On the other hand, it starts the selective ATF4 translation. Further, ATF regulates the gene expression of CHOP, GADD34, and ERO-1 α . Apart from these, I κ B synthesis is suppressed by the translation inhibition caused by p-eIF2 α . NF- κ B is disassociated from the I κ B-NF- κ B complex because of the reduction in the I κ B. Consequently, NF- κ B is activated. Next, it goes through the nucleus for initiating the pro-inflammatory cytokines transcription.

1.3.2. Mitochondria Stress

Mitochondria is an organelle with vital roles in the cell, such as balancing homeostasis and energy production throughout the ATP generation by oxidative phosphorylation, Ca²⁺ imbalance, and creating reactive oxygen species (ROS) [87]. It has a specialized, unique structure consisting of the outer mitochondrial membrane (OMM), mitochondrial matrix, inner membrane space, and inner mitochondrial membrane (IMM) [88]. While DNA replication, transcription, and citric acid cycle occur in the mitochondrial matrix, electrons that are produced due to the citric acid cycle go through the IMM [89].

Mitochondria-associated ER membranes (MAMs) are a place where the ER and mitochondria are interacted with [90]. This communication is crucial for the fate of cells since it provides a multifunctional area for regulatory processes comprising fission, Ca²⁺ homeostasis, and autophagy [91]. The most vital process of MAMs is Ca²⁺ transportation between mitochondria and ER [92]. The connection between voltage-dependent anion-selective channel protein (VDAC1) and IP3 receptor (IP3R) is necessary for pumping out Ca²⁺ into the mitochondrial matrix and the inner membrane space by the ER channels [93]. Hence, Grp75, a protein that connects

VDAC1 and IP3R, is crucial for their interaction [94]. Furthermore, a chaperone, the sigma-1 receptor (Sig-1R), is responsible for stabilizing the structure of IP3R at MAMs [95]. IP3R/Grp75/VDAC coupling enables MAMs structure to preserve stability [96]. PERK and IRE1 are other proteins that reside in abundant amounts at MAMs by keeping the ER-mitochondria interaction stable, which is essential for mitochondrial dysfunction [97], [98]. Protein folding is a crucial process for maintaining the stability of MAMs, which is performed by Calnexin (CNX), another MAM protein [91]. Therefore, ER stress and, thereby, destruction of MAMs are observed when there are abnormalities in these protein levels.

When there is an alteration in the Ca^{2+} levels, apoptosis may occur, and impairment in the mitochondrial division happens [99]. The enhancement of Ca^{2+} influx into the mitochondria from ER, leading to membrane depolarization and worsening the generation of ROS and mitochondrial dysfunction, is observed because of the ER stress condition [100]. As a result of the damage to mitochondria brought by the mtROS worsening, the NLRP3 inflammasome is activated by the IP3R opening that regulates the Ca^{2+} transportation between mitochondria and ER [101]. Overall, it is interpreted that MAMs behave as a mediator between ER stress and inflammation brought on by NLRP3.

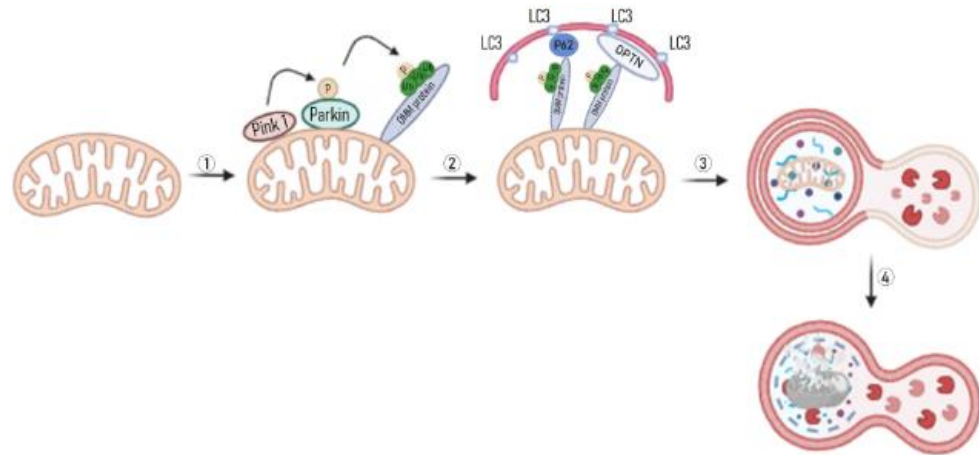
1.3.2.1. Mitophagy

Removing the dysfunctional mitochondria through mitophagy, known as mitochondrial autophagy, is a drastic remedy for misfolded protein aggregation in mitochondria [102]. Under depolarization of mitochondria and mitochondria stress, mitophagy takes place in two distinct pathways, which are the receptor-mediated pathway and ubiquitin-mediated pathway [103]. Along with mitochondrial

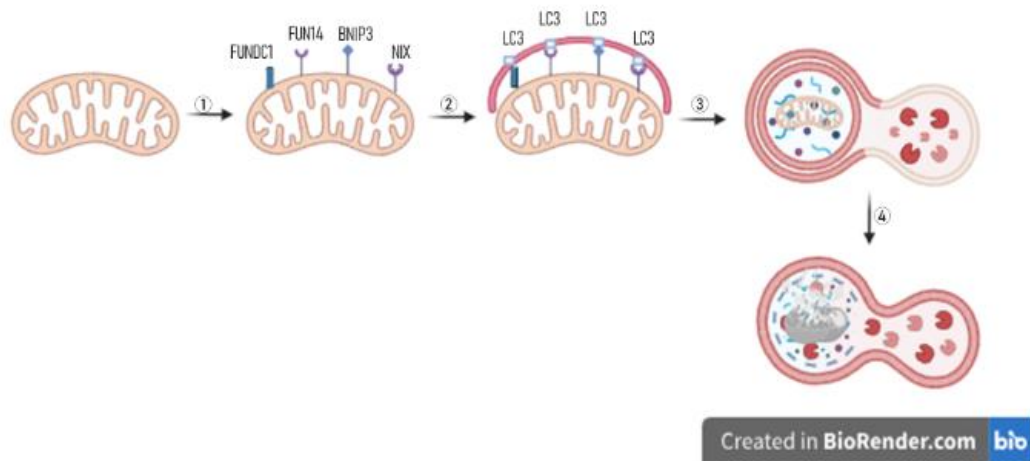
dysregulation, the receptor-mediated pathway is activated in response to cellular differentiation, membrane potential loss, and hypoxia conditions [104]. Various autophagy receptors in the OMM, FKBP8, FUN14, BNIP3/NIX (BCL2 interacting protein 3/(A homolog of the E1B 19K/Bcl-2 binding and pro-apoptotic protein Nip3), BCL2L13, and domain-containing-1 (FUNDC1), turn into the active form [105]. Thereby, phagophore is enlarged autophagosomes surround and engulf the mitochondria owing to the recruitment of LC3 by activation of such receptor proteins, and it is degraded by fusing with the lysosomes (Figure 1.3) [106].

In normal conditions, PTEN-induced kinase 1 (PINK1) is transported from the OMM to the IMM, where it is broken down by Lon Protease 1 (LONP1) [107]. In the Ubiquitin-mediated pathway, also named Pink1/RBR E3 ubiquitin-protein ligase (Parkin) dependent pathway, cleavage of Pink1 at IMM is disrupted and begins to agglomerate in OMM, which results in the recruitment of Pink1 and phosphorylation of Parkin [108]. Further, ubiquitination of mitochondria proteins such as MFN1, MFN2, and VDAC is initiated by the activated Parkin [109]. Next, this ubiquitination enables autophagy receptor proteins, which were optineurin (OPTN), nuclear domain 10 protein 52 (NDP52), and p62, to recruit, and they begin the generation of autophagosomes [110]. Formation of phagophore at the mitochondria is provided when autophagy receptor proteins attract the autophagosome pathways such as PI3K complex, ULK complex, and ATG9A to the impaired mitochondria [107]. The phagophore is enlarged, and mitochondria are surrounded by the autophagosome, then fuse with lysosomes, where it is degraded (Figure 1.3) [111]. In summary, variable pathways, including mitophagy deficiency, calcium imbalance, and mitochondrial damage, disrupt the detoxifying ROS, forming mitochondrial dysfunction.

A



B



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Figure 1.3. Mitophagy Pathways. This figure was created by BioRender. (A) Ubiquitin-mediated Mitophagy Pathway. (1) Ubiquitin-mediated Mitophagy Pathway was activated under oxidative stress and membrane potential loss. Stress promotes PINK1 on the OMM to stabilize, boosting Parkin recruitment. Next, activated parkin phosphorylated poly-Ubiquitin (Ub) chains on OMM proteins. (2) Autophagy receptor proteins, p62 and OPTN, detect and identify phosphorylated poly-Ubiquitin chains. LC3 binds to these receptors on the phagophore. (3) The phagophore first expands

before enclosing itself in an autophagosome. (4) The autophagosome surrounds the mitochondria, which fuses with lysosomes to be broken down there. (B) Receptor-mediated Mitophagy Pathway. (1) During the hypoxia and membrane potential loss, the receptor-mediated pathway was activated. (2) Some mitophagy receptors, FUNDC1, FUN14, BNIP3, and NIX transform into active form at OMM. LC3 binds to these receptors on the phagophore. (3) Phagophore starts to enlarge, then encloses with autophagosome. (4) The autophagosome complex fuses with lysosomes and mitochondria are degraded by lysosomes.

1.3.2.2. PERK Pathway, Mitophagy and Inflammasome

As mentioned in the previous parts, the PERK pathway is one of the essential branches of the UPR. In addition, PERK resides in the MAMs, and it plays a role in the structural stability of mitochondria. However, it is not known that the Pink1/Parkin-dependent mitophagy pathway is influenced significantly by the PERK pathway. Moreover, it was demonstrated that LONP1 is stimulated by ER stress during atherosclerosis, and upregulation is done by activated PERK-eIF2 α signaling, which results in a decline in Parkin, mitophagy, whereas enhancement in the IL-1 β production [112]. One study demonstrates that high levels of generation of mitochondrial ROS occur from ER stress driven by dietary lipids and PERK-eIF2 α -LONP1-activated signaling, leading to inflammasome activation. Therefore, the levels of inflammatory cytokines are enhanced due to the inflammasome activation [113]. Further, to overcome the mitochondrial dysfunction brought about by the translation suppression because of eIF2 α phosphorylation, PERK activation increases the protein expression regulated in mitochondrial proteostasis, such as LONP [114]. Additionally, the PERK pathway and mitophagy are essential to various neurodegenerative diseases such as PD and AD

[115], [116]. In the literature, studies [117], [118], [119] show that p-PERK, p-eIF2 α , LONP1, and activated caspase-3 levels are elevated in subjects with PD. Therefore, it is vital to comprehend the process so as to halt possible deterioration in the PERK-associated mitophagy process, which could cause neurodegeneration, neuroinflammation, and neuronal dysregulation.

1.4. Aim of the Thesis

Hyperlipidemia induced by HFD is the vital factor leading to inflammasome activation, which releases pro-inflammatory cytokines in the peripheral tissues. Further, brain regions can be impacted in a serious manner since astrocytes and microglial cells are activated, and inflammatory cytokines are released. Thus, neurodegenerative diseases come out owing to disrupted neuronal turnover and neuronal dysregulation, which results from neuroinflammation. It was shown that peripheral inflammation had been caused by hyperlipidemia [120], [121]. However, the molecular mechanism underlying the inflammation of the CNS, particularly the cerebral cortex's inflammation caused by hyperlipidemia, is still poorly understood. Furthermore, ER and mitochondria are influenced by the overload of nutrients in the body. Thus, these organelle stresses can lead to abnormalities in neuronal survival. For this reason, neuroinflammation and organelle stress caused by HFD-induced hyperlipidemia were investigated in the cerebral cortex tissue of C57BL/6 and Apoe^{-/-} mice model.

Hyperactivation of the PERK pathway, branches of the UPR ER Stress, suppresses the mitophagy in macrophages during atherosclerosis [113] and in podocytes [122]. Nevertheless, the molecular mechanism underlying the effects of hyperlipidemia on PERK pathway activation, inflammation, and mitophagy relationship in the cerebral

cortex is unknown. Understanding the molecular mechanism at neuroinflammation and mitophagy levels by inhibiting the PERK pathway sheds light on future therapeutic approaches to neurodegenerative diseases. Overall, the main goal of this study is to evaluate whether hyperlipidemia hyperactivated in the PERK pathway and the underlying molecular mechanism by which hyperlipidemia impacts the PERK pathway and mitophagy and the relationship between mitophagy and neuroinflammation in cerebral cortex tissue of C57BL/6 and Apoe^{-/-} mice model. The other main aim is to investigate the impacts of inhibiting the PERK pathway on mitochondrial stress, how mitochondrial stress is related to the neuroinflammation of cerebral cortex tissue, and whether neuronal abnormalities are fixed by inhibiting the PERK pathway.

CHAPTER 2

MATERIAL & METHODS

2.1. Subjects

The male C57BL/6.129P2 (C57BL/6) and ApoEtm1Unc (ApoE^{-/-}) mice were acquired from Jackson Laboratory, Bar Harbor, Maine. They reside in the controlled temperature (20°C) and humidity (50%) at Bilkent University's animal house. Animals were kept in a 12-hour dark/light period. All mice can reach water and diet *ad libitum*. All procedures related to the animal were done according to the Bilkent University Animal Ethics Committee (Ethical number:2020/18). The subject number for the non-injected animals was 7-8 animals, and there were 8 animals for the Trans-ISRIB administrated ApoE^{-/-} animals and 4 animals for the GSK-administrated ApoE^{-/-} animals.

2.2. Animal Experiments

C57BL/6 and ApoE^{-/-} mice were fed a chow diet (CD) (4.5% fat) and a Western diet (WD) (0.21% cholesterol, 21% butterfat) for 16 weeks. The weights of the animals were monitored weekly. After ten weeks of diet, a set of western-diet-fed ApoE^{-/-} mice were intraperitoneally injected with either GSK2606414 (HY-18072, MedChemExpress, NJ, USA) (30 mg/kg/day in 12.5 % Cremophor EL® (238470, Millipore Sigma, MA, USA) -Saline solution) or vehicle (DMSO with 12.5 % Cremophor-EL-Saline solution) for six weeks before the diet period ended to prohibit the PERK pathway. Furthermore, another separate group of western-diet-fed ApoE^{-/-} mice were intraperitoneally injected with either Trans-ISRIB (HY-12495, MedChemExpress, NJ, USA) (1 mg/kg/day in 16 % Cremophor EL-Saline solution) or vehicle (DMSO with 16 % Cremophor EL-Saline solution) for six weeks to suppress

the downstream of PERK pathway. A published paper by Dr. Inci Onat and Dr. Ebru Erbay [113] demonstrated the ideal dose of inhibitors given to animals to protect against atherosclerosis; therefore, the dosages of the inhibitors given to animals were determined by this paper.

After 16 weeks of the diet period ended, the animals were fasted for 16 hours. Anesthesia was performed by the isoflurane. The depth of anesthesia was controlled with the help of the toe pinch response. After ensuring that the reflexes of mice were gone entirely, the abdominal cavity and thoracic were opened, and 1 mL of blood was gathered from the pinnacle of the heart. Blood samples were put into the eppendorf tubes containing Heparin or EDTA solutions. After blood collection, the right atrium of the mice was perforated, and 10 mL of Phosphate-buffered saline (PBS) solution was injected into the pinnacle of the heart to remove the residual blood from the brain and internal organs.

Brains were extracted after the PBS perfusion and put into the ice-cold petri dish cleaned by the RNase-ZAP (R2020, Sigma-Aldrich, MO, USA). Cerebellum and olfactory bulb, amygdala, hippocampus, and hypothalamus of the brain were eliminated, and two hemispheres of the brain were separated by the sterile blade. After that, the right cortex and left cortex tissue were taken out and placed into the 1.5 mL eppendorf tubes one by one to be used in the immunoblot experiments. The right cortex was used for the western blot and RT-qPCR experiments. In addition to that, the last 2 mm of the tail tissue was taken into separate eppendorf tubes to confirm the genotype of the C57BL/6 and *Apoe*^{-/-} mice. All tissues were put into the liquid nitrogen tank. Thereafter, they were stored at -80°C until the experiments started.

2.3. Genotyping Experiments

DNA isolation from tails was done using the HotSHOT method [123]. 2-3 mm tail pieces of the C57BL/6 ve Apoe^{-/-} mice were cut and incubated in 50 µl Alkaline Lysis Buffer (Table 2.1) at 95°C for 2 hours. After 2 hours of incubation, 50 µl Neutralization Buffer (Table 2.2) was added to the samples. Then, the PCR reaction was set up by a tail DNA and a master mix including nuclease-free water (AM9937, Thermo Fisher Scientific, USA), MgCl₂, premix, dNTP (25 mM), Taq Reaction Buffer (LT-02241, Thermo Fisher Scientific, USA), and Taq polymerase enzyme (EP0402, Thermo Fisher Scientific, USA) for both the C57BL/6 and Apoe^{-/-} mice groups. The PCR reaction was set up by preparing a dilution premix for the primer. Premix for the C57BL/6 and Apoe^{-/-} groups were prepared in two separate eppendorf tubes containing common forward primer and reverse primers specific to either C57BL/6 or Apoe^{-/-} mice and nuclease-free water. In two separate eppendorf tubes (for C57BL/6 and Apoe^{-/-}), the premix was prepared by adding 2.5 µl C57BL/6 reverse primer (5'-TGTGACTTGGGAGCTCTGCAGC-3'), 2.5 µl Apoe^{-/-} reverse primer (5'-GCCGCCCCGACTGCATCT-3'), 2.5 µl forward common primer (5'-GCCTAGCCGAGGGAGAGCCG-3') and 95 µl nuclease-free water. Master mixes for C57BL/6 and Apoe^{-/-} mice groups containing 8.05 µl nuclease-free water, 0.45 µl MgCl₂, 1.25 µl premix specific to either C57BL/6 or Apoe^{-/-} mice, 0.1 µl dNTP (25 mM), 1.25 µl Taq Reaction Buffer, 0.4 µl Taq polymerase enzyme was put into the separate eppendorf tubes and tail DNA was added. After that, samples were put into the thermal cycler machine (Techne TC-512 Thermal Cycler, Keison Products, UK). PCR conditions were followed by an initial denaturation step of 3 minutes at 95°C, followed by amplification in 35 cycles of 30 seconds, denaturation at 95°C for 30 seconds, annealing at 60.2°C for 45 seconds, and elongation at 72°C for 10 minutes. 8

μl of PCR sample was mixed with 2 μl 5X DNA Loading Dye (5X DNA Loading Buffer, 5400200-1000). Then, C57BL/6 and Apoe^{-/-} PCR samples were loaded onto a 2% agarose gel with 1 μl DNA ladder (SMO243, GeneRuler 100bp, Thermo Scientific) and run at 100 volts for 40 minutes. Gel imaging was done using the ChemiDoc™ XRS+ imaging system (Biorad, CA, USA).

Table 2.1. Recipe of Alkaline Lysis Buffer

Ingredients	Amounts
0.5 M Disodium EDTA	10 μl
25 mM NaOH	62.5 μl
Double Distilled Water (ddH ₂ O)	Up to 25 mL

Table 2.2. Recipe of Neutralization Buffer

Ingredients	Amounts
1 M Tris-HCL	0.163 g
ddH ₂ O	Up to 25 mL

2.4. Total Protein Isolation from Cerebral Cortex

The weights of cerebral cortex tissues were measured. They were homogenized in 1000 μl PBS. Half of the homogenates were separated for protein isolation, and the other half were allocated for RNA isolation. Following homogenization, cerebral cortex tissues were centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatant was removed, and the pellets were suspended in a solution containing 500 μl radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl (118553, Sigma Aldrich, Germany), 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% (w/v) sodium deoxycholate, 1.0 mM EDTA (6381-92, 6 Scharlau, Spain)] and 5 μl Protein Inhibitor

Cocktail (Sigma, P8340-1ML), 5 μ l Phosphatase Inhibitor Cocktail (Sigma, P0044-1ML), 5 μ l PMSF, 5 μ l sodium orthovanadate (Na_3VO_4). Then, the ultrasonication step was done at 50 amplitudes, 0.5-1 cycle 9 times once a second. Samples were incubated on ice for 30 minutes, tapping the eppendorf tubes every 10 minutes. Next, they were centrifuged at 13000 rpm for 20 minutes at 4°C. The supernatant was taken carefully to another eppendorf tube and aliquoted. Protein samples were stored at -80°C until the experiment started.

2.4.1. Mitochondria Isolation

The weights of cerebral cortex tissues were measured. 800 μ l Homogenization Buffer (HB, 1.0 mM EDTA (6381-92-6, Scharlau, Spain), 250 mM Sucrose, 10 mM Hepes, pH=7.4) was put into the samples. Next, the homogenate was spun at 600 *g* for 5 minutes at 4°C. The 50 μ l of supernatant was allocated for the whole cell lysate and stored at -80°C for conformation experiments. The remaining supernatant was transferred to another eppendorf labeled with mitochondria. It was centrifuged at 7000 *g* for 10 minutes at 4°C. The supernatant was separated for the cytosol and put into -80°C for conformation experiments. Besides, the pellet was suspended with the 500 μ l HB. Following the resuspension of the sample, it was spun at 7000 *g* for 10 minutes at 4°C. The supernatant was discarded, and the pellet was again suspended with 500 μ l HB. The step was repeated. The sample was spun at 7000 *g* for 10 minutes at 4°C, and the supernatant was removed and resuspended with 500 μ l HB. The supernatant was again eliminated, and the pellet was lysed in a solution containing 175 μ l RIPA, 1.75 μ l protease inhibitor cocktail (PIC) (Sigma, P8340-1ML), and 1.75 μ l sodium orthovanadate (Na_3VO_4). Protein concentration was measured and kept at -80°C until the experiment began.

2.5. Bradford Assay

The measurement of protein concentration was applied by the Bradford Assay. The standard control protein was 1 mg/ml Bovine Serum Albumin (A2153, BSA, Sigma-Aldrich, USA), and the blank solution was ddH₂O. In 96 well plates, BSA standards were prepared as in Table 2.3. Next, 4.5 μ l ddH₂O and 0.5 μ l protein samples were added into the one well. Furthermore, protein samples and standards were duplicated. After all protein samples and ddH₂O were added, 250 μ l Bradford reagent (B6916, Sigma-Aldrich, USA) was put into each well, including samples. The plate was shaken and incubated for 10 minutes. Values of absorbances were read at 595 nm with the help of the microplate reader (Biotek Synergy HT Multi-Mode, BioTek® Instruments, Inc., USA). Following the reading of absorbance values, a standard curve was drawn, and protein concentrations of samples were calculated.

Table 2.3. BSA Standard Preparation

BSA (μ l)	0	0.5	1	2	3	4	5
ddH ₂ O (μ l)	5	4.5	4	3	2	1	0

2.6. Western Blot Analysis

2.6.1. Sample Preparation

Protein samples were calculated according to the Bradford Assay and mixed with ddH₂O. A loading buffer was prepared by mixing the 10% dithiothreitol (DTT) and 2X Loading dye [3% Bromophenol blue, 1 M Tris-HCl (pH=6.8), 10% SDS, Glycerol]. After adding the 2X loading dye and DTT mixture to each sample, they were heated to 95°C for 10 minutes and spun down.

2.6.2. Gel Preparation

Two gels with concentrations of 12% resolving (Table 2.4) and 5% stacking (Table 2.5) were prepared. First, 12% resolving gel was poured between the 1.5 mm glass plate and thin glass after ensuring they were squeezed without any leakage. Isopropanol was put on top of the resolving gel to prevent bubble formation. When the resolving gel was polymerized, isopropanol was removed and washed with ddH₂O to discard the residues of isopropanol. 5% stacking gel was poured onto the polymerized resolving gel, and the gel comb was located.

Table 2.4. Two gel recipes for 12% Resolving Gel

Ingredients	Amounts
ddH ₂ O	5.25 mL
1 M Tris-HCl (pH=8.8)	6.56 mL
40% Acrylamide/Bis-acrylamide (Fisher Scientific, BP-1408-1)	5.25 mL
10% SDS	175 µl
10% APS (Ammonium persulfate)	175 µl
N, N, N'N - Tetremethylethane-1,2-diamine (TEMED)	17.5 µl

Table 2.5. Two gel recipes for 5% Stacking Gel

Ingredients	Amounts
ddH ₂ O	5.66 mL
1 M Tris-HCl (pH=6.8)	940 µl
40% Acrylamide/Bis-acrylamide (Fisher Scientific, BP-1408-1)	0.75 µl
10%SDS	75 µl
10% APS (Ammonium persulfate)	75 µl
N, N, N'N - Tetremethylethane-1,2-diamine (TEMED)	7.5 µl

2.6.3. Gel Electrophoresis

Following the polymerization of gels, they were taken out from the gel holder and placed into the electrode assembly (Bio-Rad, CA, USA), which is located in the electrophoresis tank (Mini PROTEAN Tetra cell, Bio-Rad, CA, USA). The tank was filled with 1X Running buffer (Table 2.7), and gel combs were removed carefully without harm to the wells. 18 µl protein samples and 1.5 µl protein ladder (PageRuler Prestained Protein Ladder, Thermo Fisher Scientific, USA, 22619) were loaded into the well. Protein samples were run at 80 V until the proteins passed entirely through the stacking gel at mid-phase. After that, they were run at 100 V until the separation occurred.

Table 2.6. Recipe of 10X Running Buffer

Ingredients	Amounts
100 mM Trizma Base (Sigma-Aldrich, USA)	30 g
Glycine	144 g
10% SDS	100 mL
ddH ₂ O	1 L

Table 2.7. Recipe of 1X Running Buffer

Ingredients	Amounts
The stock of 10X Running Buffer	100 mL
ddH ₂ O	Up to 1 L

2.6.4. Transferring proteins to Polyvinylidene difluoride (PVDF) Membrane

At the end of the running process of the Western Blot procedure, transfer sandwich equipment, which are sponge, gel holder cassettes, and two Whatman gel blotting paper per one gel holder cassette, were wet into the 1X Transfer Buffer (Table 2.9). At the same time, the Polyvinylidene difluoride (PVDF) membranes (88518, Thermo Fisher Scientific, Ireland) were placed into the case containing 100% methanol for 1 minute to be activated. After activating the PVDF membranes, they were soaked into the 1X transfer buffer. When this process was completed, the gel was carefully removed from the gel holders, and the stacking gel was cut off and allocated from the resolving gel. The sandwich system is given in Figure 2.1. The transfer step was initiated at 100 V for 90 minutes at 4°C.

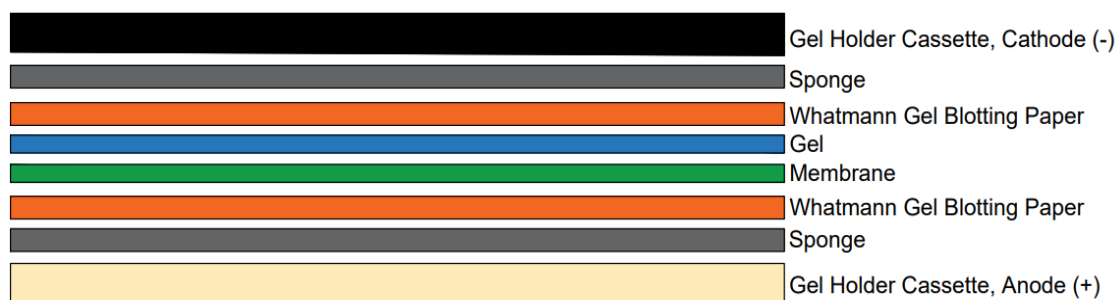


Figure 2.1. The Sandwich System for the Protein Transfer Step. The resolving gel was put into the gel holder cassette on the PVDF membrane. In the following, a Whatman gel blotting paper was placed. It was gently pressed by the rolling brush on the Whatmann gel blotting paper to prevent bubble formation between the gel and the membrane. Next, a foam pad was put in, and the gel holder cassette was closed and placed into the tank (Mini PROTEAN Tetra cell, Bio-Rad, CA, USA).

Table 2.8. Recipe of 10X Transfer Buffer

Ingredients	Amounts
100 mM Trizma Base (Sigma-Aldrich, USA)	30 g
Glycine	144 g
ddH ₂ O	1 L

Table 2.9. Recipe of 1X Transfer Buffer

Ingredients	Amounts
Stock of 10X Transfer Buffer	200 mL
Methanol	400 mL
ddH ₂ O	Up to 2 L

2.6.5. Incubation with Primary and Secondary Antibodies

When the transfer process ended, the membrane was allocated from the gel holder cassette. The membrane was cut and divided into pieces based on the size of the target protein, considering the protein ladders. Blocking solutions were made ready near the end of the transfer step. They were prepared as either 5% bovine serum albumin (BSA) or 5% non-fat dry milk in 1X Tris Buffered Saline – 1% Tween-20 (TBS-T) (Table 2.11). The blots were placed into the box containing the blocking solution and shaken for 1 hour at room temperature (RT). Therewithal, primary antibodies were prepared in 5% BSA or 5% non-fat dry milk solution in TBS-T. Following the end of the blocking step, the membrane was washed in TBS-T 2 times for 5 minutes. Then, it was incubated into the primary antibodies (Table 2.12) at 4°C overnight. After 16 hours, membranes were soaked with TBS-T for 10 minutes three times. Secondary antibody (Table 2.13) incubation was started for 1 hour at RT on a shaker. After 1 hour of secondary antibody incubation, the membranes were soaked with TBS-T thrice for 10 minutes again. In the end, chemiluminescence detection was started.

Table 2.10. Recipe of 10X Tris Buffered Saline (TBS) at pH=7.6

Ingredients	Amounts
100 mM Trizma Base (Sigma-Aldrich)	24.2 g
1500 mM NaCl	80 g
ddH ₂ O	1 L

Table 2.11. Recipe of 1X Tris Buffered Saline-Tween 20 (TBS-T)

Ingredients	Amounts
The stock of 10X TBS Buffer	100 mL

Tween-20 (Bioshop, Canada Inc., Burlington)	1 mL
ddH ₂ O	Up to 1 L

Table 2.12. List of Primary Antibodies

Used Primary Antibody	Company	Catalog Number	Conditions
LONP1	Abcam	103809	1.2000 in 5% BSA in TBS-T
PINK1 (Oligoclonal 17HCLC)	Thermo Fisher Scientific	710993	1.2000 in 5% BSA in TBS-T
Parkin	Abcam	77924	1.2000 in 3% Milk in TBS-T
VDAC	Abcam	15895	1.1000 in 5% BSA in TBS-T
Rho-GDI	Santa Cruz Biotechnology	365190	1.2000 in 5% BSA in TBS-T
NLRP3	Abcam	270449	1.2000 in 5% Milk in TBS-T
NF- κ B	Abcam	16502	1.2000 in 5% Milk in TBS-T
IL-1 β	Abcam	9722	1:2000 in 5% BSA in TBS-T
Caspase-1	Abcam	179515	1:1000 in 5% Milk in TBS-T
Tubulin	Cell Signaling Technologies	2146	1.5000 in 5% Milk in TBS-T

Table 2.13. List of Secondary Antibodies

Used Secondary Antibody	Company	Catalog Number
Anti-rabbit IgG, HRP-Linked Antibody	Cell Signaling Technology, USA	7074
Goat Anti-Mouse IgG HRP-Linked Antibody	Abcam	97023

2.6.6. Imaging

Chemiluminescent detections were performed with the help of the Supersignal West Femto Maximum Sensitivity Substrate (34095, Thermo Fisher Scientific). It was prepared with a 1:1 ratio. Femto reagent was gently placed onto the membrane, and 5 minutes were waited in the dark chamber. Blots were imaged by the ChemiDoc™ XRS+ imaging system (Biorad, CA, USA) using the ImageLab software (Biorad, CA, USA).

2.6.6.1. Image Quantification

After taking the images of the blots, they were quantified using the Image J software (National Institute of Health, MD, USA). First, the rectangular shape was clicked, and all bands were selected with the same rectangular shape. The band intensity was quantified. Then, within-gel normalization and standard normalization were performed according to the band intensity measurements. In the within-gel normalization, the band intensity of the protein of interest was divided by the average band intensity of the protein. Then, the value of the within-gel normalization of the protein of interest was divided by the value of the within-gel normalization of the housekeeping protein. Therefore, standard normalization was calculated.

2.6.7. Statistical Analysis

A two-way analysis (ANOVA) was conducted to ascertain the impact of the dietary factors (Western Diet and Chow Diet) and genotypes (C57BL/6 and Apoe^{-/-}). Moreover, a non-parametric Kruskal-Wallis Test was performed when subject variation did not fit the normal distribution and homogeneity parameters. In addition, the drug administration's statistical significance (Trans-ISRIB and GSK2606414) was

assessed using the student's t-test. Furthermore, a non-parametric Mann-Whitney test was performed where the findings showed that subject variation did not fit the normal distribution and homogeneity characteristics. Statistical analysis was completed by the IBM SPSS Statistics 26 (IBM, SPSS Inc., USA) program. GraphPad Prism 8 (Dotmatics, MA, USA) was used to create the statistical graphs that included the statistical analyses to assess the data's significance.

2.7. RNA Isolation and Quantitative Polymerase Chain Reaction (RT-qPCR)

Experiments

2.7.1. RNA Isolation

Allocated samples for the RNA were centrifuged at 11000 *g* for 10 minutes at 4°C. The supernatant was discarded, and 500 µl TRIzol reagent (19367501, Trizol Reagent, Ambion) was added to the pellet. Samples are waited at room temperature (RT) for 10 minutes. Then, 125 µl chloroform (1.02245.2500, Chloroform, Merck) was put onto them and shaken vigorously 4-5 times. After that, samples were again waited at the RT for 5 minutes to see the separation of phases. They were centrifuged at 15000 rpm for 15 minutes at 4°C. The phase separation was observed as a three-layer, which was the aqueous, interphase, and organic phases. The aqueous phase was taken out carefully and put into the new eppendorf tubes. 250 µl isopropanol was inserted and mixed with the aqueous phase. Then, samples remained in the RT for 10 minutes. They were centrifuged at 15000 rpm for 35 minutes at 4°C. When the 35 minutes ended, samples were put on ice, and the supernatant was removed. The pellet is resuspended in the 500 µl of 70% Ethanol solution and vortexed. They were centrifuged at 13000 rpm for 10 minutes at 4°C. Following the centrifugation, the ethanol solution was carefully removed from the sample, and the pellets remained air-dry for 10 minutes.

Ultimately, 35 μ l nuclease-free water was inserted into the RNA sample. After 1 hour, the RNA samples' concentration was checked using the NanoDropTM 2000 (ND-2000, Thermo Fisher Scientific, IL, USA).

2.7.2. cDNA Synthesis

The complementary DNA (cDNA) was transcribed from the isolated RNA using the iScript cDNA Synthesis Kit (1708891, Biorad, Hercules, CA, USA). To synthesize 500 ng cDNA, the amount of RNA needed to be used in the reaction was calculated using the measured RNA concentrations. Next, samples were diluted with nuclease-free water by calculating the amount of RNA to complete a total of 15 μ l. Afterward, 4 μ l of 5X buffer and 1 μ l Reverse Transcriptase Enzyme were added. 20 μ l reaction was created. Then, samples were spun. Samples were put into the thermal cycler machine (Thermal Cycler C1000, Biorad, CA, USA). Conditions were followed by 5 minutes at 25°C, at 42°C for 30 minutes, 85°C for 5 minutes, and finally at 4°C. When the samples were taken from the machine, they were put at -80°C for use in the experiments.

2.7.3. Quantitative Polymerase Chain Reaction (RT-qPCR) Experiments

Premix for the gene of interest was prepared by adding one eppendorf tube into the 2 μ l reverse primer (Table 2.14), 2 μ l forward primer (Table 2.14), and 36 μ l nuclease-free water. Afterward, the master mix for the gene of interest was prepared by adding a premix, PCR water, and SYBR Green I Master Mix (Roche, Mannheim, Germany, 04887352001). In 96-well plates (Roche, Switzerland, 04707516001), each well received 2 μ l cDNA samples and 18 μ l master mix for the gene of interest. Following plate sealing, the plate was centrifuged for 2 minutes at 1500 rpm. Then, the plate was put into the A Roche 480 Light Cycler (Roche) to perform the RT-qPCR. RT-qPCR

conditions were followed by 15 minutes at 95°C, followed by amplification in 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 10 seconds. It was calculated to express the fold changes as $2^{(-\Delta\Delta Ct)}$. Normalization was done according to the GAPDH and RPL13A transcript levels. First, two replicates of each gene of interest were done, and average Ct values were calculated from two replicates. To obtain the delta Ct value, the average Ct value of GAPDH is subtracted from the average Ct values of a gene of interest. Then, the average Ct value of the Trans-ISRIB control group animals and the GSK2606414 control group animals were calculated and subtracted from the delta Ct value of a gene of interest. As a result of these, $\Delta\Delta Ct$ was obtained, and fold changes were calculated by the $2^{(-\Delta\Delta Ct)}$ method.

Table 2.14. List of Primers used in the RT-qPCR Experiments

Gene	Forward Primer	Reverse Primer
IL-1β	5'AATTGGTCATAGCCCGCA CT 3'	5'AAGCAATGTGCTGGTGCTT C 3'
Caspase-1	5'AGGCATGCCGTGGAGAG AAACAA 3'	5'AGCCCCTGACAGGATGTC TCCA 3'
NLRP3	5'ATTACCCGCCCGAGAAAG G 3'	5'TCGCAGCAAAGATCCACA CAG 3'
GAPDH	5'GTGAAGGTCGGTGTGAAC G 3'	5'GGTCGTTGATGGCAACAA TCTC 3'
RPL13A	5'AGCCTACCAGAAAGTTTG CTTAC 3'	5'GCTTCTTCTTCCGATAGTG CATC 3'

2.7.4. Statistical Analysis

Statistical analysis was completed by the IBM SPSS Statistics 26 (IBM, SPSS Inc., USA) program. The Levene test was used to evaluate if the variance had a homogenous distribution, while the Shapiro-Wilk test was employed to establish whether the normal

distribution had been observed. A two-way analysis of variance (ANOVA) was performed to ascertain the impact of the diet (Chow Diet and Western Diet) and genotype (C57BL/6 and Apoe^{-/-}) factors. Furthermore, the Student's t-test was used to determine the statistical significance of the drug administration. In addition, where the results indicated that subject variation did not fit the parameters of a normal distribution, a non-parametric Mann-Whitney U test was conducted. The statistical analyses to determine the relevance of the data were built into graphics using the GraphPad Prism 8 (Dotmatics, MA, USA) application.



CHAPTER 3

RESULTS

3.1. Genotyping of Animals

PCR experiments were done to confirm the genotype of the mice models used in this study. As expected, it was observed that the C57BL/6 mice were at 155 bp, and the $Apoe^{-/-}$ mice were at 245 bp (Figure 3.1) (Figure 3.2) (Figure 3.3) [124].

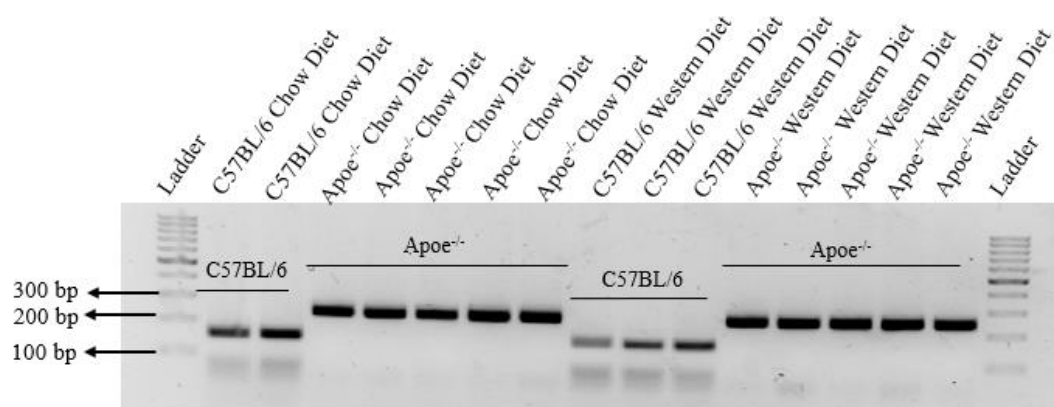


Figure 3.1. Agarose gel imaging of PCR products of C57BL/6 and $Apoe^{-/-}$ mice not injected with either GSK2606414 or Trans-ISRIB. PCR samples of C57BL/6 and $Apoe^{-/-}$ mice were loaded onto a 2% agarose gel, run, and visualized by the ChemiDoc™ XRS+ imaging system. PCR fragments of C57BL/6 mice were depicted at 155 bp, and the $Apoe^{-/-}$ mice were at 245 bp.

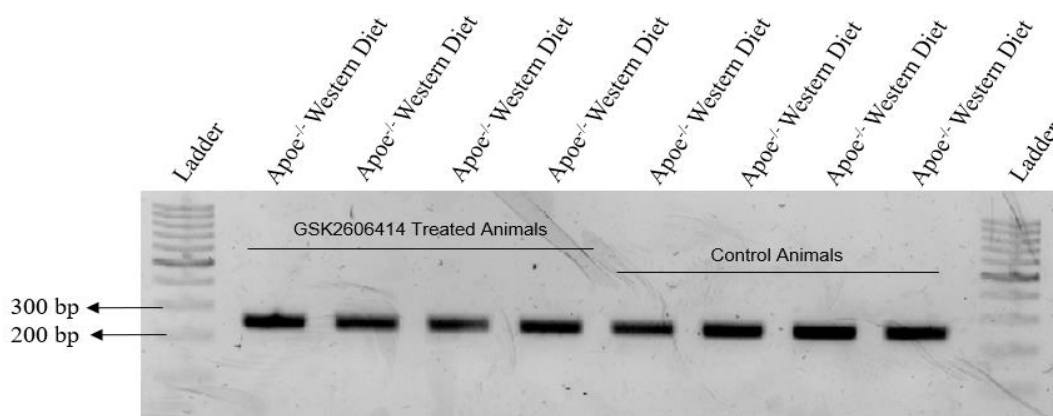
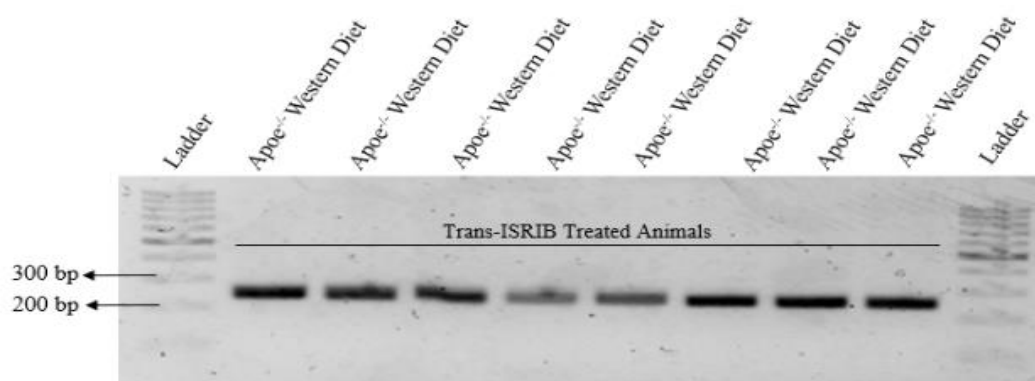


Figure 3.2. Representative agarose gel imaging of PCR products of GSK2606414 administrated $Apoe^{-/-}$ mice and the GSK2606414 control group of $Apoe^{-/-}$ mice. PCR samples of $Apoe^{-/-}$ mice were loaded onto a 2% agarose gel, run, and visualized by the ChemiDoc™ XRS+ imaging system. PCR fragments of the GSK2606414 administrated $Apoe^{-/-}$ mice and the GSK2606414 control group $Apoe^{-/-}$ mice had bands at 245 bp.

A



B

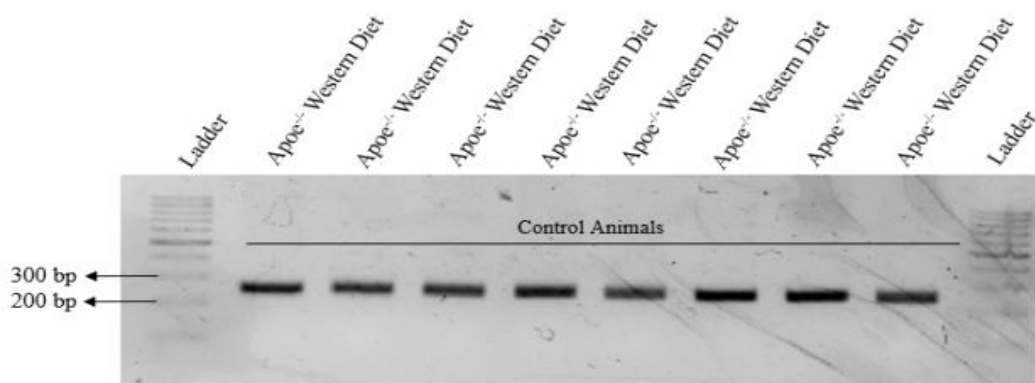


Figure 3.3. Representative agarose gel imaging of PCR products of Trans-ISRIB treated $Apoe^{-/-}$ mice and Trans-ISRIB control group $Apoe^{-/-}$ mice. PCR samples of $Apoe^{-/-}$ mice were loaded onto a 2% agarose gel, run, and visualized by the ChemiDoc™ XRS+ imaging system. (A) PCR fragments of the Trans-ISRIB administrated $Apoe^{-/-}$ mice give bands at 245 bp. (B) PCR fragments of the Trans-ISRIB control group of $Apoe^{-/-}$ mice had bands at 245 bp.

3.2. Body Weights

3.2.1. The Effects of Genotype and High Fat Diet on Cerebral Cortex

3.2.1.1. Experiments Conducted with Total Protein Lysate

While genotype had no significant alteration in body weights of animals used in experiments conducted with total protein lysates ($H(1)=0.954$ $p=0.33$), the body weight of subjects was significantly increased when fed with WD compared to CD ($H(1)=18.531$ $p\leq 0.001$). Furthermore, WD-fed C57BL/6 displayed a significantly higher trend in body weights than CD-fed C57BL/6 mice ($U=0.000$, $p\leq 0.001$). There was an enhancement in the levels of body weights in CD-fed Apoe^{-/-} mice compared to CD-fed C57BL/6 mice ($U=2.500$, $p\leq 0.001$). Moreover, the body weights of WD-fed Apoe^{-/-} mice were significantly higher than those of CD-fed Apoe^{-/-} mice ($U=8.000$, $p=0.021$) (Table 3.1).

Table 3.1. The Last Body Weights of C57BL/6 and Apoe^{-/-} mice were not injected with either GSK2606414 or Trans-ISRIB and Separated for Experiments Conducted with Total Protein Lysate.

#	CD-fed C57BL/6 mice	WD-fed C57BL/6 mice	CD-fed Apoe ^{-/-} mice	WD-fed Apoe ^{-/-} mice
N	8	8	7	7
Mean Body Weights (g)	27.63	36.25	29.63	35.711

3.2.1.2. Experiments Conducted with Mitochondrial Lysate

There were no significant alterations in body weights of mitochondrial lysates ($F(1,30)=0.836$, $p=0.37$) for the genotype group. Nevertheless, the body weight of subjects was significantly enhanced when fed with WD compared to CD ($F(1,30)=70.406$, $p\leq 0.001$). Moreover, WD-fed C57BL/6 indicated a significantly higher trend in body weights than CD-fed C57BL/6 mice ($F(1,30)=43.515$, $p\leq 0.001$). There was an enhancement in the levels of body weights of WD-fed Apoe^{-/-} mice compared to CD-fed Apoe^{-/-} mice ($F(1,30)=28.293$, $p\leq 0.001$) (Table 3.2). The post-hoc analysis revealed significant differences for both the diet and genotype group in the C57BL/6 mice ($p\leq 0.001$) were found. In addition, there were statistically significant differences between the CD-fed C57BL/6 mice and CD-fed Apoe^{-/-} mice ($p\leq 0.001$, and significant differences were detected between the WD-fed C57BL/6 mice and WD-fed Apoe^{-/-} mice ($p\leq 0.001$).

Table 3.2. The Last Body Weights of C57BL/6 and Apoe^{-/-} mice not injected with either GSK2606414 or Trans-ISRIB and Separated for Experiments Conducted with Mitochondrial Lysate.

#	CD-fed C57BL/6 mice	WD-fed C57BL/6 mice	CD-fed Apoe ^{-/-} mice	CD-fed Apoe ^{-/-} mice
N	9	9	7	7
Mean Body Weights (g)	28.33	38.11	30	38.43

3.2.2. The Effects of PERK Pathway Inhibitor on Cerebral Cortex

3.2.2.1. Experiments Conducted with Total Protein Lysate

The body weights of GSK-administrated animals that were separated for experiments done with total protein lysate were significantly lower than those of the GSK vehicle-injected animals ($U=0.000$, $p=0.02$). Besides, the body weights of Trans-ISRIB administrated animals were significantly enhanced compared to the Trans-ISRIB vehicle-injected animals ($t(14)=3.574$, $p\leq 0.01$) (Table 3.3).

Table 3.3. Last Body Weights of Trans-ISRIB and GSK2606414 administrated $Apoe^{-/-}$ mice and the Trans-ISRIB and GSK2606414 control group of $Apoe^{-/-}$ mice that were Separated for Experiments Conducted with Total Protein Lysate.

#	GSK2606414 Vehicle- Administrated $Apoe^{-/-}$ mice	GSK2606414- Administrated $Apoe^{-/-}$ mice	Trans-ISRIB Vehicle- Administrated $Apoe^{-/-}$ mice	Trans-ISRIB- Administrated $Apoe^{-/-}$ mice
N	4	4	8	8
Mean Body Weights (g)	36.5	23.5	30.5	38.13

3.2.2.2. Experiments Conducted with Mitochondrial Lysate

While there was a significant decline in body weights of GSK-injected animals that were taken for experiments used with mitochondrial lysate compared to GSK vehicle-administrated animals ($t(6)=7.406$, $p\leq 0.001$), no significant difference was observed in the body weights between Trans-ISRIB injected and Trans-ISRIB vehicle-injected group ($t(14)=0.524$, $p= 0.61$) (Table 3.4).

Table 3.4. Last Body Weights of Trans-ISRIB and GSK2606414 administrated Apoe^{-/-} mice and the Trans-ISRIB and GSK2606414 control group of Apoe^{-/-} mice that were Separated for Experiments Conducted with Mitochondrial Lysate.

#	GSK2606414 Vehicle- Administrated Apoe ^{-/-} mice	GSK2606414- Administrated Apoe ^{-/-} mice	Trans-ISRIB Vehicle- Administrated Apoe ^{-/-} mice	Trans-ISRIB- Administrated Apoe ^{-/-} mice
N	4	4	8	8
Mean Body Weights (g)	40	22.5	39.5	37.75

3.3. Protein Expression Experiments

3.3.1. The Effects of Genotype and High Fat Diet on Cerebral Cortex

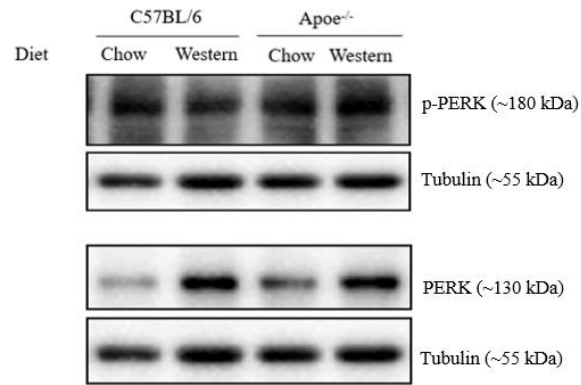
The first aim was to investigate how diet-induced hyperlipidemia in the cerebral cortex of C57BL/6 and Apoe^{-/-} subjects has an influence on the PERK pathway, mitophagy, and inflammation in the condition of both inhibitors that were given and not injected. Hence, the ER stress, mitophagy, and inflammation markers in protein levels were observed.

3.3.1.1. ER Stress Markers

3.3.1.1.1. p-PERK

Two-way ANOVA revealed that diet ($F(1,27)=1.170$, $p=0.68$) and genotype ($F(1,27)=2.207$, $p=0.15$) did not demonstrate any significant impact on p-PERK levels in the cerebral cortex [125]. Moreover, diet had no effects on genotype ($F(1,27)=0.889$, $p=0.35$) (Figure 3.4). Spearman's partial correlation analysis revealed no significant correlation was assessed between body weights and PERK/p-PERK ratio ($r(27)=0.094$, $p=0.63$).

A



B

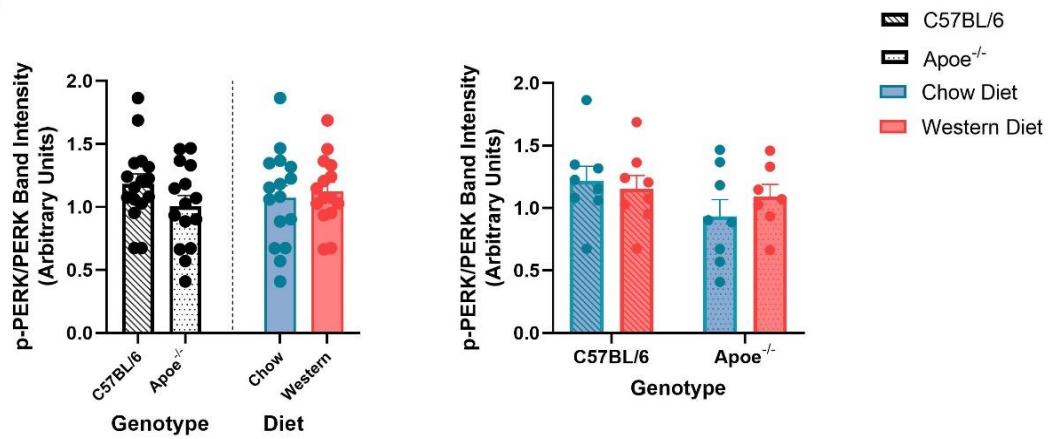
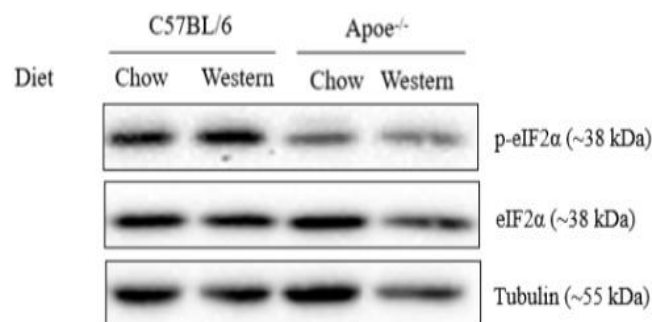


Figure 3.4. p-PERK Band Intensity in Cerebral Cortex. (A) Representative western blot images and (B) Quantification graphs of the immunoblot images. There was no significant effect of genotype and diet on p-PERK levels in the cerebral cortex of mice. (n=16 for C57BL/6 mice, n=15 for Apoe^{-/-} mice) (n=16 for Chow diet, n=15 for Western diet) (n=8 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed Apoe^{-/-} mice and n=7 for WD-fed Apoe^{-/-} mice). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with patterns demonstrated the genotype groups; the blue indicated chow diet-fed animals, and the red indicated the western diet-fed animals.

3.3.1.1.2.p-eIF2 α

Genotype ($F(1,27)=13.75$, $p\leq 0.001$) showed significant alterations in p-eIF2 α levels in cortical tissue. However, there was no significant difference in diet ($F(1,27)=3.644$, $p=0.07$). WD-fed C57BL/6 was also significantly enhanced than CD-fed C57BL/6 mice ($F(1,27)=14.72$, $p\leq 0.001$) [125]. Moreover, a significantly higher trend existed in WD-fed C57BL/6 than in WD-fed Apoe^{-/-} mice ($F(1,27)=24.57$, $p\leq 0.001$). No significant interaction was found between the Apoe^{-/-} mice ($F(1,27)=1.11$, $p=0.30$). Furthermore, the results displayed that the effect of genotype depended on the effect of the diet for p-eIF2 α levels ($F(1,27)=11.71$, $p\leq 0.01$) in the cerebral cortex (Figure 3.5) [125]. In addition to that, post-hoc analysis indicated significant differences between the diet group in the C57BL/6 mice ($p\leq 0.001$); however, there were no significant differences between the diet group in Apoe^{-/-} mice ($p=0.10$). No significant differences between the CD-fed C57BL/6 mice and CD-fed Apoe^{-/-} mice ($p=0.84$), but significant differences were detected between the WD-fed C57BL/6 mice and WD-fed Apoe^{-/-} mice ($p\leq 0.001$) [125]. Spearman's partial correlation analysis was done and no significant correlation was assessed between body weights and eIF2 α /p-eIF2 α ratio $r(27)=0.280$, $p=0.14$).

A



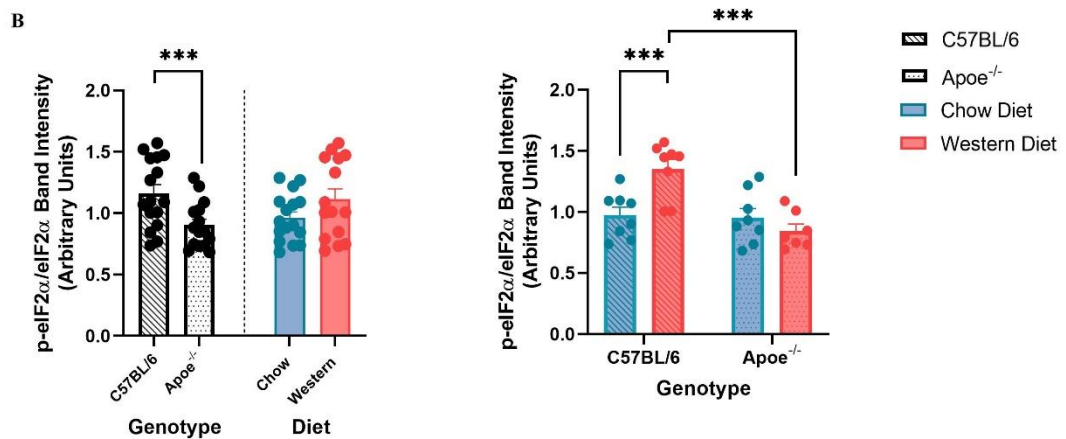


Figure 3.5. p-eIF2 α Band Intensity in Cerebral Cortex. (A) Representative western blot images and (B) Quantification graphs of the immunoblot images. While there was no significant difference between diet groups in p-eIF2 α levels, genotype showed significant alterations in the cortical tissue of mice. Furthermore, WD-fed C57BL/6 displayed a significantly higher trend in p-eIF2 α levels than CD-fed C57BL/6 mice. There was an enhancement in the levels of p-eIF2 α in WD-fed C57BL/6 compared to WD-fed Apoe^{-/-} mice. (n=16 for C57BL/6 mice, n=15 for Apoe^{-/-} mice) (n=16 for Chow diet, n=15 for Western diet) (n=8 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed Apoe^{-/-} mice and n=7 for WD-fed Apoe^{-/-} mice). Each error bar represents mean value + SEM, and the statistical significance was set to $p \leq 0.05$, *** $p \leq 0.001$. Bars with patterns demonstrated the genotype groups; the blue indicated chow diet-fed animals, and the red indicated the western diet-fed animals.

3.3.1.2. Mitophagy Markers

3.3.1.2.1 LONP1

Diet ($H(1)=0.868$ $p=0.35$) did not change the protein levels of LONP1 significantly; however, genotype ($H(1)=5.668$, $p=0.02$) indicated a statistically significant difference

in the protein levels of the LONP1 in the cerebral cortex (Figure 3.6). Furthermore, there was no significant interaction between C57BL/6 mice ($U=26.000$, $p=0.2$) and between $Apoe^{-/-}$ mice ($U=13.000$, $p=0.06$). No significant interaction was found between the CD-fed C57BL/6 mice and CD-fed $Apoe^{-/-}$ mice ($U=34.000$, $p=0.57$) and between the WD-fed C57BL/6 mice and WD-fed $Apoe^{-/-}$ mice ($U=19.000$, $p=0.19$) (Figure 3.6). A partial correlation study was conducted between the animals' recent body weight measurements and LONP1. The assessment was done using Spearman's rank partial correlation. Considering the outcomes, there was no significant correlation found between body weights and LONP1 ($r(30)=-0.122$, $p=0.51$).

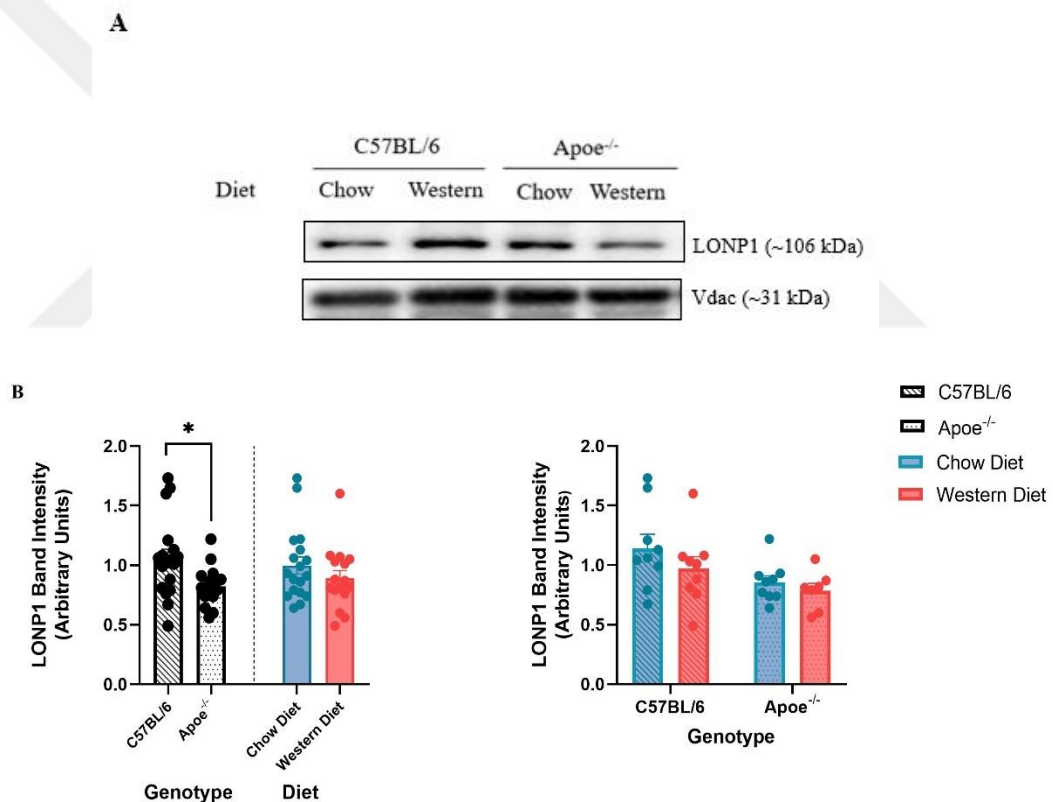


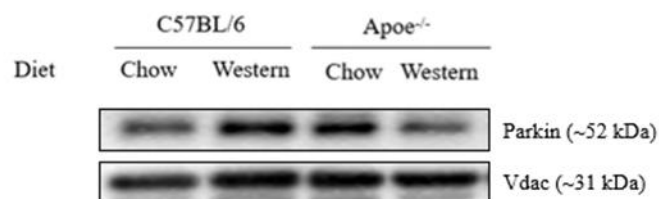
Figure 3.6. LONP1 Band Intensity in Cerebral Cortex. (A) Representative western blot images and (B) Quantification graphs of the immunoblot images. While diet had no significant impact on the LONP1 levels in the cortical tissue of mice, LONP1 levels in the C57BL/6 mice were significantly higher than in the $Apoe^{-/-}$ mice. (n=18 for C57BL/6 mice, n=16 for $Apoe^{-/-}$ mice) (n=18 for Chow diet, n=16 for Western diet)

(n=9 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed Apoe^{-/-} mice and n=7 for WD-fed Apoe^{-/-} mice). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$, * $p \leq 0.05$. Bars with patterns demonstrated the genotype groups; the blue indicated chow diet-fed animals, and the red indicated the western diet-fed animals.

3.3.1.2.2. Parkin

The results showed that the Parkin levels between genotypes significantly altered ($H(1)=4.187$, $p=0.04$). Mitochondrial Parkin protein levels in C57BL/6 mice were significantly higher than in Apoe^{-/-} mice. Furthermore, the Kruskal-Wallis test shows a significant decline in Parkin protein levels in WD compared to CD ($H(1)=4.601$ $p=0.03$) in mitochondrial lysates of cortical tissue. In addition, no significant interaction was detected between C57BL/6 mice ($U=23.000$, $p=0.12$) and between Apoe^{-/-} mice ($U=16.000$ $p=0.10$). There were no significant changes between the CD-fed C57BL/6 mice and CD-fed Apoe^{-/-} mice ($U=23.000$, $p=0.12$) and between the WD-fed C57BL/6 mice and WD-fed Apoe^{-/-} mice ($U=15.000$, $p=0.08$) (Figure 3.7). Spearman's rank correlation was assessed and it demonstrated that there was no significant correlation found between body weights and Parkin ($r(30)=-0.057$ $p=0.76$).

A



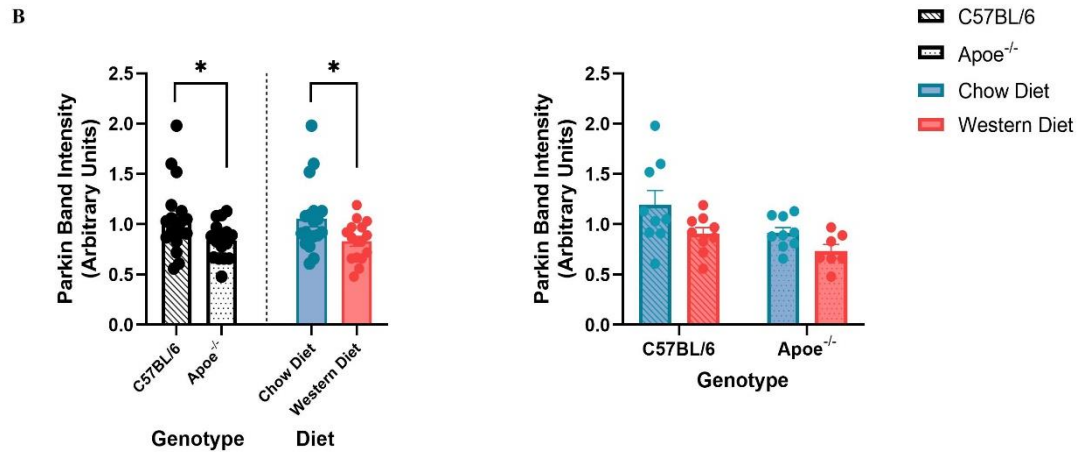
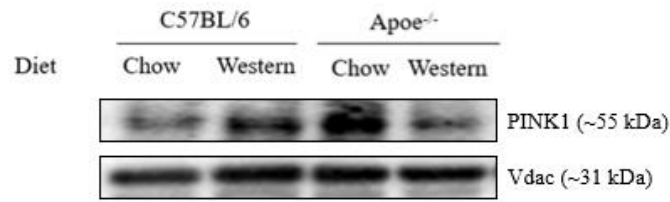


Figure 3.7. Parkin Band Intensity in Cerebral Cortex. Representative western blot images and (B) Quantification graphs of the immunoblot images. Parkin levels between genotypes and diet significantly changed. (n=18 for C57BL/6 mice, n=16 for Apoe^{-/-} mice) (n=18 for Chow diet, n=16 for Western diet) (n=9 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed Apoe^{-/-} mice and n=7 for WD-fed Apoe^{-/-} mice). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$, * $p \leq 0.05$. Bars with patterns demonstrated the genotype groups; the blue color indicated chow diet-fed animals, and the red color indicated the western diet-fed animals.

3.3.1.2.3. PINK1

When comparing the effects of genotype and diet on PINK1 protein levels, diet ($H(1)=0.03$, $p=0.86$) and genotype ($H(1)=0.525$, $p=0.47$) made no significant alterations in mitochondrial PINK1 levels in the cerebral cortex (Figure 3.8). Using Spearman's rank correlation, a partial correlation analysis was conducted between body weights and PINK1 ($r(30)=-0.019$, $p=0.92$).

A



B

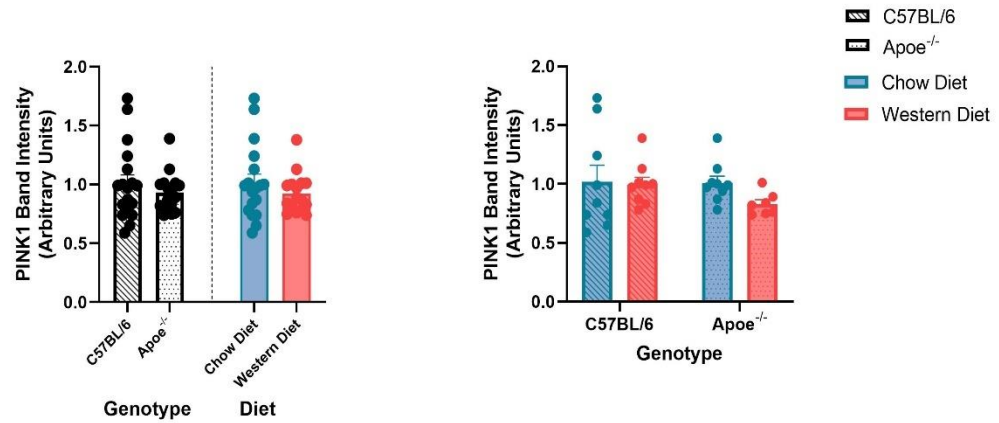


Figure 3.8. PINK1 Band Intensity in Cerebral Cortex. Representative western blot images and (B) Quantification graphs of the immunoblot images. There were no significant effects of diet and genotype in PINK1 levels on cortical tissue. (n=18 for C57BL/6 mice, n=16 for Apoe^{-/-} mice) (n=18 for Chow diet, n=16 for Western diet) (n=9 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed Apoe^{-/-} mice and n=7 for WD-fed Apoe^{-/-} mice). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with patterns demonstrated the genotype groups; the blue indicated chow diet-fed animals, and the red indicated the western diet-fed animals.

3.3.1.3. Inflammatory Markers

The important inflammatory cytokines, NLRP3, NF- κ B, IL-1 β , and Caspase-1 protein levels to assess the effects of diet-induced hyperlipidemia on inflammation in cortical tissue of C57BL/6 mice and Apoe^{-/-} mice.

3.3.1.3.1. NLRP3

A non-parametric test displayed that genotype ($H(1)=0.63$, $p=0.43$) and diet ($H(1)=0.352$, $p=0.55$) did not change significantly in the protein levels of the NLRP3 in the cerebral cortex (Figure 3.9). Furthermore, Spearman's partial rank correlation was assessed and it showed that there was no significant correlation between body weights and NLRP3 ($r(27)=0.052$, $p=0.79$).

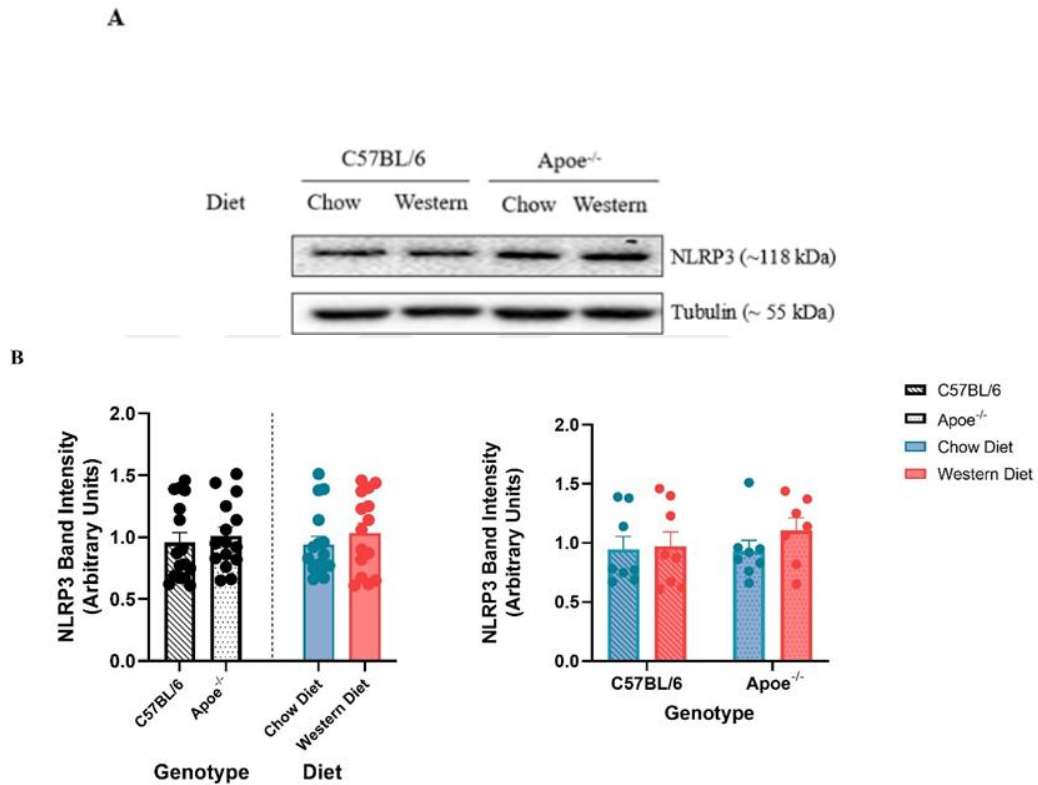


Figure 3.9. NLRP3 Band Intensity in Cerebral Cortex. Representative western blot images and (B) Quantification graphs of the immunoblot images. There were no significant effects of diet and genotype in NLRP3 levels on cortical tissue. (n=16 for C57BL/6 mice, n=15 for Apoe^{-/-} mice) (n=16 for Chow diet, n=15 for Western diet) (n=8 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed Apoe^{-/-} mice and n=7 for WD-fed Apoe^{-/-} mice). Each error bar represents mean value + SEM, statistical significance was set to $p \leq 0.05$. Bars with patterns demonstrated the genotype groups; the blue indicated CD-fed animals, and the red indicated the WD-fed animals.

3.3.1.3.2. NF-κB

A two-way ANOVA was conducted to compare the effect of genotype and diet on protein levels of the NF-κB in cortical tissue. Diet ($F(1,27)=1.093$, $p=0.31$) and genotype ($F(1,27)=1.008$, $p=0.32$) made no significant alterations in NF-κB levels. Also, the effect of genotype did not depend on the effect of the diet for NF-κB levels ($F(1,27)=0.267$, $p=0.61$) in the cerebral cortex (Figure 3.10). Spearman's rank correlation indicated that no significant correlation was detected between body weights and NFκB ($r(27)=0.014$).

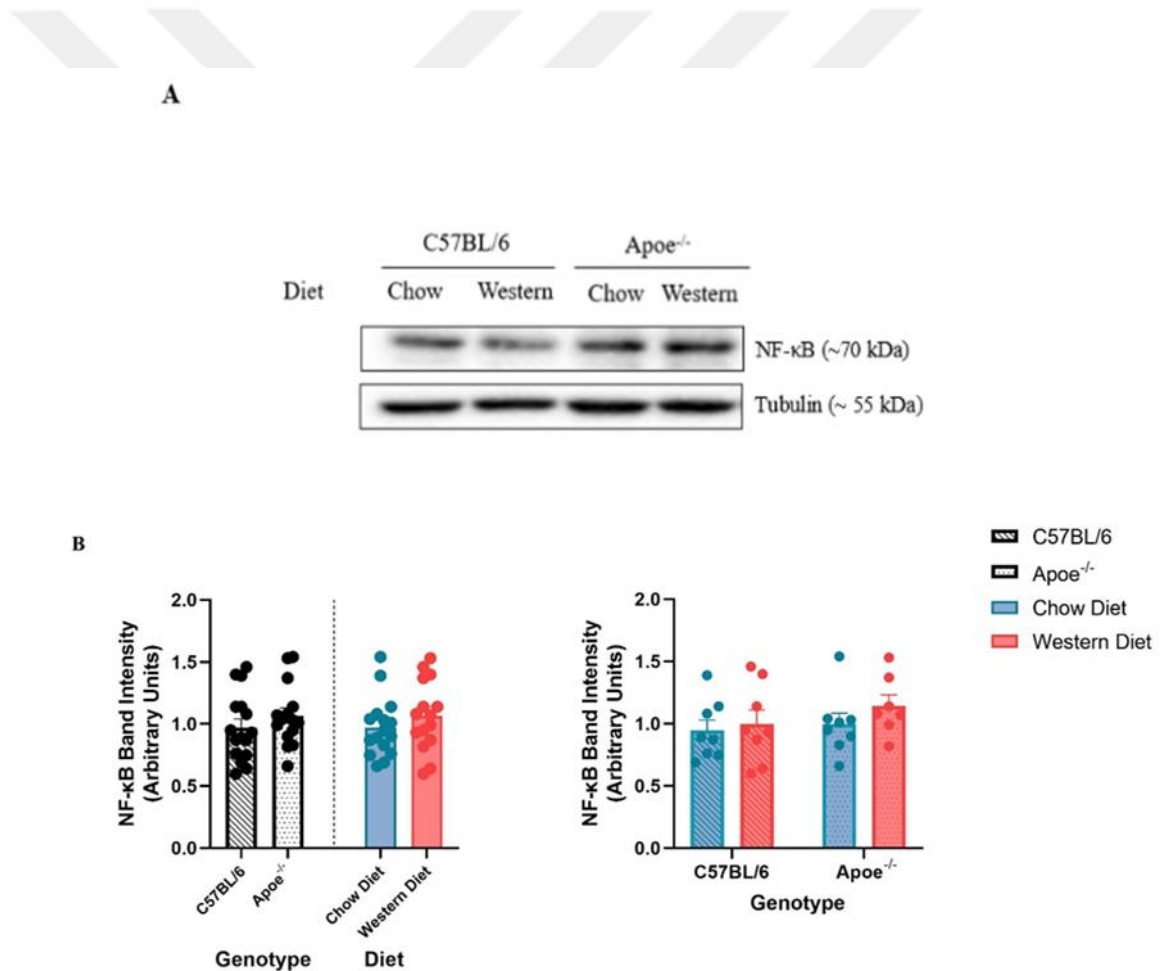


Figure 3.10. NF- κ B Band Intensity in Cerebral Cortex. Representative western blot images and (B) Quantification graphs of the immunoblot images. There were no significant effects of diet and genotype in NF- κ B levels on cortical tissue. (n=16 for C57BL/6 mice, n=15 for Apoe^{-/-} mice) (n=16 for Chow diet, n=15 for Western diet) (n=8 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed Apoe^{-/-} mice and n=7 for WD-fed Apoe^{-/-} mice). Each error bar represents mean value + SEM and statistical significance was set to $p \leq 0.05$. Bars with patterns demonstrated the genotype groups; the blue indicated chow diet-fed animals, and the red indicated the western diet-fed animals.

3.3.1.3.3. Pro-IL-1 β and Mature IL-1 β

There were no significant alterations caused by diet ($F(1,27)=0.057$, $p=0.81$) and genotype ($F(1,27)=2.155$, $p=0.15$) in the protein levels of the pro-IL-1 β in the cerebral cortex. Furthermore, the effect of genotype did not depend on the impact of the diet on pro-IL-1 β levels ($F(1,27)=0.072$, $p=0.79$). A non-parametric test was performed to compare the effect of genotype and diet on protein levels of mature IL-1 β in cortical tissue lysate. The results demonstrated that the mature IL-1 β protein levels in C57BL/6 mice were significantly decreased compared to Apoe^{-/-} mice ($H(1)=3.752$, $p=0.05$). However, no significant difference between the diets ($H(1)=0.039$, $p=0.84$) was found in the mature IL-1 β protein levels in the cerebral cortex. In addition, no significant interaction was obtained between C57BL/6 mice ($U=27.000$, $p=0.6$) and between Apoe^{-/-} mice ($U=23.000$, $p=0.56$). There were no significant changes between the CD-fed C57BL/6 mice and CD-fed Apoe^{-/-} mice ($U=28.000$, $p=0.67$) and between the WD-fed C57BL/6 mice and WD-fed Apoe^{-/-} mice ($U=6.000$, $p=0.02$) (Figure 3.11). Additionally, Spearman's partial correlation revealed that there were no

significant correlations between body weights and inflammatory markers which were pro-IL-1 β ($r(27)=0.237$, $p=0.22$) and Mature IL-1 β ($r(27)=0.013$, $p=0.95$).

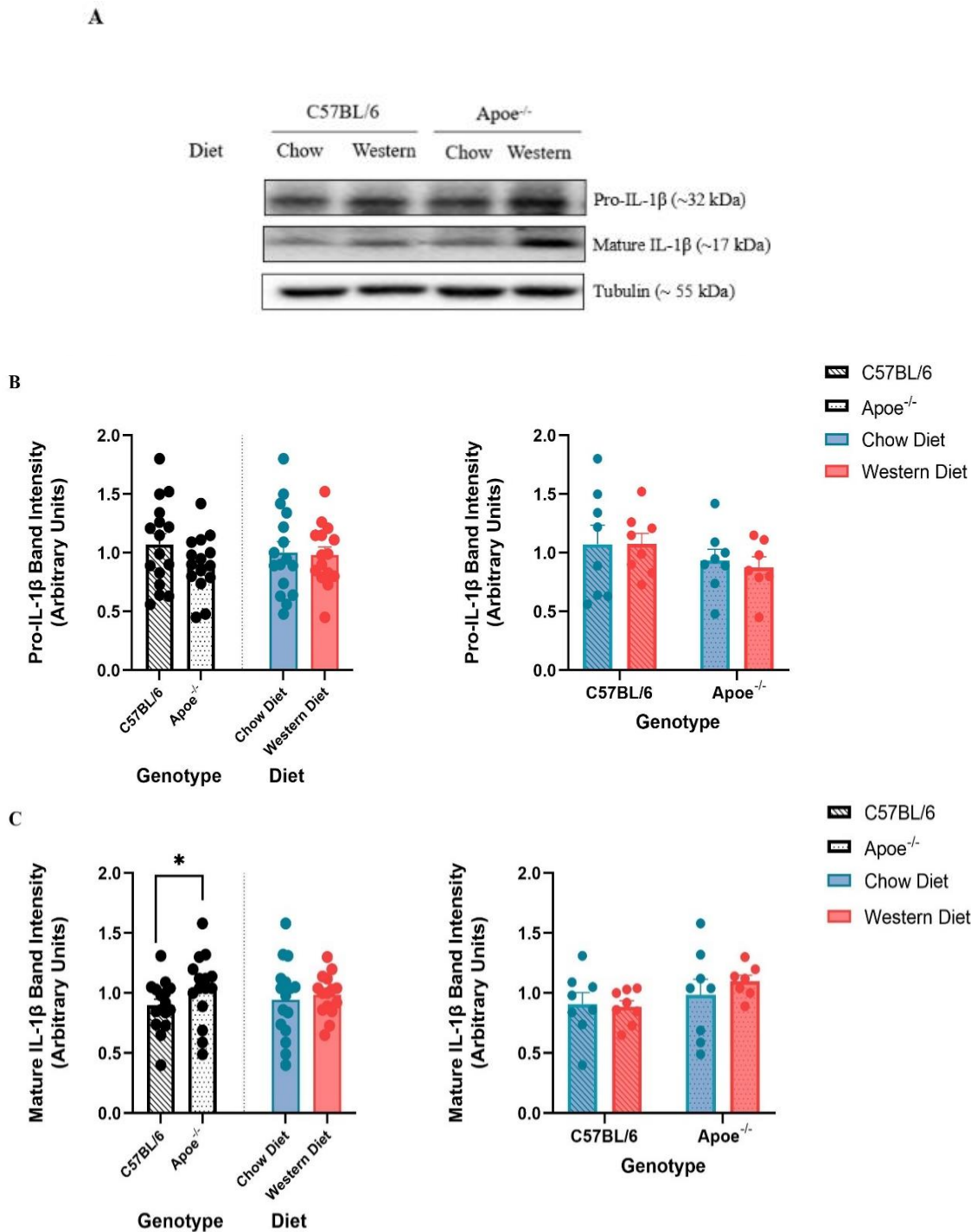


Figure 3.11. Pro-IL-1 β and Mature IL-1 β Band Intensity in Cerebral Cortex.

Representative western blot images (A) and (B), (C) Quantification graphs of the immunoblot images. Pro-IL-1 β protein levels did not significantly alter with diet or

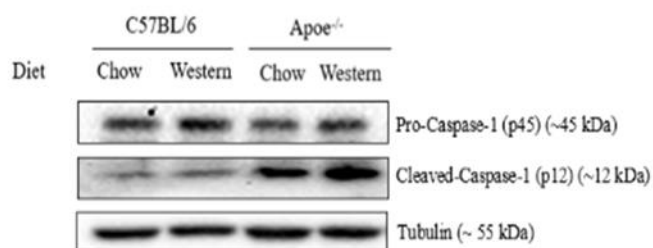
genotype. Mature IL-1 β protein levels showed a significantly higher trend in Apoe^{-/-} mice compared to C57BL/6 mice. (n=16 for C57BL/6 mice, n=15 for Apoe^{-/-} mice) (n=16 for Chow diet, n=15 for Western diet) (n=8 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed Apoe^{-/-} mice and n=7 for WD-fed Apoe^{-/-} mice). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$, * $p \leq 0.05$. Bars with patterns demonstrated the genotype groups; the blue indicated chow diet-fed animals, and the red indicated the western diet-fed animals.

3.3.1.3.4. Pro-Caspase-1 and Cleaved Caspase-1

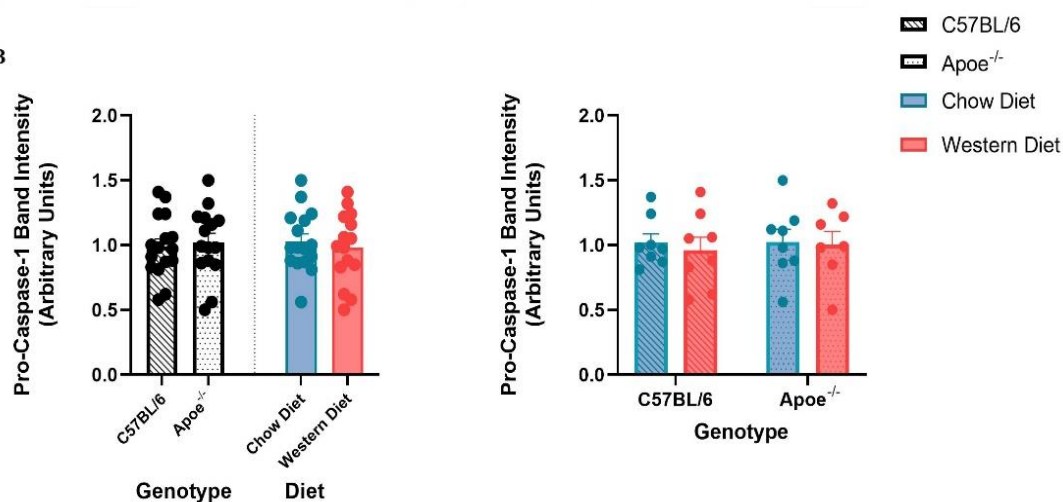
As a result of the two-way ANOVA test, there were no significant alterations on both genotype ($F(1,27)=0.79$, $p=0.78$) and diet ($F(1,27)=0.213$, $p=0.65$) on protein levels of the pro-caspase-1. No interaction was determined between the diet and genotypes for pro-caspase-1 levels ($F(1,27)=0.045$, $p=0.83$) in the cerebral cortex. A two-way ANOVA was conducted to observe the effects of genotype and diet on protein levels of cleaved caspase-1. There was a significant decline in the cleaved caspase-1 levels in the C57BL/6 mice compared to Apoe^{-/-} mice ($F(1,27)=5.473$, $p=0.03$). On the other hand, there was no significant difference between the diets ($F(1,27)=0.143$, $p=0.71$). The genotype did not depend on the diet ($F(1,27)=0.068$, $p=0.8$) (Figure 3.12). Furthermore, the post-hoc analysis indicated no significant differences between the CD-fed C57BL/6 mice and CD-fed Apoe^{-/-} mice ($p=0.07$), and there were no significant differences between the WD-fed C57BL/6 mice and WD-fed Apoe^{-/-} mice ($p=0.16$). Moreover, no significant distinction between diet groups in C57BL/6 mice ($p=0.93$) and between diet groups in Apoe^{-/-} mice ($p=0.66$). In addition, Spearman's partial correlation demonstrated that there were no significant correlations between

body weights and inflammatory markers which were pro-caspase-1 ($r(27)=0.101$, $p=0.60$), cleaved caspase-1 ($r(27)=0.346$, $p=0.07$).

A



B



C

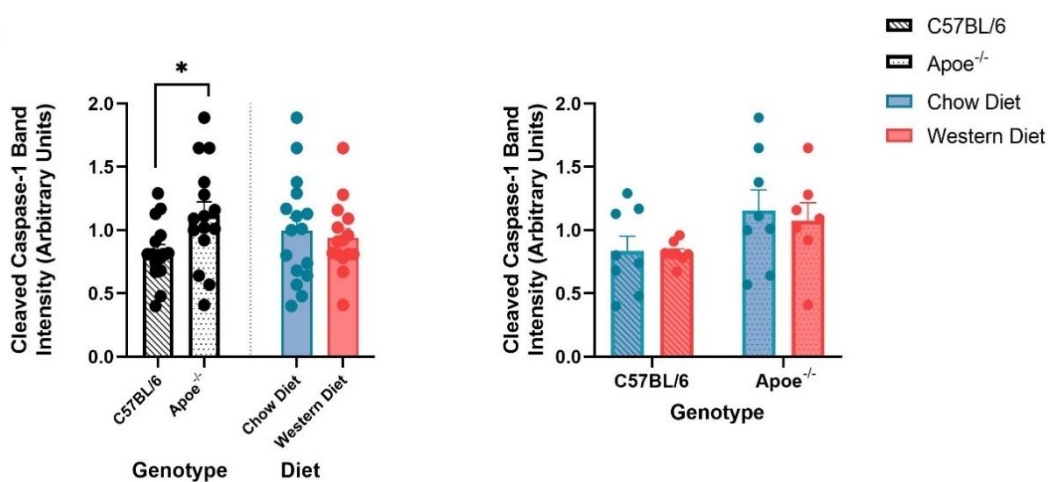


Figure 3.12. Pro-Caspase-1 and Cleaved Caspase-1 Band Intensity in Cerebral Cortex. Representative western blot images (A) and (B), (C) Quantification graphs of the immunoblot images. Pro-Caspase-1 protein levels did not significantly alter with diet or genotype. Cleaved Caspase-1 protein levels showed a significantly higher trend in Apoe^{-/-} mice compared to C57BL/6 mice. (n=16 for C57BL/6 mice, n=15 for Apoe^{-/-} mice) (n=16 for Chow diet, n=15 for Western diet) (n=8 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed Apoe^{-/-} mice and n=7 for WD-fed Apoe^{-/-} mice). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$, * $p \leq 0.05$. Bars with patterns demonstrated the genotype groups; the blue indicated chow diet-fed animals, and the red indicated the western diet-fed animals.

3.3.2. The Effects of PERK Pathway Inhibition on Cerebral Cortex

The second aim was to suppress PERK signaling, which may reduce inflammation brought on by hyperlipidemia and affect mitophagy in the cerebral cortex of WD-fed Apoe^{-/-} mice. Thereby, the ER stress markers, mitophagy markers, and inflammation markers in protein levels were observed.

3.3.2.1.ER Stress Markers

3.3.2.1.1.p-PERK

Results showed that GSK2606414 ($U=8.000$, $p=0.56$) and Trans-ISRIB ($U=8.000$, $p=0.67$) administration had no significant alterations (Figure 3.13) [125]. A partial correlation analysis was done between PERK/p-PERK ratio and the last body weights of GSK-injected and GSK-vehicle-injected animals using Spearman's rank correlation. According to the analysis, there was no substantial correlation between body weights and PERK/p-PERK ratio ($r(5)=-0.569$, $p=0.18$) for GSK2606414.

Moreover, no significant correlation was assessed between body weights and PERK/p-PERK ratio for Trans-ISRIB ($r(13)=-0.084$, $p=0.77$).

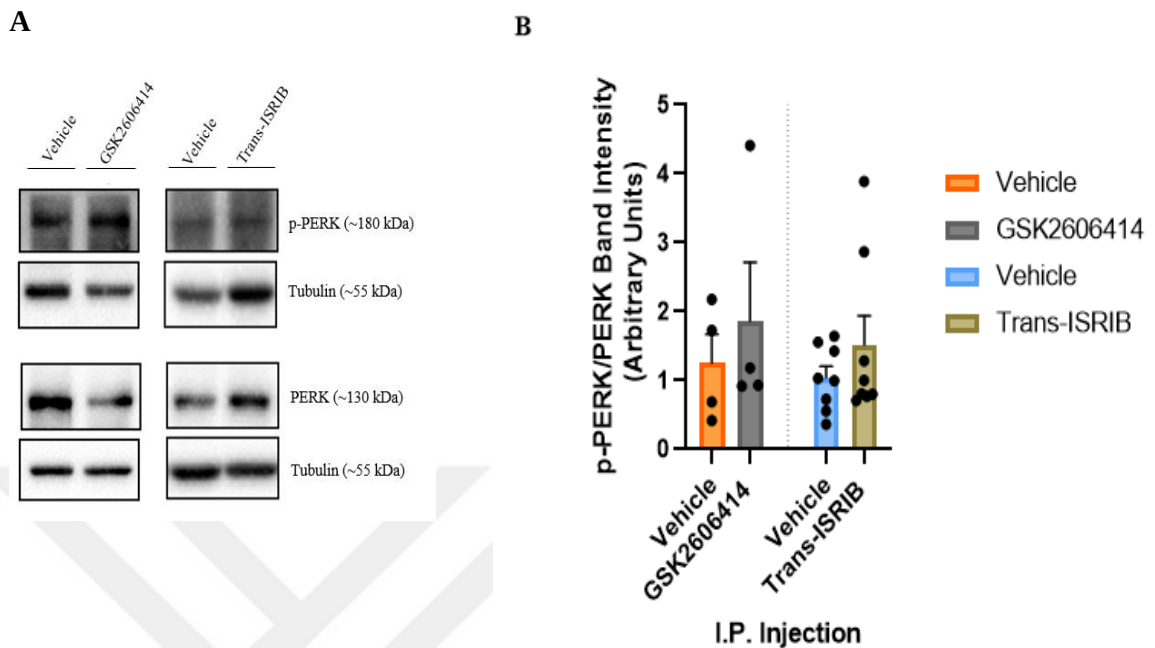


Figure 3.13. Effects of GSK2606414 and Trans-ISRIB administration on p-PERK protein levels in the cerebral cortex. Representative western blot images (A) and (B) Quantification graphs of the immunoblot images. GSK2606414 and Trans-ISRIB administration had no significant changes in p-PERK levels in the cortical tissue of WD-fed $Apoe^{-/-}$ mice. ($n=4$ for GSK2606414 vehicle and GSK2606414 administrated group) ($n=8$ for Trans-ISRIB vehicle and Trans-ISRIB administrated group). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with orange indicated the GSK2606414 vehicle group injected; the gray demonstrated the GSK2606414 administrated group; the blue indicated the Trans-ISRIB vehicle group; the green indicated the Trans-ISRIB administrated group.

3.3.2.1.2.p-eIF2 α

The student's t-test revealed that there were no significant changes in p-eIF2 α levels in cerebral cortex tissue of $Apoe^{-/-}$ subjects administrated GSK2606414 ($t(6)=-0.789$,

$p=0.46$); Trans-ISRIB ($t(14)=0.773$, $p=0.45$) also and no influence on p-eIF2 α levels in cortical tissue of Apoe^{-/-} mice (Figure 3.14) [125]. Spearman's partial correlation showed that there was no significant correlation between body weights and eIF2 α /p-eIF2 α ratio ($r(5)=0.054$, $p=0.91$) for GSK2606414. Moreover, no significant correlation was detected between body weights and eIF2 α /p-eIF2 α ratio for Trans-ISRIB ($r(13)=0.172$, $p=0.54$).

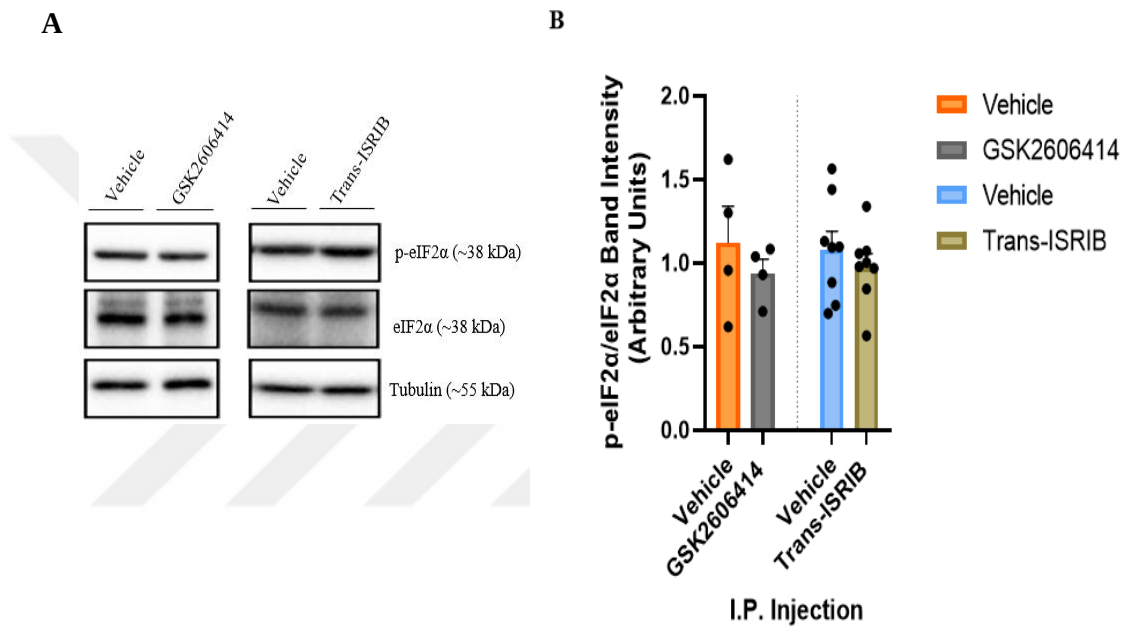


Figure 3.14. Effects of GSK2606414 and Trans-ISRIB administration on p-eIF2 α protein levels in the cerebral cortex. Representative western blot images (A) and (B) Quantification graphs of the immunoblot images. GSK2606414 and Trans-ISRIB administration had no significant changes in the levels of p-eIF2 α in cortical tissue of WD-fed Apoe^{-/-} mice. (n=4 for GSK2606414 vehicle and GSK2606414 administrated group) (n=8 for Trans-ISRIB vehicle and Trans-ISRIB administrated group). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with orange indicated the GSK2606414 vehicle group injected; the gray demonstrated the GSK2606414 administrated group; the blue indicated the Trans-ISRIB vehicle group; the green indicated the Trans-ISRIB administrated group.

3.3.2.2. Mitophagy Markers

3.3.2.2.1. LONP1

No significant changes in LONP1 protein levels were observed when GSK2606414 administration ($t(6)=0.38$, $p=0.72$) happens (Figure 3.15). In addition to that, there was no change in the mitochondrial LONP1 levels caused by Trans-ISRIB administration ($t(14)=-0.58$, $p=0.57$) (Figure 3.15). Moreover, Pearson's partial correlation was done and it showed that no significant correlation was obtained between body weights and LONP1 for GSK2606414 ($r(5)=-0.248$, $p=0.59$) and Trans-ISRIB ($r(13)=0.174$, $p=0.53$).

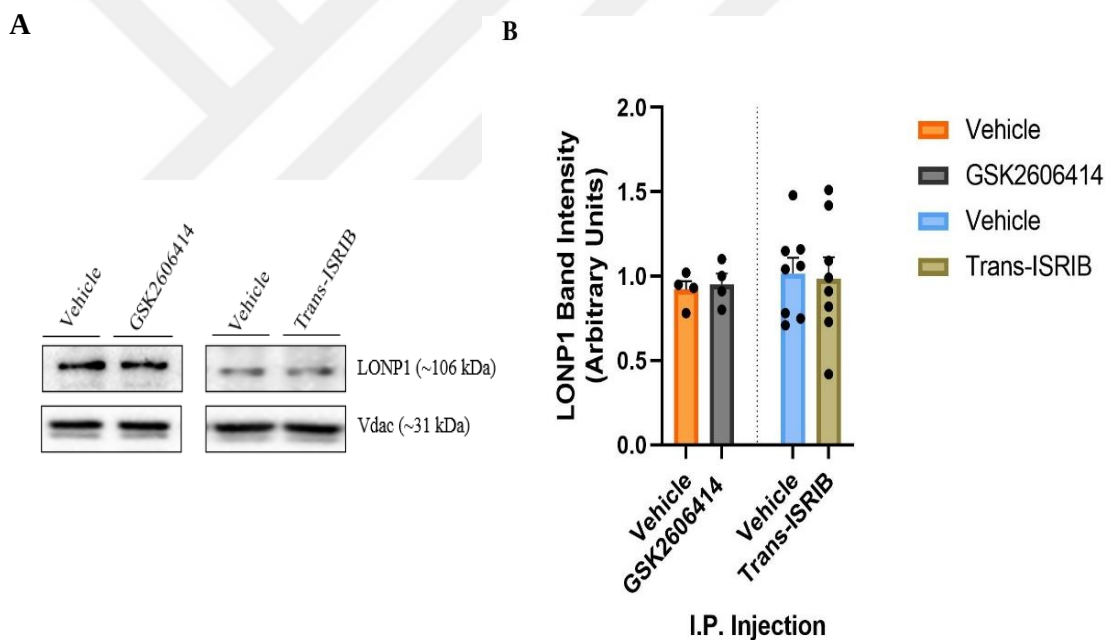


Figure 3.15. Effects of GSK2606414 and Trans-ISRIB administration on LONP1 protein levels in the cerebral cortex. Representative western blot images (A) and (B) Quantification graphs of the immunoblot images. The protein levels of LONP1 didn't display any significant alterations in the cortical tissue of WD-fed *Apoe*^{-/-} mice administrated with GSK2606414 and Trans-ISRIB. (n=4 for GSK2606414 vehicle and GSK2606414 administrated group) (n=8 for Trans-ISRIB vehicle and Trans-ISRIB

administrated group). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with orange indicated the GSK2606414 vehicle group injected; the gray demonstrated the GSK2606414 administrated group; the blue indicated the Trans-ISRIB vehicle group; the green indicated the Trans-ISRIB administrated group.

3.3.2.2.2. Parkin

GSK2606414 administration ($t(6)=-0.25$, $p=0.81$) did not show significant alteration in the Parkin protein levels (Figure 3.16). Nevertheless, a significant increase in the protein levels of Parkin in the Trans-ISRIB injected $Apoe^{-/-}$ mice was observed compared to Trans-ISRIB vehicle administrated $Apoe^{-/-}$ mice in cortical tissue ($t(14)=2.304$, $p=0.04$) (Figure 3.16). Furthermore, Pearson's partial correlation was conducted and there was no significant correlation between body weights and Parkin for GSK2606414 ($r(5)=0.619$, $p=0.14$) and Trans-ISRIB ($r(13)=0.078$, $p=0.78$).

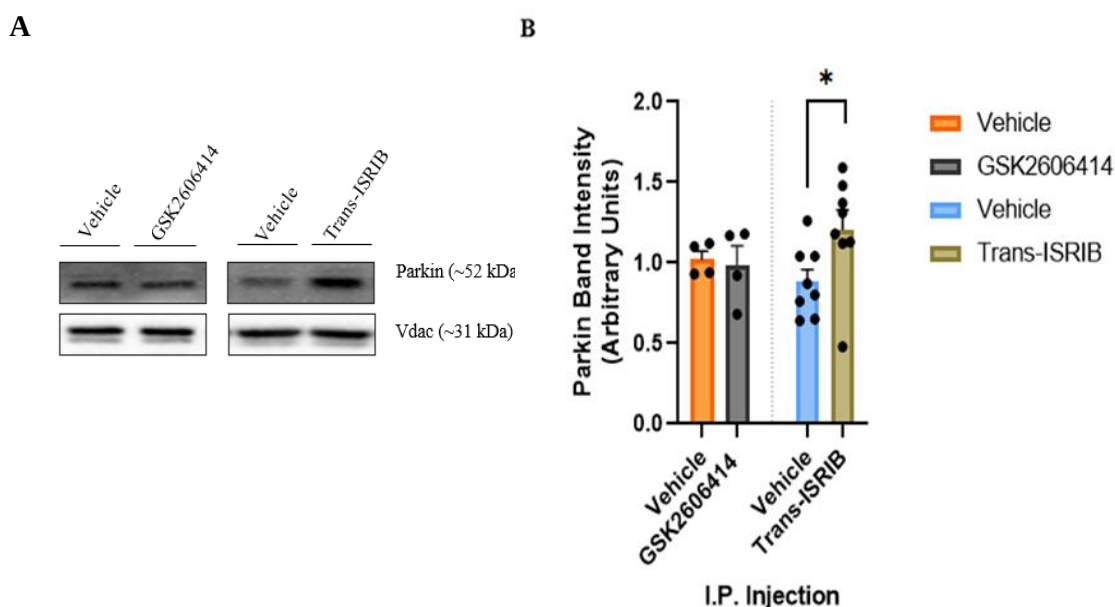


Figure 3.16. Effects of GSK2606414 and Trans-ISRIB administration on Parkin protein levels in the cerebral cortex. Representative western blot images (A) and (B)

Quantification graphs of the immunoblot images. While GSK2606414 administration did not show significant alteration in the Parkin protein levels, Trans-ISRIB administrated Apoe^{-/-} mice had a significantly increasing trend in Parkin levels compared to Trans-ISRIB vehicle administrated Apoe^{-/-} mice in cortical tissue. (n=4 for GSK2606414 vehicle and GSK2606414 administrated group) (n=8 for Trans-ISRIB vehicle and Trans-ISRIB administrated group). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$; * $p \leq 0.05$. Bars with orange indicated the GSK2606414 vehicle group injected; the gray demonstrated the GSK2606414 administrated group; the blue indicated the Trans-ISRIB vehicle group; the green indicated the Trans-ISRIB administrated group.

3.3.2.2.3. PINK1

PINK1 demonstrated that there was a significant enhancement in cortical tissue of Apoe^{-/-} mice fed with WD in the condition of GSK2606414 administration compared to vehicle administration ($t(6)=2.85$, $p=0.03$) (Figure 3.17). However, the protein levels of mitochondrial PINK1 were not significantly changed by Trans-ISRIB administration ($t(14)=0.829$, $p=0.42$) in the cerebral cortex (Figure 3.17). Additionally, Pearson's partial correlation was conducted and it showed that no significant correlation between body weights and PINK1 for GSK2606414 ($r(5)=-0.217$, $p=0.64$) and Trans-ISRIB ($r(13)=-0.147$, $p=0.61$).

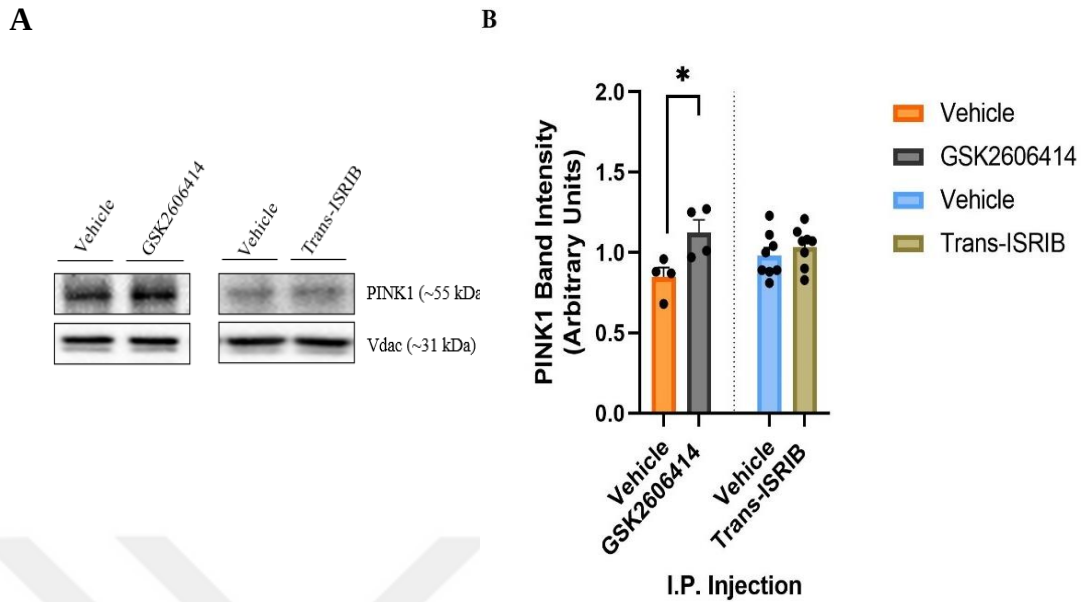


Figure 3.17. Effects of GSK2606414 and Trans-ISRIB administration on PINK1 protein levels in the cerebral cortex. Representative western blot images (A) and (B) Quantification graphs of the immunoblot images. While Trans-ISRIB administrated $Apoe^{-/-}$ mice did not show significant alteration in the PINK1 protein levels, GSK2606414 administration had a significantly increasing trend in PINK1 levels compared to GSK2606414 vehicle-administrated $Apoe^{-/-}$ mice in cortical tissue. (n=4 for GSK2606414 vehicle and GSK2606414 administrated group) (n=8 for Trans-ISRIB vehicle and Trans-ISRIB administrated group). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$, * $p \leq 0.05$. Bars with orange indicated the GSK2606414 vehicle group injected; the gray demonstrated the GSK2606414 administrated group; the blue indicated the Trans-ISRIB vehicle group; the green indicated the Trans-ISRIB administrated group.

3.3.2.3. Inflammatory Markers

To conduct the impacts of PERK pathway inhibitors on inflammation in cortical tissue of C57BL/6 mice and *Apoe*^{-/-} mice, inflammatory cytokines, NLRP3, NF- κ B, IL-1 β , and Caspase-1 protein levels were investigated.

3.3.2.3.1. NLRP3

GSK2606414 ($t(6)=0.351$, $p=0.74$) and Trans-ISRIB ($t(14)=0.50$, $p=0.53$) administration displayed no significant changes on NLRP3 levels in cerebral cortex (Figure 3.18). Spearman's partial correlation was done and it showed that no significant correlation between body weights and NLRP3 for GSK2606414 ($r(5)=-0.407$, $p=0.37$). On the other hand, partial correlation analysis between body weights and NLRP3 for Trans-ISRIB was done using Pearson's correlation. The results revealed that no significant correlation was obtained ($r(13)=-0.115$, $p=0.68$).

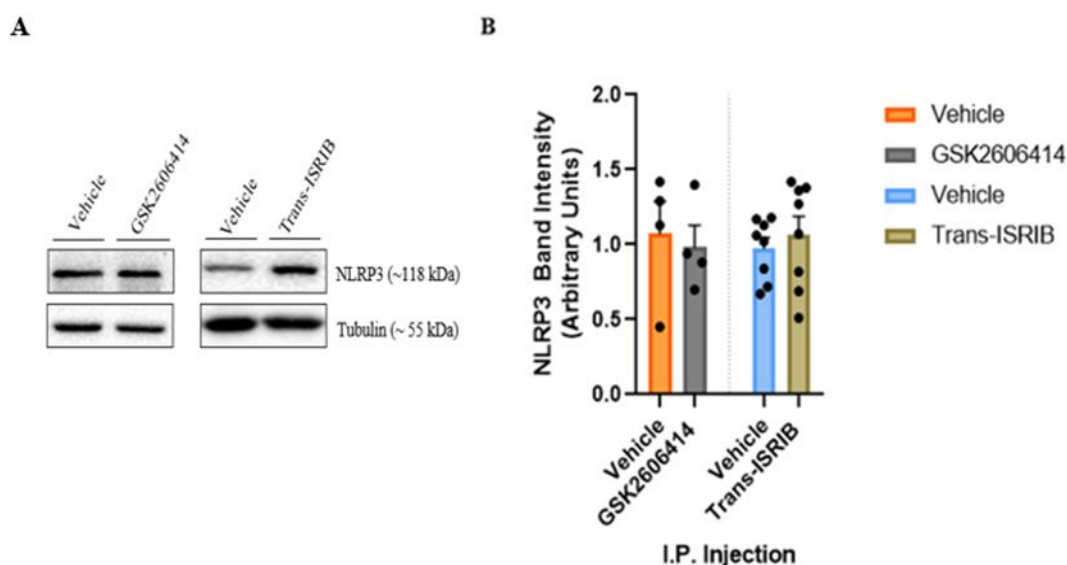


Figure 3.18. Effects of GSK2606414 and Trans-ISRIB administration on NLRP3 protein levels in the cerebral cortex. Representative western blot images (A) and (B) Quantification graphs of the immunoblot images. GSK2606414 and Trans-ISRIB administration had no significant changes in the levels of NLRP3 in cortical tissue of WD-fed Apoe^{-/-} mice. (n=4 for GSK2606414 vehicle and GSK2606414 administrated group) (n=8 for Trans-ISRIB vehicle and Trans-ISRIB administrated group). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with orange indicated the GSK2606414 vehicle group injected; the gray demonstrated the GSK2606414 administrated group; the blue indicated the Trans-ISRIB vehicle group; the green indicated the Trans-ISRIB administrated group.

3.3.2.3.2. NF- κ B

There were no significant influence of GSK2606414 ($t(6)=1.337$, $p=0.23$) (Figure 3.17) and Trans-ISRIB ($t(14)=1.735$, $p=0.11$) administration on the protein levels of NF- κ B in cortical tissue of Apoe^{-/-} mice fed with WD (Figure 3.17). Moreover, Spearman's partial correlation was conducted and there was no significant correlation between body weights and NF- κ B for GSK2606414 ($r(5)=0.500$, $p=0.25$). Besides, Pearson's partial correlation analysis between body weights and NLRP3 for Trans-ISRIB was done and no significant correlation was detected ($r(13)=0.114$, $p=0.69$).

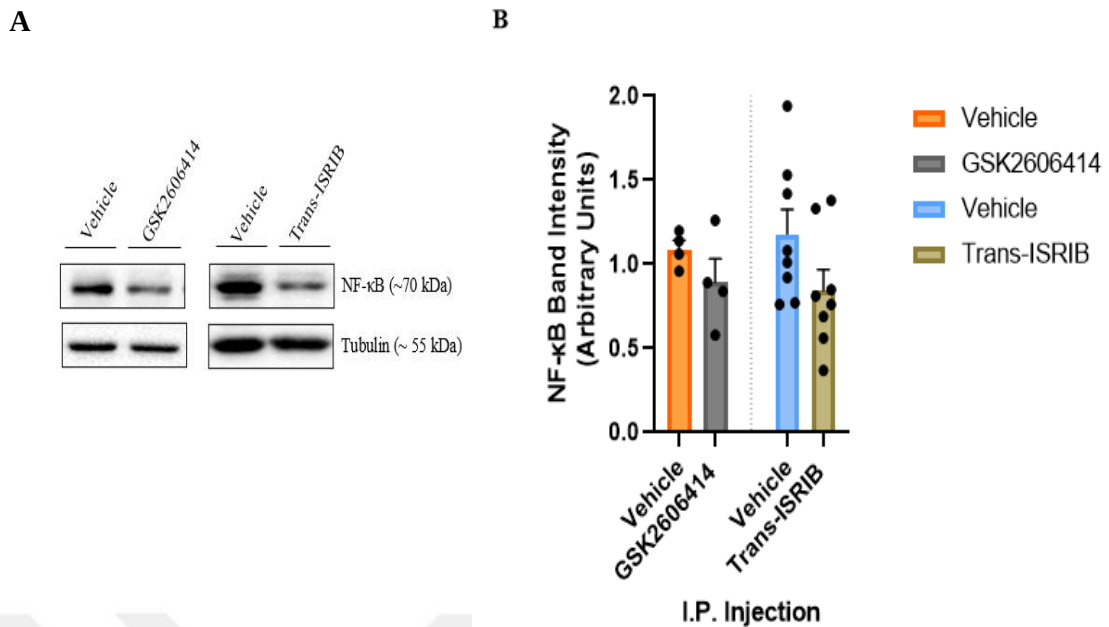


Figure 3.19. Effects of GSK2606414 and Trans-ISRIB administration on NF-κB protein levels in the cerebral cortex. Representative western blot images (A) and (B) Quantification graphs of the immunoblot images. GSK2606414 and Trans-ISRIB administration had no significant changes in the levels of NF-κB in cortical tissue of WD-fed Apoe^{-/-} mice. (n=4 for GSK2606414 vehicle and GSK2606414 administrated group) (n=8 for Trans-ISRIB vehicle and Trans-ISRIB administrated group). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with orange indicated the GSK2606414 vehicle group injected with DMSO with 16 % Cremophor-EL-Saline solution; the gray demonstrated the GSK2606414 administrated group; the blue indicated the Trans-ISRIB vehicle group injected with DMSO with 12.5 % Cremophor-EL-Saline solution; the green indicated the Trans-ISRIB administrated group.

3.3.2.3.3. Pro-IL-1β and Mature IL-1β

Both GSK2606414 ($t(6)=-0.651$, $p=0.19$) and Trans-ISRIB ($t(4)=0.793$, $p=0.44$) had no significant influence on mature IL-1β protein levels in cerebral cortex of Apoe^{-/-}

mice (Figure 3.18). Similarly, there was no significant difference in the pro-IL-1 β protein levels in cortical tissue of Apoe^{-/-} mice when GSK2606414 ($U=7.000$, $p=0.77$) Trans-ISRIB ($t(4)=-1.923$, $p=0.08$) administration was done (Figure 3.20). Spearman's partial correlation was done and there was no significant correlation between body weights and inflammatory markers which were pro-IL-1 β ($r(5)=0.506$, $p=0.25$), mature IL-1 β ($r(5)=-0.029$, $p=0.95$) for GSK2606414. On the other hand, Pearson's partial correlation analysis demonstrated that there was no significant correlation between body weights inflammatory markers which were pro-IL-1 β ($r(13)=0.313$, $p=0.26$), mature IL-1 β ($r(13)=-0.048$, $p=0.87$) for Trans-ISRIB.

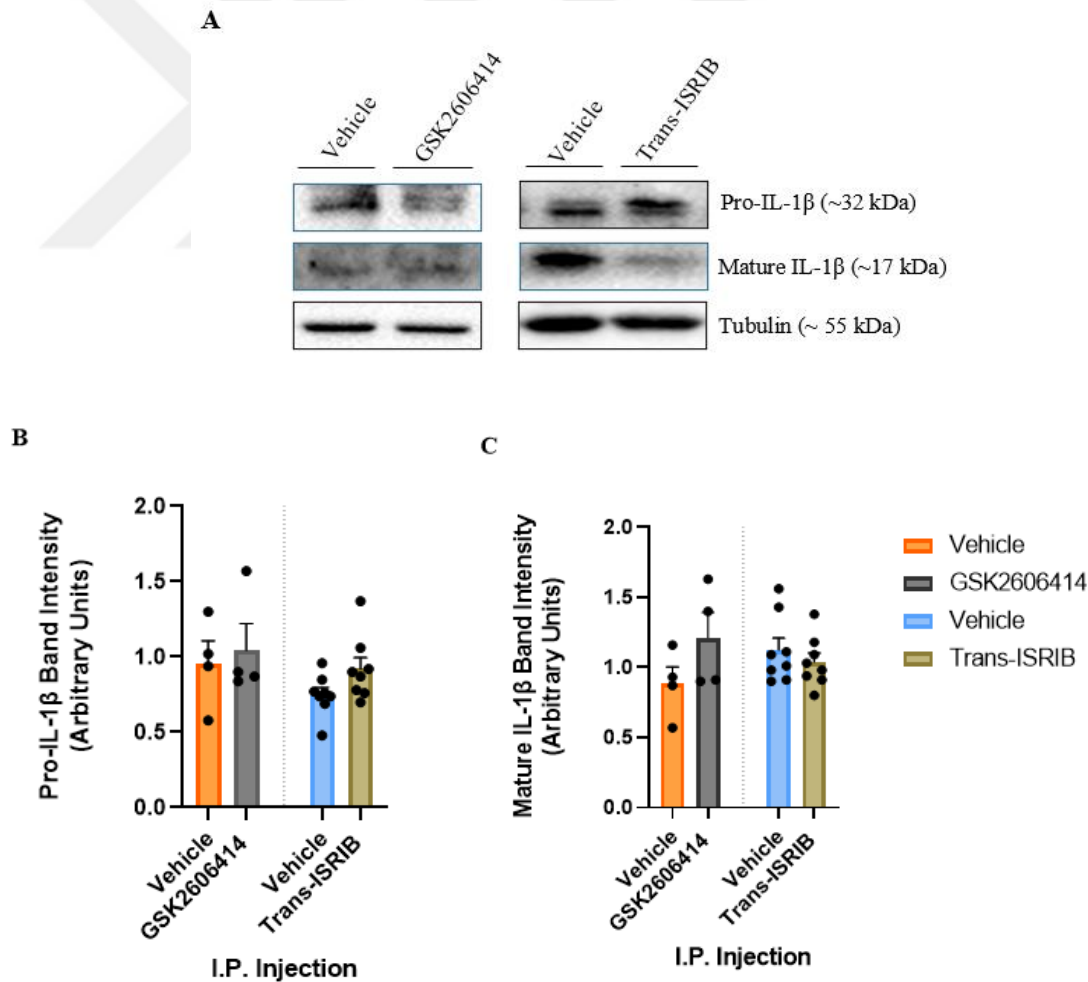


Figure 3.20. Effects of GSK2606414 and Trans-ISRIB administration on Pro-IL-1 β and Mature IL-1 β protein levels in the cerebral cortex. Representative western blot images (A) and (B), (C) Quantification graphs of the immunoblot images. GSK2606414 and Trans-ISRIB administration had no significant changes in the levels of Pro-IL-1 β and Mature IL-1 β in cortical tissue of WD-fed Apoe^{-/-} mice. (n=4 for GSK2606414 vehicle and GSK2606414 administrated group) (n=8 for Trans-ISRIB vehicle and Trans-ISRIB administrated group). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$, * $p \leq 0.05$. Bars with orange indicated the GSK2606414 vehicle group injected; the gray demonstrated the GSK2606414 administrated group; the blue indicated the Trans-ISRIB vehicle group; the green indicated the Trans-ISRIB administrated group.

3.3.2.3.4. Pro-Caspase-1 and Cleaved Caspase-1

While GSK2606414 had a significant decline in the protein levels of cleaved caspase-1 ($t(6)=7.337$, $p \leq 0.001$) compared to GSK2606414 vehicle group, Trans-ISRIB treated Apoe mice had no significant alterations in the cleaved caspase-1 levels ($t(14)=-1.946$, $p=0.07$) (Figure 3.21). On the other hand, Trans-ISRIB administrated Apoe^{-/-} mice demonstrated a significant enhancement in the pro-caspase-1 levels ($U=8.000$, $P \leq 0.01$) compared to the vehicle group of Trans-ISRIB; however, no significant alterations were seen when GSK2606414 ($t(6)=2.139$, $p=0.08$) administered in Apoe^{-/-} mice (Figure 3.21). Moreover, Spearman's partial correlation revealed that no significant correlation was found between body weights and inflammatory markers which were pro-caspase-1 ($r(5)=0.210$, $p=0.65$), cleaved caspase-1 ($r(5)=0.705$, $p=0.08$) for GSK2606414. Spearman's rank correlation was done only to detect the correlation between body weights and pro-caspase-1 ($r(13)=-0.301$, $p=0.28$). On the

other hand, Pearson's partial correlation analysis demonstrated that there was no significant correlation between body weights cleaved caspase-1 ($r(13)=-0.210$, $p=0.45$) for Trans-ISRIB.

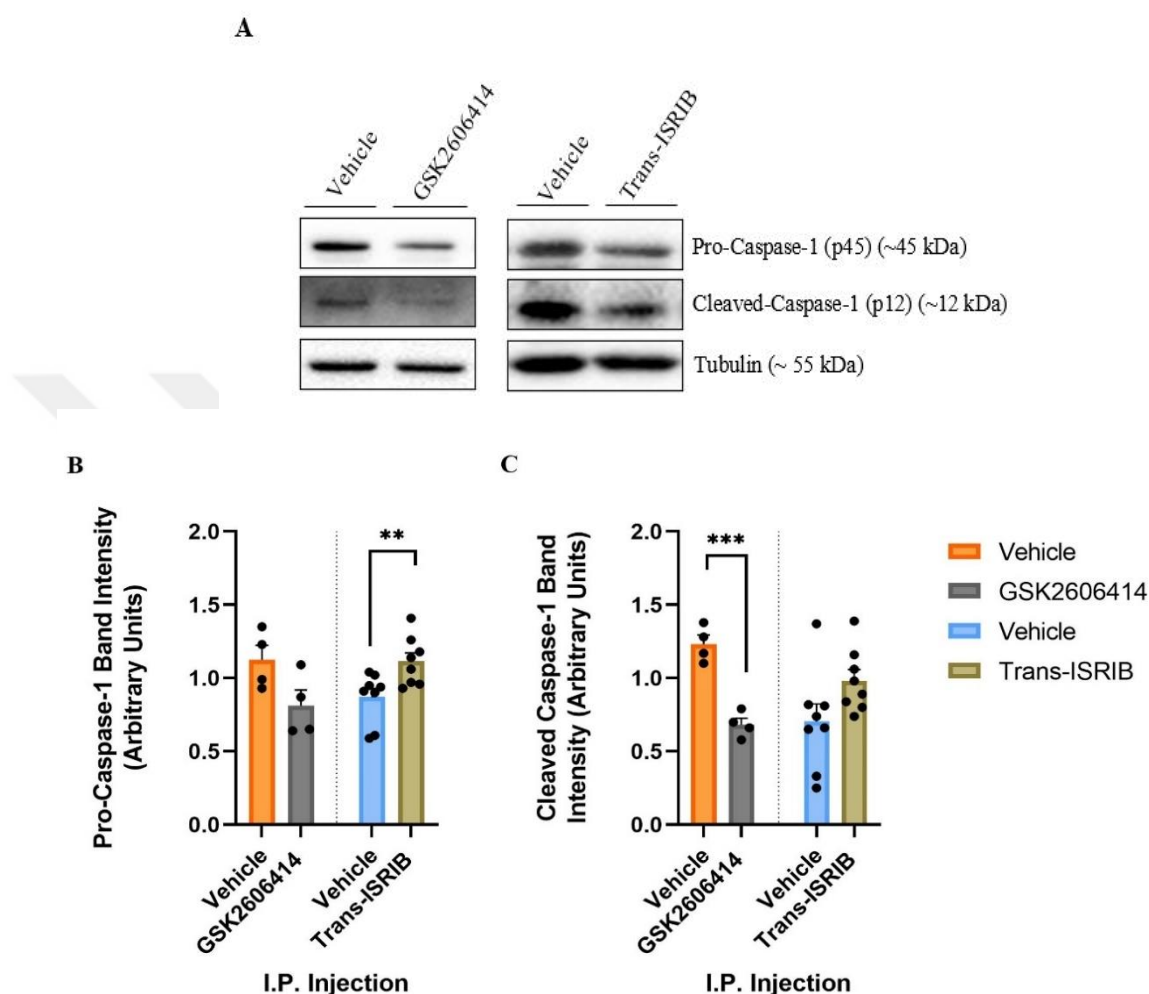


Figure 3.21. Effects of GSK2606414 and Trans-ISRIB administration on Pro-Caspase-1 and Cleaved Caspase-1 protein levels in the cerebral cortex. Representative western blot images (A) and (B), (C) Quantification graphs of the immunoblot images. While GSK2606414 administration did not show significant alteration in the Pro-Caspase-1 protein levels, Trans-ISRIB administrated *Apoe*^{-/-} mice had a significantly increasing trend in Pro-Caspase-1 levels compared to Trans-ISRIB vehicle administrated *Apoe*^{-/-} mice in cortical tissue. On the other hand, while Trans-ISRIB administrated *Apoe*^{-/-} mice did not show significant alteration in the Cleaved

Caspase-1 protein levels, GSK2606414 vehicle-administrated *Apoe*^{-/-} mice had a significantly increasing trend in Cleaved Caspase-1 levels compared to GSK2606414 administrated *Apoe*^{-/-} mice. (n=4 for GSK2606414 vehicle and GSK2606414 administrated group) (n=8 for Trans-ISRIB vehicle and Trans-ISRIB administrated group). Each error bar represents mean value + SEM, and statistical significance was set to $p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. Bars with orange indicated the GSK2606414 vehicle group injected; the gray demonstrated the GSK2606414 administrated group; the blue indicated the Trans-ISRIB vehicle group; the green indicated the Trans-ISRIB administrated group.

3.3.3. Confirmation of Mitochondria Isolation

Rho-GDI, a cytosolic marker [126], VDAC, a mitochondrial marker [127], and Tubulin markers were examined in the whole cell lysate (WCL), cytosol, and mitochondrial lysate to confirm mitochondria were isolated from the cerebral cortex lysate of the non-injected animals, and GSK2606414 and Trans-ISRIB administrated animals (Figure 3.22) (Figure 3.23).

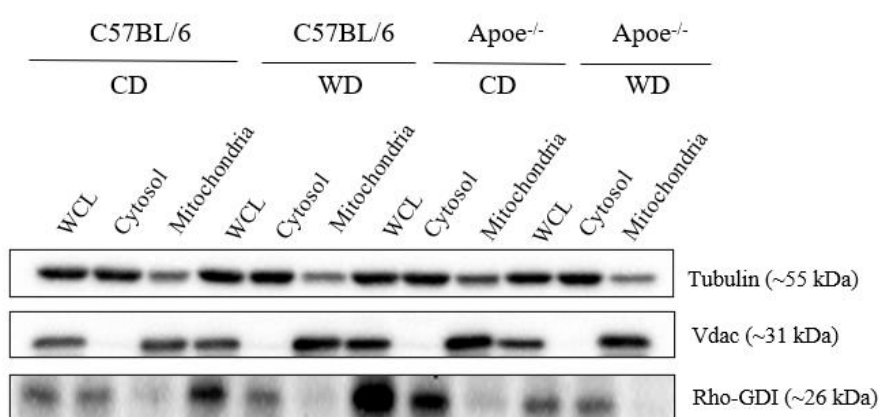


Figure 3.22. Confirmation of Mitochondria Isolation of Non-injected Animals.

Representative western blot images of Tubulin, VDAC, and Rho-GDI. VDAC,

mitochondrial marker, Rho-GDI, cytosolic marker, and Tubulin markers were used to confirm mitochondria isolation in WCL, cytosolic and mitochondria lysates of cerebral cortex tissue of C57BL/6 and Apoe^{-/-} mice either fed with CD and WD.

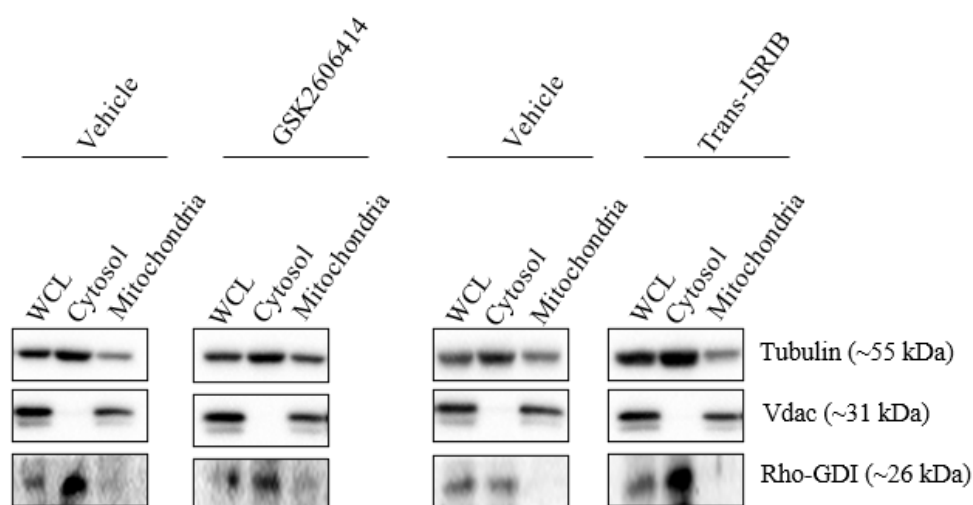


Figure 3.23. Confirmation of Mitochondria Isolation of GSK2606414 and Trans-ISRIB administrated animals. Representative western blot images of Tubulin, VDAC, and Rho-GDI. Representative western blot images of Tubulin, VDAC, and Rho-GDI. VDAC, mitochondrial marker, Rho-GDI, cytosolic marker, and Tubulin markers were used to confirm mitochondria isolation in WCL, cytosolic and mitochondria lysates of cerebral cortex tissue of Apoe^{-/-} mice administrated with GSK2606414, Trans-ISRIB and GSK2606414 vehicle group, Trans-ISRIB vehicle.

3.4. Gene Expression Experiments

Inflammatory markers, NLRP3, and IL-1 β , Caspase-1 were also examined at the mRNA levels for further investigation.

3.4.1. The Effects of Genotype and High Fat Diet on Cerebral Cortex

3.4.1.1. Inflammatory Markers

3.4.1.1.1. NLRP3

The diet ($F(1,8)=0.631$, $p=0.15$) and genotype ($F(1,8)=0.044$, $p=0.68$) differences did not significantly change the expression level of NLRP3 (Figure 3.24). Furthermore, the effect of genotype did not depend on the effect of the diet for NLRP3 expression in the cerebral cortex ($F(1,8)=1.070$, $p=0.33$) (Figure 3.24). Furthermore, partial correlation analysis was done between NLRP3 and the last body weights of non-injected animals by using Spearman's rank correlation. No significant correlation was assessed between body weights and any of NLRP3 ($r(8)=-0.240$, $p=0.51$).

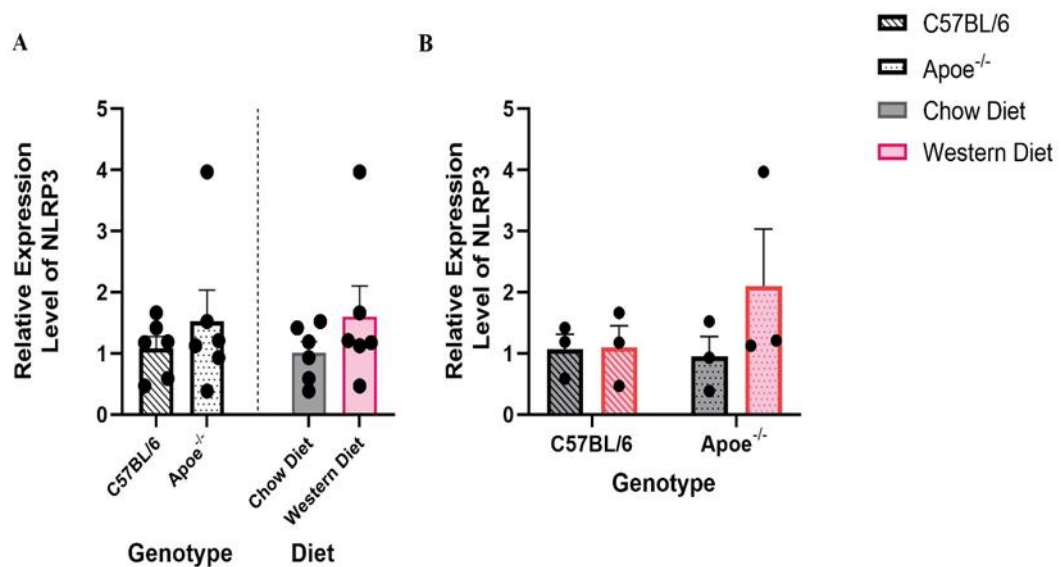


Figure 3.24. Relative Expression Levels of NLRP3 in the cerebral cortex. (A) (B) Quantification graphs of the expression levels of NLRP3. There were no significant effects of genotype and diet on NLRP3 expression levels in the cerebral cortex of mice. (n=6 for C57BL/6 mice and Apoe^{-/-} mice) (n= 6 for Chow diet and Western diet) (n=3

for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed *Apoe*^{-/-} mice and WD-fed *Apoe*^{-/-} mice). Error bars demonstrate mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with dots patterned showed C57BL/6 mice, line patterned showed *Apoe*^{-/-} mice, dark gray indicated CD-fed animals, and pink indicated the WD-fed animals.

3.4.1.1.2. IL-1 β

The diet ($F(1,8)=0.187$, $p=0.54$) and genotype ($F(1,8)=0.007$, $p=0.9$) made no significant changes in IL-1 β relative gene expression (Figure 3.25). Moreover, the effect of genotype did not depend on the effect of the diet for IL-1 β expression in the cortical tissue ($F(1,8)=1.319$, $p=0.28$) (Figure 3.25). Furthermore, Spearman's rank partial correlation was conducted and it showed that there was no significant correlation between body weights and IL-1 β ($r(8)=0.180$, $p=0.62$).

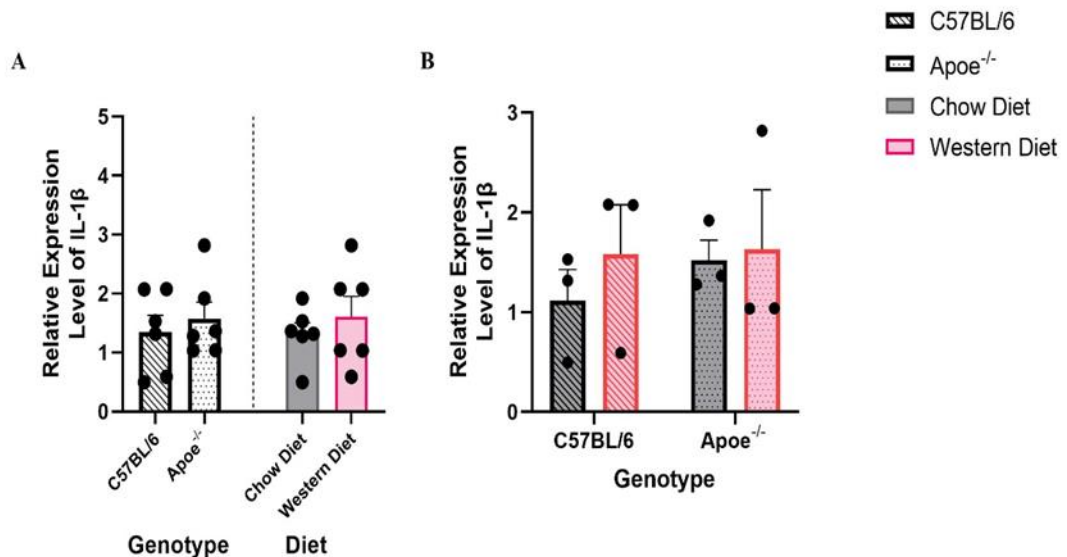


Figure 3.25. Relative Expression Levels of IL-1 β in the cerebral cortex. (A) (B) Quantification graphs of the expression levels of IL-1 β . There were no significant effects of genotype and diet on IL-1 β expression levels in the cerebral cortex of mice. (n=6 for C57BL/6 mice and *Apoe*^{-/-} mice) (n= 6 for Chow diet and Western diet) (n=3 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed *Apoe*^{-/-} mice and WD-fed

Apoe^{-/-} mice). Error bars demonstrate mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with dots patterned showed C57BL/6 mice, line patterned showed Apoe^{-/-} mice, dark gray indicated CD-fed animals, and pink indicated the WD-fed animals.

3.4.1.1.3. Caspase-1

A two-way ANOVA was performed to compare the effect of genotype and diet on gene expression levels of Caspase-1. Two-way ANOVA revealed that diet ($F(1,8)=0.044$, $p=0.69$) and genotype ($F(1,8)=0.055$, $p=0.65$) did not demonstrate a statistically significant difference in the expression levels of the caspase-1 in cerebral cortex brain tissue (Figure 3.26). No interaction was determined by diet or genotype for Caspase-1 ($F(1,8)=0.071$, $p=0.8$) expression in the cerebral cortex (Figure 3.26). Additionally, Spearman's rank correlation was done to assess correlation between body weights and Caspase-1. Results showed that no significant correlation was found between body weights and Caspase-1 ($r(8)=-0.034$, $p=0.93$).

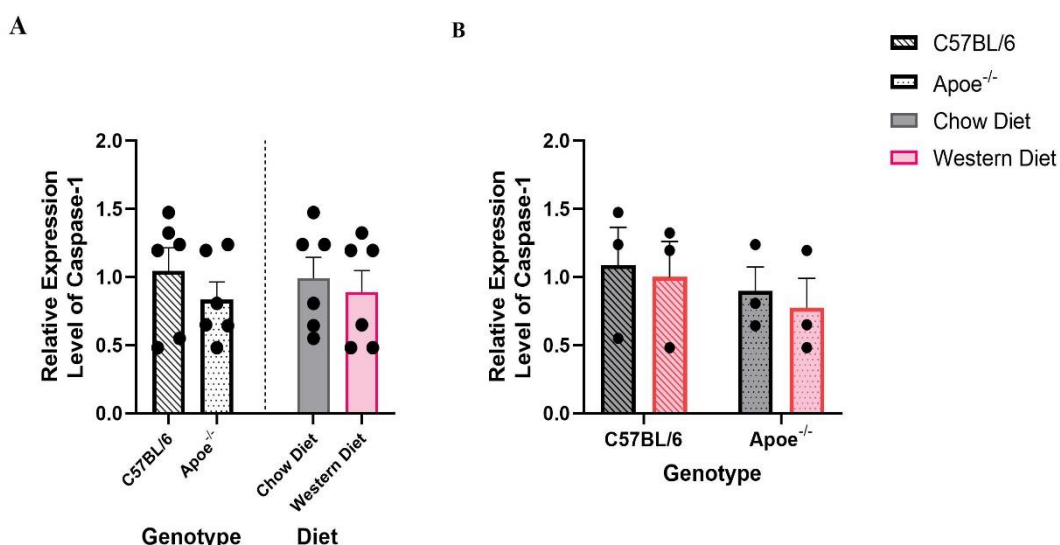


Figure 3.26. Relative Expression Levels of Caspase-1 in the cerebral cortex. (A) (B) Quantification graphs of the expression levels of Caspase-1. There were no significant effects of genotype and diet on Caspase-1 expression levels in the cerebral

cortex of mice. (n=6 for C57BL/6 mice and Apoe^{-/-} mice) (n= 6 for Chow diet and Western diet) (n=3 for CD-fed C57BL/6 mice, WD-fed C57BL/6 mice, CD-fed Apoe^{-/-} mice and WD-fed Apoe^{-/-} mice). Error bars demonstrate mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with dots patterned showed C57BL/6 mice, line patterned showed Apoe^{-/-} mice, dark gray indicated CD-fed animals, and pink indicated the WD-fed animals.

3.4.2. The Effects of PERK Pathway Inhibition on Cerebral Cortex

3.4.2.1. Inflammatory Markers

Transcript levels of Caspase-1, NLRP3, and IL-1 β were not significantly affected by the Trans-ISRIB or GSK2606414 administration. There was no change in the expression levels of Caspase-1 caused by Trans-ISRIB administration ($t(4)=0.50$, $p=0.82$) or GSK2606414 administration ($U(3)=2.67$, $p=0.4$) (Figure 3.27). In addition to that, no significant alterations in mRNA levels of NLRP3 were observed for Trans-ISRIB administration ($t(4)=2.14$, $p=0.92$) and GSK2606414 administration ($t(4)=2.14$, $p=0.32$) (Figure 3.27). In parallel with the previous results, the transcript levels of IL-1 β were not significantly changed by either Trans-ISRIB ($U(3)=2.67$, $p=0.4$) or GSK2606414 ($t(4)=1.2$, $p=0.16$) administration (Figure 3.27). Furthermore, Spearman's rank correlation partial correlation analysis was done between inflammatory markers and the last body weights of GSK-injected and GSK-vehicle-injected animals. Results revealed that no significant correlation was detected between body weights and any of the inflammatory markers (NLRP3: $r(3)=-0.146$, $p=0.81$ IL-1 β : $r(3)=-0.220$, $p=0.72$, Caspase-1: $r(3)=-0.146$, $p=0.81$). Besides, Pearson's partial correlation analysis between inflammatory markers, and the last body weights of Trans-ISRIB-injected and Trans-ISRIB vehicle-injected animals was done.

Spearman's rank correlation was done only to detect the correlation between IL1B and body weights. The analysis demonstrated that there was no significant correlation between body weights and inflammatory markers, which were NLPR3: $r(3)=-0.752$, $p=0.14$, and IL-1 β : $r(3)=-0.798$, $p=0.11$, but Caspase-1 was significantly negatively correlated in Trans-ISRIB-injected animals ($r(3)=-0.919$, $p=0.03$).

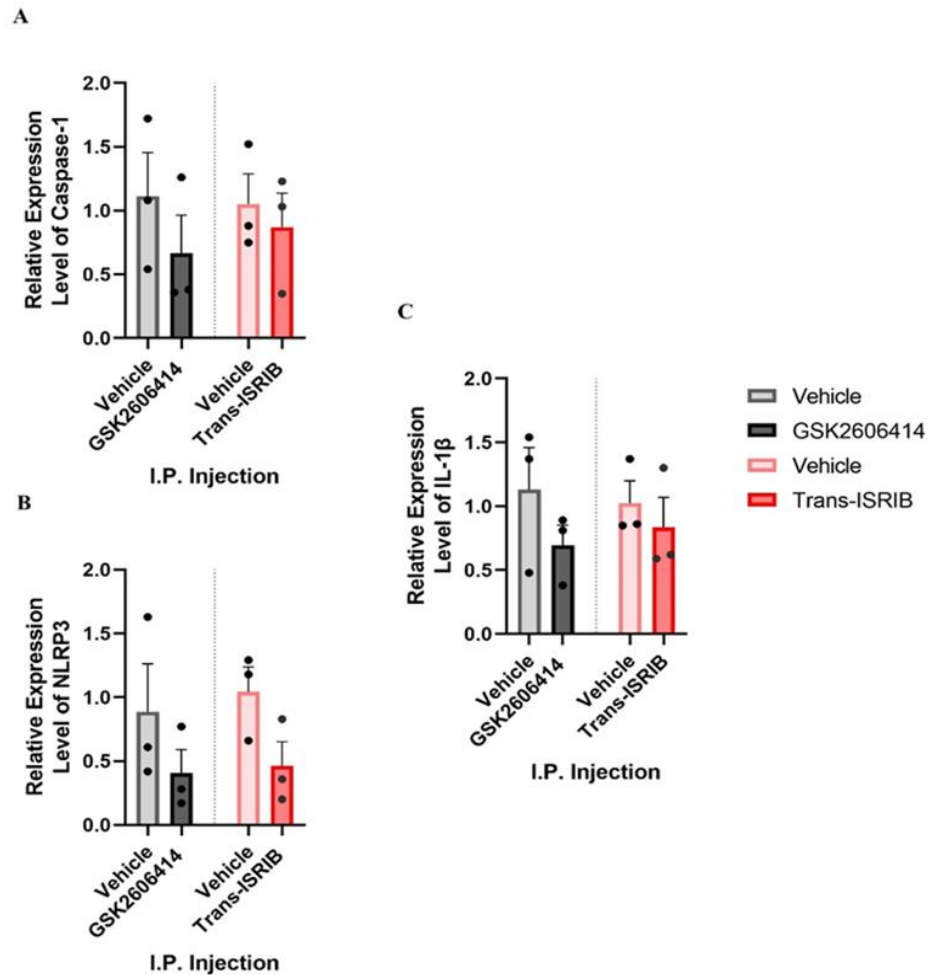


Figure 3.27. Effects of GSK2606414 and Trans-ISRIB administration on Relative Expression Levels of Caspase-1, NLRP3, and IL-1 β in the cerebral cortex. (A) Quantification graphs of the expression levels of Caspase-1, (B) Quantification graphs of the expression levels of NLRP3, (C) Quantification graphs of the expression levels of IL-1 β . GSK2606414 and Trans-ISRIB administration had no significant changes in the expression levels of NLRP3, IL-1 β , and Caspase-1 in cortical tissue of WD-fed Apoe^{-/-} mice. (n=3 for GSK2606414 vehicle and GSK2606414 administrated group)

(n=3 for Trans-ISRIB vehicle and Trans-ISRIB administrated group). Error bars demonstrate mean value + SEM, and statistical significance was set to $p \leq 0.05$. Bars with gray indicated the GSK2606414 vehicle group; the dark gray demonstrated the GSK2606414 administrated group; the light pink indicated the Trans-ISRIB vehicle group; the red indicated the Trans-ISRIB administrated group.



CHAPTER 4

DISCUSSION

In this study, C57BL/6 and Apoe^{-/-} male mice were fed with the CD or WD. In contrast to C57BL/6 (Wild type mice), Apoe^{-/-} mice have high cholesterol levels [128]. Besides the genotype of mice, WD has a high-fat and high-cholesterol content diet compared to CD, leading to neuroinflammation in the brain [129]. Hence, the first goal of this study was to investigate the influence of hyperlipidemia on the PERK pathway, one of the branches of ER stress, mitochondria stress, and inflammation in the cortical tissue of C57BL/6 and Apoe^{-/-} mice. When enhancement in the lipid metabolism is detected, the PERK pathway is hyperactivated [130], and its role in causing mitochondrial dysregulation and a possible relationship between inflammation and mitochondrial stress was conducted. In the literature, enhancement in the expression levels of LONP1 is observed in the adipose tissue of obese women [131] and liver and skeletal muscle [132]. Moreover, a study [133] has investigated the preclinical and clinical models for examining ER stress and UPR, especially the PERK pathway, in neurological disorders, highlighting the significance of the cognitive tests to verify the model. Furthermore, because it has been studied that LONP1 is stimulated by the PERK in macrophages [113], it was expected that p-PERK, p-eIF2 α activation should have been higher during the hyperlipidemia; in turn, enhancement in the LONP1 levels. Considering all of this, p-PERK, p-eIF2 α , and LONP1 levels were expected to be higher during the increased lipid metabolism. Remarkably, there were no significant changes in the p-PERK levels in the cortical tissue for both diet and genotype in this study, suggesting that the PERK pathway was not activated by hyperlipidemia. Nevertheless, the p-eIF2 α and LONP1 levels were significantly higher in the C57BL/6 mice than in Apoe^{-/-} mice. Although there were no changes in p-PERK levels for both

diet and genotype, LONP1 and p-eIF2 α levels were higher in C57BL/6 mice compared to Apoe^{-/-} mice. In the literature, it was shown that the expression of proteins controlled in mitochondrial proteostasis, such as LONP, counteract the mitochondrial malfunction caused by the translation suppression brought about by eIF2 α phosphorylation [134]. For this reason, LONP1 levels may show a significant trend in C57BL/6 mice with the same pattern as p-eIF2 α to tackle mitochondrial dysfunction. Heme-regulated Inhibitor (HRI) [135] and General control nonderepressible 2 (GCN2) [136] phosphorylate the eIF2 α since they are other activators of phosphorylation of eIF2 α . p-eIF2 α levels were significantly higher in WD-fed C57BL/6 mice than in CD-fed C57BL/6 mice, suggesting the WD's high fat and high cholesterol content may have activated the HRI and GCN2, which caused phosphorylation of eIF2 α . Therefore, such stress mechanisms enhance the phosphorylation of eIF2 α in an attempt by the body to counteract the diet-induced metabolic stress in the cortical tissue of C57BL/6 mice.

The results also showed that p-eIF2 α levels were significantly enhanced in WD-fed C57BL/6 mice compared to WD-fed Apoe^{-/-} mice. Since studies showed that Apoe^{-/-} mice, which have higher lipid metabolism, lead to enhancement in p-eIF2 α in human umbilical vein endothelial cells [137] and LONP1 levels in adipose tissue [131], higher levels of these markers in cortical tissue of C57BL/6 mice compared to Apoe^{-/-} mice may be due to tissue-specific responses. Indeed, compared to other tissues, the cerebral cortex may have a unique reaction to responses and defense systems. This may suggest that LONP1, p-PERK, and p-eIF2 α levels are differentially regulated and not directly correlated in the cerebral cortex. BiP is a transcription factor triggered by eIF2 α phosphorylation and is a downstream target of ATF4 [138]. To recruit protein folding ability and diminish ER stress, upregulation of BiP expression is activated by ATF4

because of elevated p-eIF2 α levels [139]. Thus, elevated levels of BiP expression are an approach to tackle ER stress brought by altered lipid metabolism, which may be associated with significantly enhanced p-eIF2 α levels observed in C57BL/6 mice fed a Western diet. On the contrary, consistent with the expectation, Parkin levels at the cortical tissue of mice during feeding with WD are significantly lower than those of feeding with CD. It is possible that diets rich in fat and cholesterol lead to metabolic stress and dysregulation in mitochondria, which could restrict the accessibility or efficiency of mitophagy markers like Parkin. Furthermore, there was a significant decline in Parkin protein levels in the Apoe^{-/-} mice compared to C57BL/6 mice. It may be due to the fact that mitochondrial dysfunction and oxidative stress are implicit in Apoe deficiency. Moreover, there were no significant alterations between the diet groups for mitochondrial LONP1 and diet and genotype for PINK1 levels. Overall, it was not inferred that PERK pathway hyperactivation occurs due to hyperlipidemia in the cerebral cortex. These results imply that, whereas activation of the PERK pathway is constant, diet and genotype have mildly unique effects on mitochondrial quality control systems. PERK pathway markers such as ATF4, CHOP, and eIF2B, as well as apoptotic markers like p62 and BDNIP, can be investigated at protein levels to understand hyperlipidemia-induced PERK pathway hyperactivation and whether it contributes to mitochondrial dysfunction or not further. Furthermore, the AMPK (AMP-activated protein kinase) - mTOR (mechanistic target of rapamycin) signaling pathway is crucial for mitochondrial homeostasis and regulates mitophagy [140]. Therefore, other signaling pathways, such as the AMPK-mTOR axis, IRE1, one of the ER Stress UPR branches, that may be involved in the mitophagy regulation could be investigated in more research.

Additionally, there were no significant alterations for the inflammatory markers such as NLRP3, NF κ B, pro-IL-1 β , and pro-caspase-1 levels between both diet and genotype groups. Diet impacts were not detected in any inflammatory markers. A 16-week diet period may not be sufficient to observe meaningful, significant alterations in terms of dietary intake. On the contrary, there were significant differences between the genotype groups. Mature IL-1 β and cleaved caspase-1 demonstrated a rise in the Apoe^{-/-} mice compared to C57BL/6 mice. Since caspase-1 is responsible for converting IL-1 β to a mature form [141], these results suggest that cleaved caspase-1 turns IL-1 β into a mature form in Apoe^{-/-} mice, and inflammation levels were enhanced. As mentioned before in the introduction, because Apoe is crucial for lipid transport and metabolism, Apoe^{-/-} mice are known to be more susceptible to oxidative stress and lipid dysregulation [142]. Even a study displayed that Apoe^{-/-} animals show deferred lipoprotein clearance, leading to inflammation [25]. The elevated amounts of mature IL-1 β and cleaved caspase-1 suggest that this tendency, regardless of diet consumption, had a role in a heightened inflammatory response in the cerebral cortex of Apoe^{-/-} mice subjects. Furthermore, mature IL-1 β and cleaved caspase-1 protein levels were high because mitophagy clearance was not detected, which proves that Parkin levels were significantly low in Apoe^{-/-} mice and PINK1 protein levels were not significantly altered with genotype. Therefore, this mitochondrial dysfunction and mtROS production may also lead to inflammatory markers reaching high levels. Abnormalities in lipid metabolism with Apoe deficiency in Apoe^{-/-} mice may lead to hyperlipidemia and worsen mitochondrial dysfunction and, in turn, increase inflammation by highlighting the vital role of Apoe in preserving cellular homeostasis. Furthermore, although NLRP3 and NF κ B levels have no significant changes in Apoe^{-/-} mice, the cleaved caspase-1 levels, in turn, mature IL-1 β levels are significantly

increased levels in Apoe^{-/-} mice. It suggests that as an alternative to upregulating NLRP3 protein levels, this can happen by means of improved activation of the already-existing NLRP3 inflammasome. In other words, activation of the already-existing inflammasome components in Apoe^{-/-} mice may cause enhanced cleaved caspase-1 levels and, in turn, mature IL-1 β levels. It is evident from these protein expression results that during the study timeframe, the genetic background (Apoe^{-/-} vs. C57BL/6) had a more significant influence on inflammation activation than the dietary intervention.

As mentioned in the earlier chapters, PERK pathway inhibitors (GSK2606414) and downstream inhibitors (Trans-ISRIB) were intraperitoneally injected into Apoe^{-/-} mice fed with WD. The second aim of this study was to examine whether an inhibition in PERK signaling would reverse the abnormalities brought on by inflammation stimulated by HFD and affect mitophagy through the blocking of the PERK pathway. Owing to the concerns about adverse negative consequences, two distinct inhibitors, GSK2606414 and ISRIB, target the different locations of the PERK pathway. GSK2606414 inhibitor can lead to severe weight loss, pancreatic toxicity, increased blood sugar level, and even mortality [143], [144]. Conversely, ISRIB has not been reported to have adverse side effects like pancreatic toxicity in the literature. Even it has memory-enhancing properties [145]. The findings of this study are crucial for determining potential targets that may be addressed to alleviate neuronal dysfunction resulting from HFD and hyperlipidemia, which in turn causes high levels of neuroinflammation, mitochondrial dysfunction, and ER stress. When looking at the results, surprisingly, there were no significant alterations in the p-PERK and p-eIF2 α levels in cortical tissue in either treatment with the GSK2606414 and Trans-ISRIB. No changes in p-eIF2 α levels in the cerebral cortex of Apoe^{-/-} mice administrated with

Trans-ISRIB indicated that ISRIB acts downstream of p-eIF2 α . Moreover, results demonstrated a significant trend in the protein levels in PINK1 when the GSK2606414 treatment was done compared to the control group. On the other hand, there was a significant alteration in the Parkin levels; it was higher in the Trans-ISRIB injected mice than in mice who weren't injected. Findings demonstrated that GSK2606414 administration leads to the prohibition of PINK1 degradation, PINK1 levels become high, and mitophagy is modulated. Similarly, Parkin levels were high during the administration of the ISRIB inhibitor despite the lack of PERK pathway inhibition. It somehow may be the target of mitophagy stimulation. The detected results could be influenced by the time and duration of inhibitor delivery. In a comparable manner, mitophagy markers may react to inhibition more quickly or sensitively than p-PERK and p-eIF2 α levels, which may need a longer duration of inhibitor administration. Additionally, findings suggest that inhibitors impact the cellular stress responses and mitochondrial regulation independent of the PERK inhibition. Specifically, the reason why Parkin levels were higher in the GSK2606414 administrated Apoe^{-/-} mice and PINK1 was enhanced in the ISRIB administrated Apoe^{-/-} mice may be the PERK pathway inhibitors have variable effects on mitophagy indicators depending on the particular inhibitor utilized.

Inflammatory markers such as NLRP3, NF κ B, pro-IL1 β , and mature IL-1 β protein levels didn't demonstrate significant changes in WD-fed Apoe^{-/-} mice administered with either GSK2606414 or ISRIB. Only pro-caspase-1 was significantly higher in the ISRIB-administrated mice, and cleaved caspase-1 was enhanced in the control group compared to the GSK2606414 administrated mice. It can be said that due to the rising levels of mitochondrial PINK1, mitophagy activity was stimulated so that cleaved caspase-1 levels appeared in the low amount in GSK2606414 administrated Apoe^{-/-}

mice. Similarly, since Parkin levels were high in ISRIB-administrated *Apoe*^{-/-} mice, mitophagy is induced compared to the control group; in turn, inflammation levels declined, and pro-caspase-1 may have started to accumulate. Therefore, its level was enhanced because it was not turned into the active form, cleaved caspase-1.

Besides the protein level experiments, gene expression experiments demonstrated that statistically significant alterations were not seen on NLRP3, caspase-1, and IL-1 β inflammatory markers in either genotype or diet on cortical tissue. Moreover, there were no significant differences in inflammation markers on protein and mRNA expression levels of GSK2606414 and Trans-ISRIB administrated *Apoe*^{-/-} mice. This may be due to the fact that the sample size was very low. MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines are generally recognized as the established guidelines for maintaining accuracy and consistency in qPCR research. According to MIQE, at least three to five are required to acquire consistent and accurate results [146]. Therefore, further experiments may be conducted in bigger sample sizes. Furthermore, DNase treatment was not done during the RNA isolation procedure, which is the first step of the RT-qPCR experiments. To make sure that the RNA is free of contaminating genomic DNA, DNase treatment is necessary during the RNA isolation. This contamination might end up in false-positive results and lead to misleading Ct values in RT-qPCR experiments [147]. As a result, DNase treatment is one of the essential steps for enhancing the accuracy of the RT-qPCR. However, no-template controls (NTC) and negative controls were included in the experimental setup to ensure that there was no contamination. Additionally, A260/A280 ratios were controlled with the help of the NanoDrop measurements to assess the purity of isolated RNA. Following RT-qPCR, double melting temperature (T_m) values were not detected. In addition to these, the

primers used in these experiments were designed to target the exon-exon junctions to prevent any contamination.

As mentioned in the Materials&Methods part, cytosolic and whole cell lysate fragment was also allocated for the conformation experiments. To verify the cellular fractions, the presence of VDAC, a mitochondrial marker [126], and Rho-GDI, a cytosolic marker [127] was investigated in WCL, cytosolic lysates, and mitochondrial lysates. While Rho-GDI was not present in the mitochondrial lysates, it was found in the WCL and cytosolic lysates. Conversely, VDAC was found in the mitochondrial and WCL lysates but not in the cytosolic lysates. Consequently, the results of the conformation experiments demonstrated that mitochondria were successfully isolated from the cerebral cortex tissue of all experimental animal groups (Figure 3.22) (Figure 3.23).

Mice models are frequently employed in neuroscience research since they can be genetically modified, their behavior is testable, and their physiology is similar to that of people, thereby rendering them useful tools for studying the function of the human brain [148]. Besides, mice models are economical and feasible options for both applied research owing to their short lifespan [149].

Consequently, ER stress was not generated, and the PERK pathway was not activated yet, as evidenced by the lack of significant change in p-PERK, p-eIF2 α , PERK pathway markers, under the condition of hyperlipidemia. It may suggest that 16 weeks of diet innervation was insufficient to stimulate the cerebral cortex's PERK pathway. Although inhibition of ER stress has been studied as a potential therapeutic approach for neurodegenerative diseases [150], [151], inhibitors did not significantly impact the p-PERK and p-eIF2 α levels but did some significant alterations on mitophagy and inflammatory markers. Since the PERK pathway was not hyperactivated by

hyperlipidemia, it is possible that neither of the inhibitors in this study suppressed the PERK pathway's activity below basal levels. Besides, we tested the dosage amount of inhibitors in the total brain tissue and saw that there was an inhibition in the previous. Nevertheless, in this study, the dosage amount of both inhibitors may be insufficient to effectively inhibit the PERK pathway in the cerebral cortex region of the brain. For instance, in a study [78], a GSK2606414 inhibitor was given 150 mg/kg of body weight in mice. Another study [152] treated 50 mg/kg GSK2606414 twice a day by oral gavage to mice with prion disease. Thus, the dosage amount may be increased at a rate that does not cause toxicity in future studies. Since the cerebral cortex is a wide area of the brain [153], investigating the other distinct brain regions, such as the hypothalamus and hippocampus, may be another topic for further investigation. Moreover, the experiment started when the mice were 6-8 weeks old. It is plausible that the older mice may exhibit different protective pathways in response to hyperlipidemia conditions and distinct reactions to inhibitors.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

This study aimed to shed light on the impact of hyperlipidemia on the PERK pathway, mitochondrial stress, and inflammation in the cerebral cortex tissue of C57BL/6 and Apoe^{-/-} mice. In addition, it was sought to investigate whether inhibition of PERK signaling potentially ameliorates hyperlipidemia-induced inflammation and has an influence on mitophagy in the cortical tissue of WD-fed Apoe^{-/-} mice.

Remarkably, results demonstrated that p-PERK, PINK1, NLRP3, NFκB, pro-IL1β, and pro-caspase-1 levels were not significantly changed during hyperlipidemia by genotype or diet. Surprisingly, p-eIF2α and LONP1 levels were significantly enhanced in C57BL/6 mice compared to Apoe^{-/-} mice. Only Parkin levels were high in C57BL/6 mice compared to Apoe^{-/-} and high in CD rather than WD. Also, mature IL-1β and cleaved caspase-1 have enhanced levels in Apoe^{-/-} mice compared to C57BL/6 mice. mRNA transcript levels of inflammatory markers also displayed no significant change by genotype or diet. Overall, it can be inferred from this study that hyperlipidemia did not induce the PERK pathway; however, mitochondria dysregulation was mildly observed, and inflammation was influenced mildly by hyperlipidemia independent from the PERK pathway. These results suggest that a neuroprotective compensatory mechanism against hyperlipidemia was induced in the cerebral cortex.

Additionally, there were no changes in p-PERK, p-eIF2α, LONP1, NLRP3, NFκB, pro-IL1β, and mature IL-1β protein levels of both GSK2606414 and ISRIB-injected Apoe^{-/-} mice. In addition, expression levels of inflammatory markers didn't significantly alter during the administration of PERK inhibitors. PINK1 protein levels were statistically significantly enhanced in GSK2606414 injected Apoe^{-/-} mice

compared to the vehicle, but no significant changes were observed in the ISRIB-administrated mice. Similarly, while Parkin levels demonstrated a statistically significant increasing trend in ISRIB-administrated Apoe^{-/-} mice compared to vehicle, there were no significant alterations in the GSK2606414 administrated mice. Both GSK2606414 and ISRIB-injected Apoe^{-/-} mice suggest that PERK pathway inhibitors have distinct effects on mitophagy indicators, depending on the specific inhibitor employed. Pro-caspase-1 levels were statistically significantly enhanced in the ISRIB injected group compared to the vehicle group, and cleaved caspase-1 levels were markedly increased in the vehicle group compared to the GSK2606414 injected mice group. Consequently, these results suggest that the PERK pathway was not inhibited, but mitophagy was stimulated, and inflammation was reduced mildly. Results displayed that PERK pathway inhibitors influenced the cellular stress and inflammation independent of the PERK pathway. Extensive research is required to figure out the direct target of inhibitors responsible for mitophagy stimulation and mild inflammation.

In this study, targeting the PERK pathway from the cerebral cortex would not be a therapeutic approach for neurodegenerative diseases. The results propose that the neuroprotective benefits of inhibitors of the PERK pathway are not attributable to the suppression of PERK signaling. Other PERK pathway inhibitors or ER Stress inhibitors could be used as a therapeutic approach for suppressing hyperlipidemia-induced ER stress, boosting mitochondrial regulation, and decreasing inflammation in the cerebral cortex. Furthermore, other brain regions would be investigated to see whether the PERK pathway inhibitors and hyperlipidemia have region-specific effects. Additionally, markers of the AMPK-mTOR axis and IRE1, one of the ER Stress UPR branches, PERK pathway markers such as ATF4, CHOP, eIF2B, as well as apoptotic

markers like p62, BDNIP and inflammatory markers such as IL-6 could be investigated in protein levels to understand hyperlipidemia-induced ER Stress activation and whether it contributes to mitochondrial dysfunction and enhances the neuroinflammation or not. Moreover, microglial, astrocyte and neuronal activity can be investigated with immunohistochemistry (IHC) experiments to examine the physiological state of them during hyperlipidemia, and the impacts of PERK pathway inhibitors. Astrocyte activity and structural changes can be measured by using astrocyte markers such as glial fibrillary acidic protein (GFAP) [154]. Following activation, they undergo hypertrophy, exhibiting thicker connections and larger cell bodies [155]. Therefore, IHC provide to assess these structural alterations. Also, neuronal nuclei (NeuN) and doublecortin (DCX) can be examined with the help of the IHC to assess neurogenesis, and the development and neurons [156]. Additionally, morphological changes of microglia are seen in the activated microglia when they turn from resting to active state; therefore, these alterations can be seen by utilizing the microglial marker such as ionized calcium-binding adapter molecule 1 (IBA-1) in brain regions [157]. To summarize, investigating their activities by IHC can be crucial for comprehending how hyperlipidemia and PERK pathway inhibitors affect the physiological status of neurons, astrocytes, and microglia.

In addition, some cognitive tests, such as the Morris Water Maze [158] and Barnes Maze [159], could be done to assess potential aspects of hyperlipidemia on the cognitive function of mice. Besides, there were some limitations, such as the sample size in this study. RT-qPCR experiments will be required to be conducted at a larger sample size. Furthermore, instead of using only male mice, female mice could also be included to detect gender-specific effects in future investigations.

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