PREVENTION OF FATTY ACID-INDUCED INFLAMMASOME ACTIVATION BY A BIOACTIVE LIPOKINE

A THESIS
SUBMITTED TO THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

BY

SEDA KOYUNCU

AUGUST 2014

I certify that I have read this thesis and that in my opinion it is fully adequate,

in scope and in quality, as a thesis for the degree of Master of Science.

_____

Assist.Prof.Dr. Ebru ERBAY (Advisor)

I certify that I have read this thesis and that in my opinion it is fully adequate,

in scope and in quality, as a thesis for the degree of Master of Science.

_____

Assist. Prof. Dr. Ali GÜRE

I certify that I have read this thesis and that in my opinion it is fully adequate,

in scope and in quality, as a thesis for the degree of Master of Science.

_____

Assoc. Prof. Dr.Sreeparna BANERJEE

Approved for the Graduate School of Engineering and Science

_____

Prof. Dr. Levent Onural

Director of the Graduate School

ABSTRACT

Prevention of Fatty Acid-Induced Inflammasome Activation by a Bioactive Lipokine

Seda Koyuncu

M.Sc.in Molecular Biology and Genetics

Supervisor: Assist. Prof. Dr. Ebru Erbay

August 2014, 82 Pages

Exposure to excess lipids such as fatty acids and cholesterol leads to cellular stress, release of reactive oxygen species (ROS), inflammation dysfunction and death. These responses have important role in the pathogenesis of chronic metabolic and inflammatory diseases such asobesity, diabetes and atherosclerosis. Recent studies show that fatty acids also cause the formation of inflammatoryprotein complexes that are called inflammasome. Inflammasome promotesthe activation of caspase-1 protein and cleavage of inactive interleukin-1 beta(IL-1β) and interleukin-18 (IL-18) into their active, secreted forms.Two signals are required for the activation of inflammasome. The first signal (also known as priming step) is needed for the inducing the expression ofthe proinflammatory factors pro-IL-1β and pro-IL-18 andthe nucleotide-binding oligomerization domain receptor (NOD-like receptor) family, pyrin domain containing-3 (NLRP3) proteins through the activation of nuclear factor kappa beta (NF-κB),a transcription factor. The second signal is needed for the activation of caspase-1 and formation of the inflammasome complex. Saturated free fatty acids such as palmitate are known to cause the activation of inflammasome through generation of mitochondrial ROS as a second signal for inflammasome complex formation. In this study, I investigated the effect of palmitoleate, a bioactive monounsaturated fatty acidpreviously shown to counteract lipid-induced ER stress and enhance insulin sensitivity, on palmitate-induced activation of

inflammasome complex. I observed that palmitoleate lead to a significant reduction in palmitate-stimulatedIL-1β transcription. Moreover, it reduced the expression of palmitate-stimulated secondary proinflammatory factors such as tumor necrosis factor alpha(TNF-α).Palmitoleate also diminished the palmitate induced activation of caspase-1, maturation and secretion of IL-1β in macrophages.

To further understand the mechanism of the protective role of palmitoleate on palmitate induced inflammasome activation,I analyzed the effect of palmitoleate on palmitate-induced mtROS in macrophages cells were analyzed. These studies showed palmitoleate decreased palmitate induced mitochondrial oxygen species in macrophages. Moreover, I investigated the effect of palmitoleate on palmitate-induced inactivation of 5' AMP-activated protein kinase (AMPK)controls autopaghy and clearance of dysfunctional mitochondria, thereby reducing mtROS formation. Palmitoleate blocked the suppression of AMPKphosphorylation that was suppressed by palmitate, suggesting PAO's impact on mtROS secretion and inflammasome activation occurs by this upstream mechanism.

Saturated fatty acids like palmitate are also known to cause endoplasmic reticulum (ER) stress, whichbe counteracted by palmitolate treatment. When ER stress happens, cells try to solve stress by activating the unfolded protein response (UPR). There are three arms of the UPR are Protein Kinase R-resemble like protein kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activating factor 6 (ATF6). In this thesis, contribution of PERK and IRE1 on palmitate-induced activation of inflammasome was investigated. First, the relation between PERK and IRE1 with palmitate-stimulated mtROS was analyzedusing PERK- and IRE1-deficient mouse embryonic fibroblast cells (MEFs).The results of this study show PERK leads to a marked reduction in the formation of mtROS while IRE1 enhanced palmitate induced mtROS.Furthermore, palmitoleate suppressed palmitate-induced autophosphorylation of IRE1 while there was no effect of palmitoleate on palmitate-induced phosphorylation of PERK.

In summary, palmitate-induced inflammasome activation can be inhibited by palmitoleate in boththe priming step and the second step.Palmitoleate blocks palmitate-induced suppression of phosphorylation of AMPK, leading to its reactivation and subsequent reduction in mtROS formation. Palmitate is a well-known inducer of ER stress, which can be counteracted by palmitoleic acid. Moreover, the IRE1 branch of the UPR has been shown to control inflammasome activationby regulating of mtROS production under lipotoxicity. The outcome of my studies show that the UPR branches initiated by PERK and IRE1 regulate mtROS production and therefore, palmitoleate may also block inflammasome activation induced by palmitate through this alternative mechanism.These findings imply that palmitoleate may have therapeutic applications inthe management of diseases where inflammasome activation has been shown to play a causal role such as obesity, diabetes and atherosclerosis.

Key words: Inflammasome, Palmitoleate, Palmitate, UPR, ER stress, Atherosclerosis

ÖZET

Serbest yağ asidlerinin neden olduğu inflammazom aktivasyonunun bir biyoaktif lipokin tarafından önlenmesi

Seda Koyuncu

Moleküler Biyoloji ve Genetik Yüksek Lisans

Tez Yöneticisi: Yrd. Doç. Dr. Ebru Erbay

Ağustos 2014, 82 Sayfa

Aşırı derecede, doymuş yağ asidi ve kolesterol gibi yağlara maruz kalmak, hücresel strese, aktif oksijen türlerinin salınmasına, inflamasyon bozukluğuna ve ölüme neden olur.Bu yanıtların obezite, diyabet, atheroskleroziz gibi kronik metabolic ve inflammatori hastalıklarda önemli rolü vardır. Son günlerdeki çalışmalar, yağ asitlerinin aynı zamanda inflammazomolarak adlandırılan inflammatori protein kompleksinin oluşumuna neden olduğunu göstermiştir. İnflammazom kaspaze-1 proteinin aktifleşmesini ve inaktif interlökin-1 beta (IL-1β) ve interlökin-18(IL-18)'in aktif ve salınan formuna kesilmesini sağlamaktadır.İnflammazom aktivasyonu için iki sinyale ihtiyaç vardır. İlk sinyal (ilkil sinyal olarakta bilinen) öncülinflammatori faktörlerin öncül-IL-1βve öncül-IL-18 ve nükleotid-bağlanan oligomerizasyon bölge reseptör (NOD- benzeri reseptör) ailesi, pyrin bölge taşıyan-3 (NLRP3) proteinin, bir transkripsiyon faktörü olan nüklear faktör kappa beta (NF-κB) aktivasyonu üzerinden ekspres olması için gereklidir. İkinci sinyal, kazpase-1 aktivasyonu ve inflammazom kompleks oluşumu için gereklidir.Palmitat gibi doymuş yağ asitlerinin inflammazom aktivasyonuna, inflammazomun ikinci sinyali olan mitokondriyal ROT oluşumunu sağlayarak neden olduğu bilinmektedir. Bu çalışmada, bir biyoaktif tekli doymamış yağ aside olan ve daha önce yağların neden olduğu ER stresi önlediği ve insulin duyarlılığını artırdığı gösterilmiş olan palmitoleatin palmitatin neden olduğu inflammazomkompleks aktivasyonuna etkisini araştırdım. Palmitoleatin palmitatin neden olduğuIL-1β transkripsiyonunu önemli ölçüde azalttığını gözlemledim.Ayrıca, palmitatin

neden olduğu tümör nekrozis factor alpha (TNF-α)gibi ikincil öncül-inflammatori faktörlerin ekspresyonunu da azaltmaktadır.Bunun yanında, palmitoleat palmitatin neden olduğu kaspaze-1 aktivasyonu ve IL-1β'ın olgunlaşması ve salınımını makrofajlarda azaltmaktadır.

Palmitoletin palmitatin neden olduğu inflammazom aktivasyonu üzerindeki koruyucu rolünün mekanizmasını daha iyi anlamak için, palmitoleatin palmitatin sebep olduğu mtROT üzerindeki etkisini makrofajlar hücrelerinde inceledim.Bu çalışmalar, palmitoletin palmitatin sebep olduğu mitokondriyal oksijen türlerinin makrofajlarda oluşumunu azalttığını gösterdi.Ayrıca, palmitoletin palmitin neden olduğu otofajiyi düzenleyen ve bozulmuş mitokondrileri temizleyerek mtROT oluşumunu azaltan 5' AMP-aktifliyen protein kinaz (AMPK) 'ın inaktivasyonuna etkisiniinceledim.Palmitatin indirgediği AMPK fosforulasyonunu palmitoleatin engellemesi, PAO'in etkisinin mtROT salınımı ve inflammazom aktivasyonu üzerindeki etkisinin üst mekanizmalarla olduğunu ileri sürmüştür.

Palmitat gibi doymuş yağ asidleri ayrıca endoplasmic reticulum (ER) stresi uyarmakta ve bu etki palmitoleat ile engellenmektedir. ER stress oluştuğu zaman, hücreler bu stresi katlanmamış protein yanıtını aktive ederek çözmeye calışırlar. Protein KinazR'yi anımsatan kinaz (PERK), inositol-gerektiren enzim-1 (IRE1) ve aktifleştiren transkripsiyon faktör 6'dır (ATF6). Bu tezde, PERK ve IRE1'in palmitatin neden olduğu inflammazom aktivasyonuna katkıları araştırılmıştır. İlk olarak, PERK ve IRE1 ile palmitatin uyardığı mtROT arasındaki ilişki IRE ve PERK eksik fare embriyonik fibroblast hücreleri(MEFs) kullanılarak analiz edildi. Bu çalışmanın sonuçları, PERK'ün mtROT oluşumunun ciddi oranda azalmasına neden olduğu ancak IRE1'in palmitatin uyardığı mtROT'u arttırdığını göstermiştir. Bunların yanında palmitoleat palmitatin uyardığı IRE1'ın otofosforulasyonunu önlemiş ancak palmitatin uyardığı PERK fosforulasyonu üzerinde etki göstermemiştir.

Özet olarak, palmitatin uyardığı inflammazom aktivasyonu palmitoleat tarafından hem birincil hemde ikinci basamakta baskılanabilmektedir. Palmitoleat palmitatin uyardığı

AMPKin fosforulasyonun baskılanmasını engellemte ve böylese bu tekrar aktivasyonuyla mtROT oluşumunu azaltmaktadır. Palmitoleat iyi bilinen bir ER stres uyaranı olan palmitat etkisizleştirmektedir. Ayrıca KPY'ın bir kolu olan IRE1 kolunun mtROT oluşumu lipotoksik durumda düzenleyerek, inflammazom oluşumunu kontrol ettiği gözlenmiştir. Benim çalışmalarımın sonucu, PERK ve IRE1'ın başlattığı KPR'un mtROT üretimin düzenlediği ve bunun sonucu olarak palmitoletin palmitatin neden olduğu inflammasome aktivasyonunu bu alternatif mekanizmayla engelleyebileceğini göstermiştir. Bu bulgular palmitoletin inflmamazomun nedensel rol oynadığı obezite, diabet ve atheroskleroziz gibi hastalıkların tedavisinde trepatik uygulamaları olabileceğini göstermiştir.

Anahtar sözcükler: İnflammasome, Palmitoleat, Palmitat, KPY, ER stres,Atheroskleroziz

ACKNOWLEDGEMENT

I would like to extend my thanks to my my supervisor Assit.Prof.Dr.Ebru Erbay not only for her help and constructive advices during my master studies but also for support and encouragement for my academic life. I am really lucky as I had chance to work with her and I learned a lot from her.

I want to thank Assist.Prof.Dr.Ali Güre and Assoc.Prof.Dr.Sreeparna BANERJEE for being members of my thesis committee andhelpful suggestion for my thesis.

I am very thankful to our group, Özlem Tufanlı, Erdem Murat Terzi, İnci Onat, Buket Gültekin, İsmail Çimen and Begüm Kocatürk, Hamid Syed for their friendship, support and helps during my studies. In addition, I would like to thanks to the former member of our group Büşra Yağabasan for her friendship and support. Also, I want to thanks to our undergraduate students Nil Aygün and Çağla Cömert for helping me during my experiment and their good friendship.

Additionally, I am very thankful to Banu Bayyurt, Sıla Özdemir, Yusuf İsmail Ertuna, Seçil Demirkol, Merve Deniz Abdülselamoğlu for their friendship and moral support. Moreover, I want to also thank to Berna Şentürk, Gülcihan Gülseren.

I would like to extend my thanks to MBG lab groups for allowing me to use their materials and equipments for my studies. Also, thanks to both MBG lab groups and UNAM nanobiotechnology group because they provided supportive and peaceful environment for my research.

I would like to express my gratitude to TUBITAK for financial support with a scholarship during my thesis study. TUBITAK supports my graduate education with BIDEB 2210 program.

I also wish to thank my friends Kübra Narcı, Alişan Kayabölen, Ceren Garip, Elif Arslan, Didem Mumcuoğlu, Yasin Tümtaş, Öncay Yaşa, Berke Gürkan, for their great friendship.

Finally, I would like to express my special thanks to my family due to their infinite love, support and helps. They always believe and encourage me and their support gives me power to my academic life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. Introduction

1.1 Inflammasome

The innate immune system is a complex system that has evolvedfor sensing a variety of pathogen and host derived signals called pathogen-associated molecular patterns (PAMPs) or endogenousdanger-associated molecular patterns (DAMPs) through a number of pattern-recognition receptors including Toll-like, the nucleotide-binding oligomerization domain receptors (Nod-like) and retinoic acid-inducible gene 1 **(**RIG-I) like receptors[1].One of the key role players in this system is the inflammasome. The inflammasome is a cytosolic protein complex that is formed by inflammasome sensor molecules that are a number of pattern recognition receptor (PRR) proteins including the nucleotide-binding domain and leucine-rich repeat containing proteins(NLRs) orthe IFI200 family member absent in melanoma 2 (AIM2), the adaptor protein ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD)and caspase-1[2]. In addition to them,caspase-5 also participates in the formation of one type of inflammasome known as NLR family, pyrin domain containing 1(NLRP1)[3].NLR proteins have three main domains consisting of a C terminal leucine-rich repeat (LRR) domain, an intermediate nucleotide binding and oligomerization domain (NOD), which is also called as neuronal apoptosis inhibitor protein (NACHT) domain, and an N-terminal pyrin (PYD), caspase activation and recruitment domain (CARD) or baculovirus inhibitor of apoptosis repeat domain (BIR)[4]. The LRR domain has a role in ligand sensing and autoregulation of NLR proteins but the exact mechanism is not known. Besides, NOD domain oligomerization is a critical step for the formation of active form of inflammasome complex. The N-terminal effectors domain can be CARD or pyrin domain (PYD) according to the NLR type. This N-terminal effectors domain is required for activation of inflammatory caspases

orNF-κB by the inflammasome[2].Recognition of the pathogen or any other danger signals by the inflammasome leads to the assembly of inflammasome complexes which ultimately provides maturation and secretion of interleukin-1 (IL-1) family proinflammatory cytokines, interleukin-1β (IL-1β) and interleukin-18 (IL-18)[5]. NF-κB activates the transcription of the pro- (or inactive) forms of these ILs, but for full activation posttranslational regulation is required. For example, the maturation of interleukin 1 family factors occurs upon processing by active caspase-1 protein[3].Caspase-1 is produced as a zymogen and upon activation its aspartic residues are exposed to autoproteolytic cleavage, which subsequently gives rise to 20 kDa(p20) and 10 kDa (p10) subunits of caspase-1. These then come together as two heterodimers of p20 and p10. As such the activated caspase-1 interacts with ASC and has a role in the formation of the active inflammasome complex[6]. The activation of inflammasome results in inflammatory programmed cell death that is called pyroptosis. Unlike apoptosis, pyroptosis needs caspase-1 activity and it results in inflammation[7].

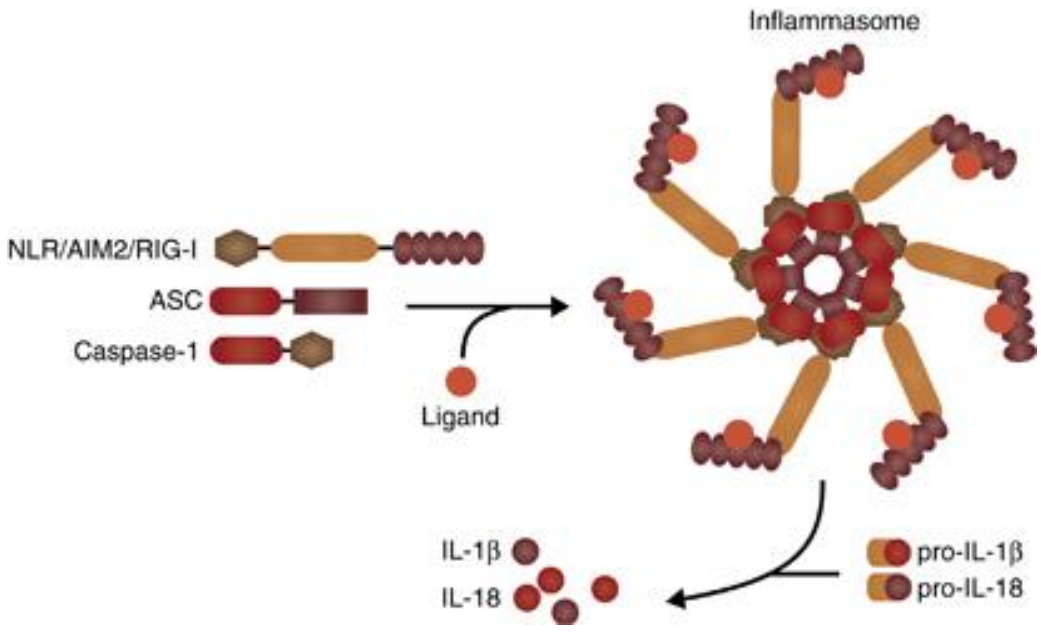


Figure 1.1. The Inflammasome Structure

The inflammasome is a multiprotein complex and it is important part of the innate immune response. The inflammasome complex is consist of mainly three parts which are adaptor protein ASC, NLRs or AIM2 and caspase-1.When inflammasome complex is activated, it provides cleavage and activation of

caspase-1 which promotes posttranslational modification of IL-1β and IL-18. This modificated forms of IL-1β and IL-18 are active and they are secreted from cells and cause inflammation. (The inflammasome: in memory of Dr. Jurg Tschopp. *Cell DeathDiffer* 19: 5–12, 2012[8]. Reprinted with permission from Dagenais M, Skeldon A, Saleh M. (2012).

1.2 Types of Inflammasome

Until now, mainly four types of inflammasome have been identified. They are the NLRP1/NALP1b inflammasome[9], the NLRP3/NALP3 inflammasome[10], the NLRC4/IPAF inflammasome[11], and the AIM2 (absent in melanoma 2) inflammasome[12].

Unique to this type of inflammasome, NLRP1 inflammasome contains additional C-terminal CARD domains that mediate the recruitment of caspase-5 or a second caspase-1 to the inflammasome complex[3,13]. The best-known inducer of NLRP1 inflammasome is the bacterial peptidolygan derivative muramyl dipeptide (MDP). Another inducer known to activate the mouse NLRP1 is the anthrax lethal toxin produced by *Bacillus anthracis*. The regulation of NLRP1 activation is additionally mediated by anti-apoptotic proteins Bcl-2 and Bcl-x(L). In the resting cells, these proteins bind and suppress the activation of NLRP1[14].

NLRC4 inflammasome does not have PYD domain to activate pro-caspase-1 to caspase-1 directly.The ASC part is required for this caspase-1 activation by PYD domain, but the main role of ASC for NLRC4 activation is not known yet. Among the inducers of NLRC4 inflammasome is the bacterial flagellar protein flagellin[15]. Through engaging this flagellin protein the inflammasome is able tomediate the recognition of *Salmonella typhimurium*and *Shigella flexneri* [2].

Finally, the NLRP3 inflammasome is the best studied inflammasome. It controls the activation of pro-caspase-1 upon interaction with ASC. NLRP3 has a broad range of activators containing extracellular ATP[15], gout-associated uric acid crystals[10], cholesterol

crystals[17], free fatty acids[18] and potassium efflux[19]. This broad range of activators of NLRP3 suggests that the activation of this inflammasome is not directly by the sensing of these activators.Generally these activators result in the production of reactive oxygen species (ROS) that leads to the activation of NLRP3, so this suggests that ROS may be the common activator of NLRP3. Further studies are needed to clarify the identity of general NLRP3 activator[20].

AIM2 is not a member of NLR proteins and it was originally identified as tumor suppressor gene in melanoma[21]. It has an N-terminal pyrin domain and a C-terminal hemopoietic expression, interferon-inducibility, nuclear localization(HIN) 200 domain. For activation, pyrin domain of ASC interacts with pyrin domain of ASC and also with caspase-1 to form the complex. The main activator of AIM2 inflammasome is cytosolic bacterial or viral double stranded DNA in the cells[12].

1.3 The Two Signals Required for The Activation of NLRP3 Inflammasome

Given the drastic consequences of erroneous activation of the inflammasome, this process is tightly controlled.This tight regulation is provided by a two signal requirements in the cells. The first signal (signal 1), also called the priming step,is required for the expression ofproinflammatory factors proIL-1β and pro-IL-18 and NLRP3 through the activation of NF-κB by PRR, cytokine receptors or other factors that can activate the NF-κB pathway[22]. For example, lipopolysaccaride (LPS) promotes the activation of TLR4 pathway by the myeloid differentiation primary response gene 88 (MyD88) pathway[23] .This step is important because in the resting cells NLRP3 level limits the activation of inflammasome. The second signal is required for the activation of caspase-1 and formation of the inflammasome complex[22]. The activation pattern of NLRP3 is a hot topic and the so far known activators include reactive oxygen species(ROS), ion flux and lysosomal destabilization[5]. However, all NLRP3 activators result in ROS production, the role of ROS for formation of inflammasome complex

is more general. Besides, sequestration of ROS with chemical scavengers prevents inflammasome activation by some of the mentioned activators[24-25].The source of ROS for activation of inflammasome is still debated. According to Dostert et al. the source of ROS for activation of inflammasome are phagosome-associated NADPH oxidases, however, another study has shown that despite the deficiency in the subunits of four different NADPH oxidases (NOX1, NOX2, NOX3, NOX4) the inflammasome complex could still be activated[26-27]. This demonstrates there must be another ROS source that can activate the inflammasome independent of the NADPH oxidase activity. Recent studies have indicated that the main source of ROS for activation of NLRP3 inflammasome is mitochondrial ROS[18,28]. During in vivo activation of NLRP3 inflammasome, signal 1 and signal 2 may be separate or joined into a common molecular event that behaves as two signals. In line with the latter thought, Sheedy et al. demonstrated that cluster of differentiation 36 (CD36)plays a role in both the priming and the activation of NLRP3 inflammasome in atherosclerosis. The recognition of oxidized low density lipoprotein (oxLDL)by CD36 provides both signals for NLRP3 activation by activating TLRs and NLRs[29]. CD36 is one of the member of B scavenger receptor family and it can bind a variety of ligands such as oxidized low density lipoprotein, long chain fatty acids and collagen[30-32].Several studies indicated that CD36 is involved in pathologies of other complex disease such as diabetes, atherosclerosis and Alzheimer disease[33].

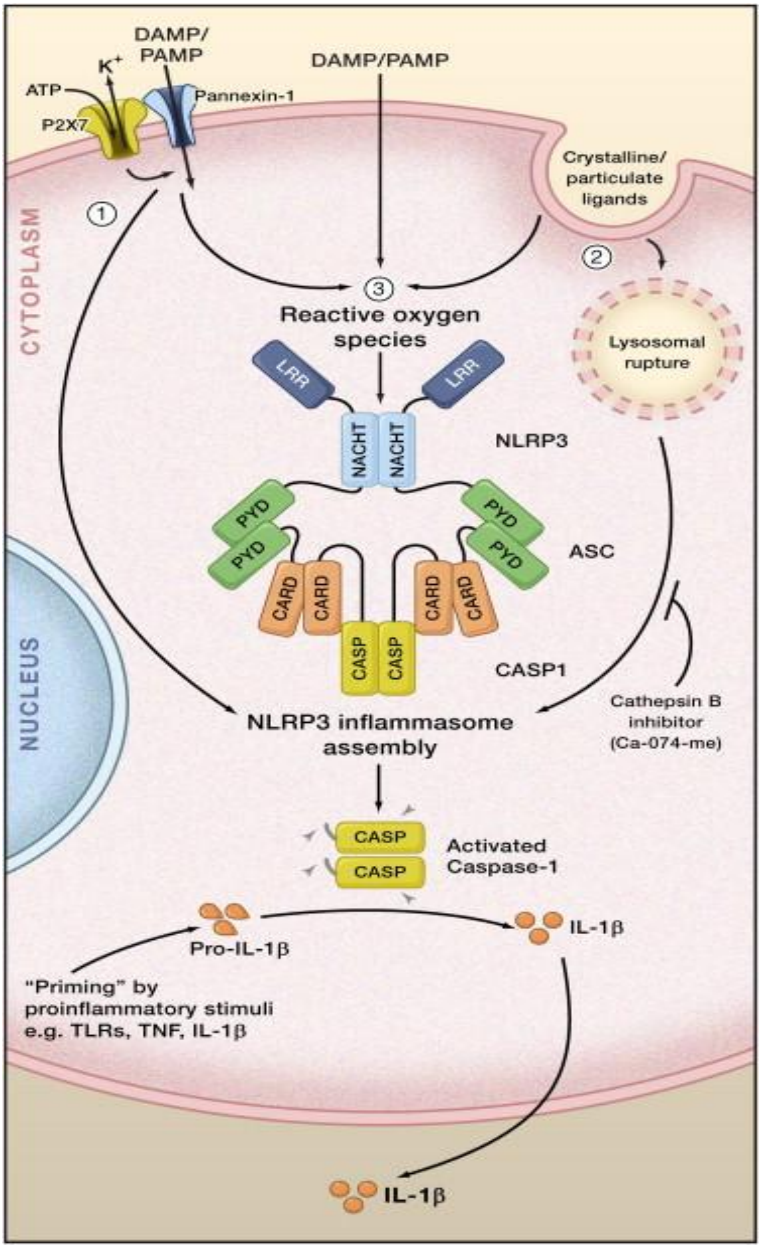


Figure 1.2 The Requirement of Two Signals for NLRP3 Inflammasome Activation

For the activation of NLRP3 inflammasome, two signals are required. The first signal is called the priming signal and is needed for the activation of NF-κB and subsequent transcription of interleukin-1β and interleukin-18. The second signal is required for the formation of the inflammasome complex, activation of caspase-1 and posttranslational processing of interleukin-1β and interleukin-18. Some of the known second signals include reactive oxygen species (ROS), ion flux and lysosomal destabilization. (reprinted with permission from Schroder K, and Tschopp J. (2010). The inflammasomes.*Cell* 140: 821-832.).

1.4 The Role of Inflammasome in Metabolic Disorders

In recent years, metabolic disorders such as obesity, atherosclerosis,non-alcoholic fatty liver disease and type-2 diabetes (T2DM) have increased drastically in both the developed and under developed countries. One important feature of these metabolic disorders is chronic, low grade inflammation, particularly in the metabolically active tissues[34-35].IL-1β and IL-18 are among the proinflammatory factors that rise in these diseases and contribute to their pathogenesis and complications. These pro-inflammatory factors result in fever, stimulation of other secondary cytokines' production and the recruitment and activation of immune cells[36]. The role of these two cytokines in the pathogenesis of metabolic disorders has been documented earlier. In type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus IL-1β has a role in inflammation-induced β cell destruction[37-38]. IL-1β inhibits insulin signaling by direct serine phosphorylation of the insulin receptor substrate-1 and also causes an increase in the amount of tumor necrosis-factor-α.An increase in the activation of the NLRP3 inflammasome in the adipose and liver tissue of the obese mice was observed and the expression rise in the main components of NLRP3 inflammasome correlated with the severity of the type 2 diabetes mellitus (T2DM) in the obese individuals[39]. In addition, NLRP3 activation was observed in the adipose tissue infiltrating macrophages, which were stimulated with saturated free fatty acids through disruption in autophagy mechanism and increase in mitochondrial ROS production. Furthermore, it was shown the activation of NLRP3 inflammasome interfered with insulin signaling[18]. Also, the accumulation of amyloid polypeptide(IAPP)in pancreatic islets activates NLRP3 in T2DM[40].In vivo studies show that Nlrp3−/−, Asc−/− and Casp1−/− mice display reduced body weight and fat mass, and also, their insulin resistance levels decrease. Moreover, these mice are also resistant to obesity that is induced by diet[41].

Another disease causally related to the activation of NLRP3 inflammasome is atherosclerosis. Atherosclerosis is a chronic inflammatory disease where lipid depositsform plaques and recruit immune cells to atherosclerotic lesions[42]. There are several endogenous inducers of atherosclerosis such as elevated serum glucose, free fatty acids and oxidized LDL. These stressors are known to cause increased production of pro-inflammatory cytokines and contribute the inflammatory state of atherosclerosis[43].In human atherosclerotic plaques, IL-1β and IL-18 are found highly expressed in macrophages[44-45].The cross of Apolipoprotein E-deficient ($ApoE^{-/-}$)mice, a mouse model of atherosclerosis,is crossed with a mouse model of genetic-deficiency for IL-1β lead to reduction of atherosclerosiswhile the genetic deficiency for IL-18 also provides protective effect to atherosclerosis[46-47].Whenmice are deficient in IL-1-receptor, they are more resistant to atherosclerosis, strongly supporting a causal role for inflammasomes inatherosclerosis[48].A direct role of inflammasome in atherosclerosis has been established by showing the role of components of inflammasome such as caspase-1, ASC and NLRP3 in atherosclerosis progression by using knock out mice.The ruptured plaque from patients with sudden coronary death display high level of activated caspase-1[49].It was also shown that cholesterol crystals accumulate in arterial vessels and activate NLRP3 in the recruited macrophages.Also, reconstitution of the LDL receptor $^{-/-}$ mice with bone marrow from mice which are deficient for various inflammasome components such as NLRP3, ASC and IL-1α/βreduced the development of atherosclerosis. In the vitro part of the same study, it was observed that cholesterol crystal-activated NLRP3 inflammasome could be suppressed by cytochalasinD and bafilomycin treatment, suggesting activation of inflammasome in this scenerios is involves phagocytosis and lysosome acidification[50]. Therefore, it is observed that cholesterol activates inflammasome through lysosomal damage.

1.5 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease and lesions are characterized with lipid deposits,innate immune cell infiltration and vascular smooth muscle cells (VSMC) proliferation in the vessel[42].

Atherosclerosis has a very complex pathogenesis. The first step in this disease pathogenesis is endothelial dysfunction[52]. The increased amount of cholesterol transported with apolipoprotein B100 (Apo-B100) containing low density lipoprotein (LDL)in circulation potentiates endothelial dysfunction at vascular sites experiencing hemodynamic sheer stress, such as arterial branch points, and leads to atherosclerosis formation[53]. The lipoproteins that accumulate on the arterial wall are prone to various modifications such as oxidation by reacting with ROS, enzymatic reaction like lipoxygenases and non-enzymatic cleavage[51]. These modified lipoproteins, especially oxidized LDL (oxLDL) promotes proinflammatory response and activation of endothelial cells. This activation results in the secretion of chemoattractants from endothelial cells. This immune response continues with intimal immune cell infiltration. The monocytes are recruited into subendothelial space. These monocytes originate from the bone marrow-derived progenitors and their differentiation is determined by the cholesterol content of the lesion[54]. Studies indicate that a distruption in cholesterol efflux, such as through a deficiency in transporters ATP-binding cassette transporter (ABCA1) and ATP-binding cassette sub-family G member 1 (ABCG1), can increase atherosclerosis in mice [55]. The monocytes are differentiated to macrophages by the action of macrophage colony stimulating factor (M-CSF). These macrophages uptake lipids and they transform into foam cells[54]. This lipid uptake occurs by scavenger receptors such as CD36; type A scavenger receptors and one member of the type B scavenger receptors[56]. However, the studies in mice show that also the absence of the lipoprotein clearing protein such as ApoE is an important factor in foam cell formation[57].The disruption in the lipid

metabolism changes macrophage characteristic and promotes a pro-inflammatory phenotype. Macrophages lose their migration capacity and this increases inflamation. The accumulated macrophages in the atherosclerosis plaques promote lesions to more complex and advanced plaques. At this level, other immune cells such as dendritic cells and vascular smooth muscle cells also take part in inflammation[58]. Macrophages cause further inflammatory response via secreting proinflammatory factors and proteases and finally cell deaths can occur by either necrosis or apoptosis. After macrophages die, cellular debris causes the formation of a necrotic core. The necrotic core forms the main part of unstable plaques and is shielded from the circulation by a fibrous cap that contains collagen and smooth muscle cells. Rupture of the fibrous cap can cause thrombotic events leading to myocardial infranction and stroke[59].

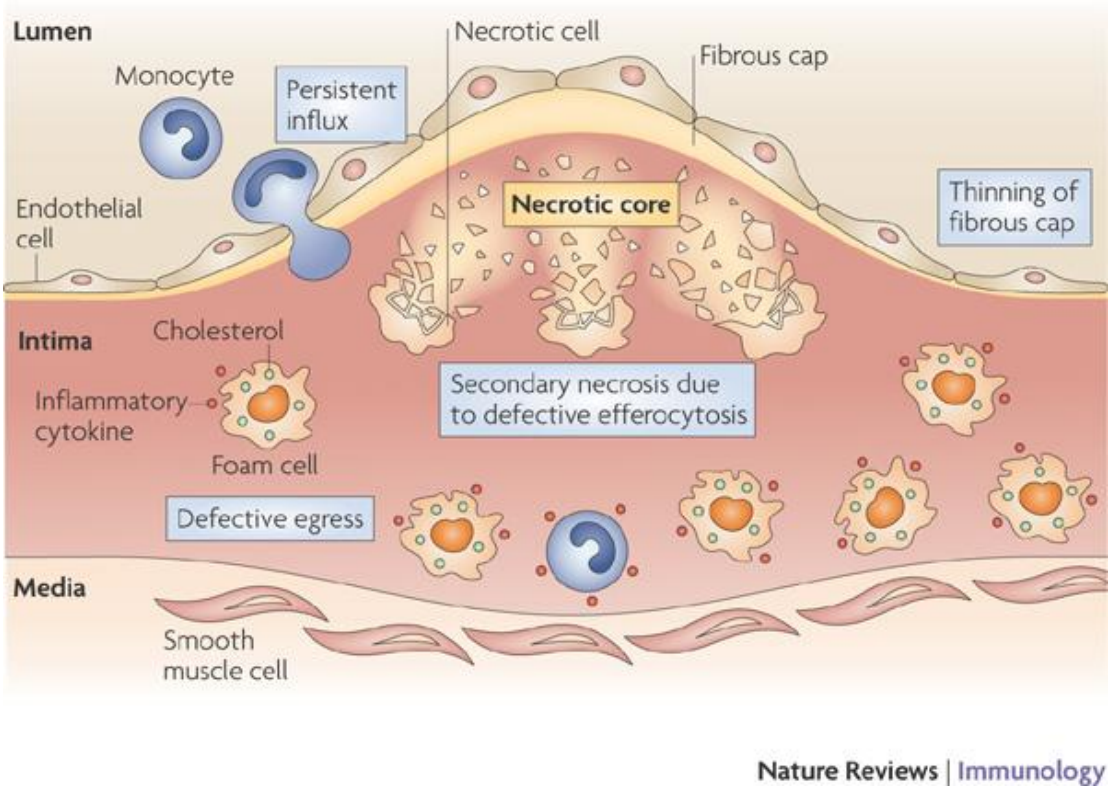


Figure 1.3 The Molecular Mechanism of Atherosclerosis

Atherosclerosis is characterized with chronic inflammation and starts with the accumulation of lipids and activation of endothelial cells. Activated endotheial cells secrete chemoattractants and cause the

accumulation of monocytes. Monocytes scavange the lipids, transform the cholesterol-filled foam cells and cause more inflammation. Cell death, formation of the necrotic core, accumulation of collagen and vascular smooth muscle in a fibrous cap follow. (Reprinted from Tabas I.(2010). Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol*. 2010;10:36–46.[60])

1.6 Endoplasmic Reticulum Homeostasis and Stress

The endoplasmic reticulum (ER) is one of the organelle which is formed by an interconnected network of flattened, membrane-enclosed sacs cisternae and microtubules[61]. According to its various stuctures, ER is classified into the ribosome-studded rough endoplasmic reticulum (RER) and the ribosome-free smooth endoplasmic reticulum (SER)[62].This organelle has a broad range of activities in the cells.The main role of ER is protein folding and controlling of quality of newly synthesized proteins[61]. In addition, ER plays also a role in synthesis of lipids and deposition of intracellular calcium (Ca^{2+})[63-64].Moreover, it is involved in the biogenesis of autophagosomes and peroxisomes. Disruption in any of these functions such as in cellular energy levels or Ca^{2+} concentration causes decrease in protein folding capacity and results in ER stress[65]. Cells activate an adaptive mechanism that is known as unfolded protein response(UPR) to cope with the decrease in protein folding capacity.The activation of UPR fights with stress that is caused by the accumulation of unfolded protein through several pro-survival mechanisms. These mechanisms are consist of attenuation of translation, selective production of key components that regulate protein folding and controlling of quality of newly synthesized proteins,expanding the ER membrane and elimination of the unfolded proteins by activation of endoplasmic-reticulum-associated protein degradation(ERAD).However, if the ER stress is not solved and homeostasis does not return to normal, the UPR leads to apoptosis[66].

The UPR has three branches each initated by ER transmembrane proteins including the protein kinase R-like ER kinase (PERK), inositolrequiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). In resting cells, chaperone Bip binds to ER luminal domain of these proteins and keeps them inactive[67].The inositol-requiring enzyme-1 (IRE1) is a transmembrane protein localized in the ER, which has several domains: an N-terminal luminal sensor domain, a single transmembrane domain and a C-terminal cytosolic effector region that contains both kinase and endoribonuclease activities[68].Under ER stress, IRE oligomerization and transphosphorylation is observed[69].After oligomerization, endoribonuclease (RNase) function is activated and cleaves X-box binding protein (XBP1)mRNA in mammalian cells and Hac1 mRNA in yeasts. This cleavage converts the transcription factor XBP1s to active form[67]. The target of IRE is not only XBP1, but it also regulates its expression by cleavage of own mRNA[70].The activation of IRE1 induces activation of several signaling pathways.Firstly, the cleavage and activation of XBP1 by IRE1 is fundamental to cells due to XBP1 being a transactivator with important role in the regulation of ER protein synthesis, folding, lipid biogenesis, glycosylation, ERAD, autopahgy, redox metabolism, and vesicular trafficking[69]. Second, C-Jun N-terminal kinase (JNK)is activated by IRE1 with tumor necrosis factor (TNF) receptor-associated adapter protein TRAF2[71].JNK has several roles in the cells such as regulation of cell death, and by activating c-Jun, promotion of survival[72-73]. IRE1 also activates nuclear kappa-light-chain-enhancer of activated B cells (NF-κB)[74].PERK is another ER transmembrane and kinase protein.Under ER stress conditions, it is activated via oligomerization and autophosphrylation.Then PERK phosphorylates and inhibits translation initiation factor eIF2alpha (eIF2α) mRNA translation[75].On the other hand, the translation of some mRNAs such as the mRNA for transcription factor, activating transcription factor 4 (ATF4)increasesthrough an open reading frame (ORF) in their 5′-untranslated region[76].ATF4 regulates C/EBP homologous protein (CHOP)gene that is an ER stress-induced proapoptotic

factor and GADD34 (growth arrest and DNA damage–inducible 34) that dephosphorylates eIF2α[68].Another important role of PERK is the phosphorylation of nuclear factor-like-2 (Nrf2)transcription factor that then dissociates fromnuclear factor-like-2/Kelch-like ECH-associated protein 1 (Nrf2/Keap1)complex, leading to its translocation into the nucleus[77]. ATF6 is the third and only arm of UPR that is regulated by a transcription factor. ATF6 is produced firstly as an ER-resident transmembrane protein[68]. Under ER stress, ATF6 translocates to the Golgi apparatus from the ER. There, it is cleaved by two proteases, site-1(S1P) and site-2 (S2P)proteases[78].Then the N-terminal fragment of ATF6 is localized to the nucleus and induces transcription of UPR genes, like CHOP, the chaperones BiP/GRP78 andheat shock protein 90kDa beta member 1 (GRP94) and XBP1[79-81]. The mechanism of how ATF6 is activated under ER stress is not well understood, however, this factor is bound to BiP and BiP release under ER stress may lead to ATF6 activation[68].

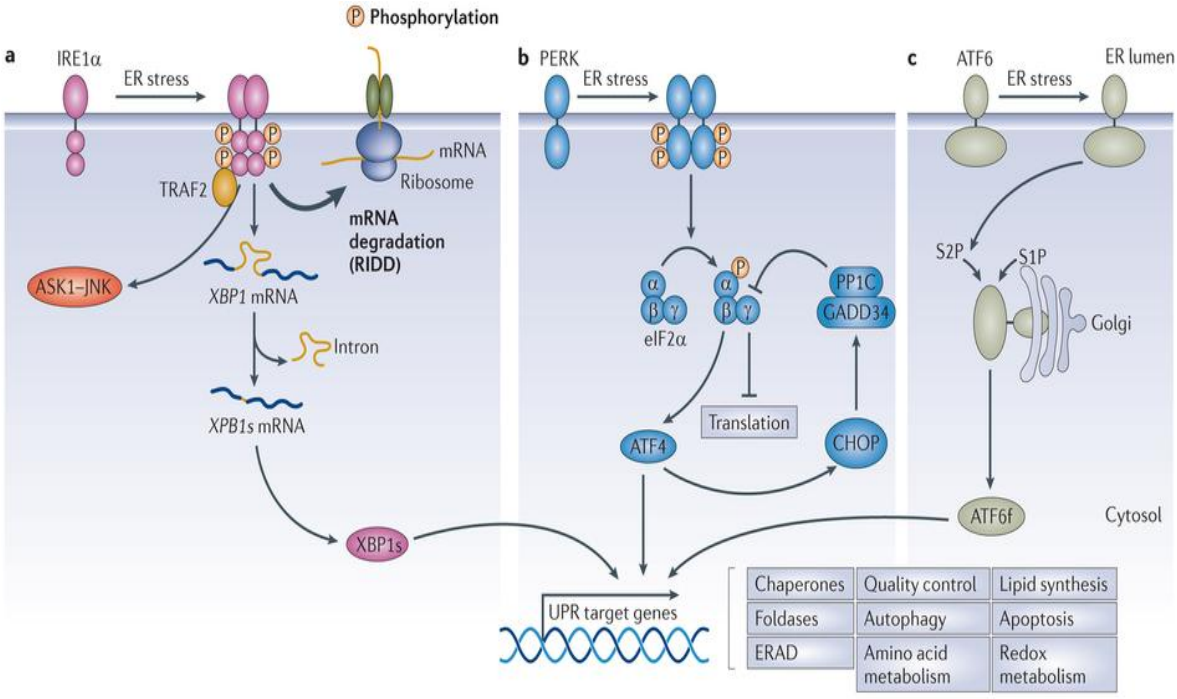


Figure 1.4The Arms of Unfolded Protein Response (UPR)

Unfolded Protein Response (UPR) has three main arms that are PERK (PKR resemble ER kinase), IRE1 (Inositol requiring enzyme 1) and ATF6 (Activating Transcription Factor 6). Under ER stress, these there arms of UPR are activated to cope with stress conditions. The activation of IRE1 and PERK occur with autophosphrylation while ATF6 is activated by cleavage with S1P and S2P proteases. The activated IRE1 provides splicing of XBP1 mRNA with its RNAse domain and promotes its translation. This regulated XBP1 protein upregulates chaperones gene expression via acting as transcription factors. Activated PERK phosphorylates eIF2alpha and promotes inhibition of translation and specificly activation of ATF4 mRNA. Activated ATF6 upregulates UPR target genes by acting as transcription factors.(reprinted from Hetz C, Chevet E, Harding HP. (2013). Targeting the unfolded protein response in disease.*Nat. Rev. Drug Discov.*, 12 (2013), pp. 703–719[82].)

1.7 The Unfolded Protein Response and Apoptosis

UPR tries to resolve the protein unfolding and aggreagaration problems but if UPR does not resolve these, apoptotic pathway is activated. This cell death can be mitochondria-dependent or mitochondria independent under ER stress. There are several different apoptotic signals which are raised by ER.

The first mechanism is proapoptotic transcription factor CHOP that is induced by PERK/eIF2 or ATF6[83]. CHOP/GADD153 is a Bzip transcription factor[68]. CHOP provides activation of several genes transcription containing Gadd34, Ero1, *Trb3* (Tribbles homolog 3), *Dr5* (death receptor 5), and carbonic anhydrase VI.[84] These expressed genes differently affect pathways and activate apoptosis. To illustrate, expressed Gadd34 causes dephosphrylation of eIF2 and so increase in protein synthesis. This results in chronic activation of UPR and causes apoptosis[85]. On the other hand, Dr5 may result in activation of caspase cascades[86]. Another important role of CHOP is decreasing expression of antiapoptotic BCL2 protein and both increasing expression of genes that has role in activating apotosis and decreasing antiapoptotic gene, CHOP activation promotes apoptosis[87].The second mechanism is BCL2

induced apoptosis under ER stress. BCL-2 can activate caspase-12 by translocation of BIM (BCL2- interacting mediator of cell death) to ER membrane under ER stress while BCL-xL (BCL2-like 1) inhibits binding of BIM and prevent entrance to apoptotic pathway[88].The third mechanism is IRE1 regulated cell death pathway. The interaction of IRE1 with TRAF2 promotes phosphorylation and activation of JNK and stress-activated protein kinase (SAPK) by mitogen-activated protein kinase kinase kinase[89]. IRE1-TRAF2 may also activate caspase-12 and ATF3 and so promotes apoptosis[84]. The final mechanism is caspase-mediated cell death under ER stress. The different members of caspase family such as caspase 2,3, 9 and 12 are involved in cell death is known[90-92].Caspase-12 is found as associated with ER membrane and under ER stress, it is activated by calpain and also BAX and BAK[84,90]. After this activation for enterence of apoptotic pathway, caspase-12 activates caspase-9 and then caspase-3 [93].

1.8 ER Stress and the Formation of Reactive Oxygen Species

Generation and accumulation of ROS in the cells is important cause of inflammation and this is called as oxidative stress[94].In the protein folding process in ER, oxidizing conditions are needed to formation of disulphide bonds[95]. For electron transport for formation of these bonds, there are two ER enzymes which are protein disulphide isomerase (PDI) and ER oxidoreductin 1 (ERO1). The role of PDI is accepting electrons directly and forming disulfide bonds via oxidation of cysteine residues while ERO1 provides transformation of electrons from PDI to oxygen. The usage of oxygen as a final terminal electron recipient results in formation of ROS[96]. In addition to them, decrease in reduced gluathione is also another contributor for production of ROS[97]. The increase in ROS under protein load also causes inflammation. To prevent accumulation of ROS, cells improve mechanisms. One of them is PERK arm of UPR which may activate antioxidant mechanism by activating expression of the

bZIP-containing transcription factor ATF4 and by phosphorylating NRF2 (nuclear factor-erythroid-derived 2 (NF-E2)-related factor 2)[96]. Phosphorylated NRF2 translocates to nucleus and promotes activation of expression of several antioxidant and oxidantdetoxifying enzymes such as glutathione *S*-transferase[98]. In addition to them, PERK antioxidant role is proved by research that indicates tunicamycin, a well known ER stress inducer promoted ROS is more in PERK lack cells when comparing wild type cells[99]. As a result, production of ROS is directly related with ER stressandone arm of UPR that is PERKhas a protective role on regulation of ROS production.

1.9 Endoplasmic Reticulum Stress and Inflammasome

Up to now, there is not enough information between ER stress and inflammasome formation. However, recent studies gave some clues about their relationship. From previous studies, it is known that reactive oxygen species (ROS) have a serious role in activation of NLRP3 inflammasome[18,100].It was indicated that the thioredoxin (TRX)-interacting protein (TXNIP) is a NLRP3 ligand and it is ROS sensitive[101].Under stress condition, by production of ROS, TXNIP dissociates from TRX and TXNIP binds NLRP3 from LRR and NACHT domains and provides processing of IL-1β [100,102].In a recent study, two arms of UPR that are PERK and IRE pathways have role in regulation of TXNIP expression. PERK regulates it at transcription control level while IRE1 regulates it at the posttranscriptional level[103].Also another study supports that both PERK and IRE1αare requirement for TXNIP induction in ER stress condition and this induction is important for production of IL-1β and beta cell death[104].In addition to them, a recent study shows that endoplasmic reticulum stress is an activator factor for NF-KB and provides production of IL-1β via this pathways[105].Previously it is known that UPR signaling pathway activate NFκB pathway andso it is predicted results[106]. Also, another study shows that ER stress behaves as a second signal that is needed for NLRP3

inflammasome activation in macrophages that are primed with LPS or phorbol esters. In this study, it is suggested that NLRP3 activation occurs independently from classic unfolded protein response (UPR) consists of PERK, IRE1 and ATF6 and it proposes that ER stress downstream part could be involved in NLRP3 activation[107]. In addition to them, another important kinase which is Protein Kinase R is important for activation of inflammasome. PKR is not direct arm of UPR but it is observed that under ER stress, PKR is also activated by well known ER stress inducers that are thapsigargin and palmitate[108]. In the Lu et al. study,the role of PKR in inflammasome activation is identified.In this study, it is observed that inhibition of PKR activity with different methods such as using PKR inhibitors or shRNA results in reduction of the secreted IL-1β, IL-18 and HMGB1. Moreover, direct interaction between different infammasome type and PKR is detected[109]. In summary, the important role of PKR in inflammasome activation is obvious.However, for understanding UPR and inflammasome activation, these researches are not enough and further investigation on ER stress and inflammasome relationship is needed.

1.10 The Monounsaturated Fatty Acids and Their Role in Health

Monounsaturated fatty acids(MUFA) contain a single double bond between carbon atoms on the fatty acid backbone. On the other hand, polyunsaturated fatty acids have two or more double bonds while saturated fatty acids do not have any double bonds.The geometric configurations of MUFA are identified by the location of the hydrogen atoms around the double bond. If the hydrogen atoms are on the same side, this is called as cis-MUFA while if the hyrogen atoms are on the opposite site, they are called as trans-MUFA[110]. The most common dietary MUFA is oleic acid (OLA; 18:1n-9), which is a cis MUFA[111]. On the other hand, the most common trans-MUFA in diet is elaidic acid (trans18:1n-9)[112].

A broad range of studies shows that saturated fatty acids(SFA) have deleterious effects on health such as perturbation ofglycemic control, insulin sensitivity and they contribute to obesity[113-114]. Therefore, the replacement of SFA with MUFA in diet is beneficial.The studies indicate that MUFA has a variety of beneficial effects on health, especially on type-2 diabetes and cardiovascular diseases[111-115]. Moreover, the replacement of SFA with MUFA leads to maintaining body mass and fat mass at a constant level and eliminating weight gain in healthy subjects[116]. To illustrate more specifically, providing oleic acid is in diet leads to decrease in low density lipoprotein (LDL) cholesterol and increase in the beneficial, high density liporpotein (HDL).In addition, it is known that oleic acids decreases DNA oxidation and ROS. This ability is important for protection from different type of cancers[117]. It has been shown that MUFA has a preventive effect on metabolic disease and cardiovascular disease via decreasing blood pressure and increasing insulin sensitivity. Whether MUFAs should preferentially replace SFA instead of polyunsaturated fatty acids (PUFA) is important question.

1.10.1Palmitoleate, a Bioactive Lipokine

Palmitoleic acid (C16:1) is an omega-7 monounsaturated fatty acid and it is found in different sources such as plant and marine species[118-119]. A recent study has shown that palmitoleic acid behaves as an adipose tissue-derivedlipid hormone or lipokine to communicate between the adipose tissue and other distant organs in order to maintain metabolichomeostasis.In the same study, palmitoleate raised insulin response of muscle and suppressed hepatosteatosis [120].Unlike saturated fatty acids that can stimulate insulin resistance, palmitoleate has been shown to increase insulin sensitivity in both mice and human[121-122]. Another interesting result shows that antidiabetic thiazolidinediones increases suppression of a stearoyl- CoA desaturase 1 (SCD 1) gene in adipose tissue, a rate limiting enzyme in palmitoleate production[123].In

addition to the effect of palmitoleate on insulin sensitivity, research in human and mouse cells showed that palmitoleate can also reduce endoplasmic reticulum stress and protect cells from apoptosis[122,124-127].

2. OBJECTIVES AND RATIONALES

Palmitoleic acid (C16:1) is an omega-7 monounsaturated fatty acid that was shown to have a beneficial role in several human diseases[118]. Palmitoleic acid behaves as an adipose tissue-derived lipid hormone or lipokine that mediates communication between the adipose tissue and other organs that are important for metabolic homeostasis[120].In mouse studies, it was observed that palmitoleate can raise insulin sensitivity[120-121].Increased palmitoleate levels have been associated with insulin sensitivity aswell[120-122].Moreover, palmitoleate could suppress hepatostetosis in another study conducted in mice[120-121]. In addition, palmitoleate has been shown to reduce endoplasmic reticulum stress and protect cells from apoptosis[124-127]. Although these beneficial effects may promote a healthier life, further investigation is required to understand the exact mechanism of action of palmitoleate in preventing complex and chronic metabolic and inflammatory diseases. Recent studies suggest that NLRP3 inflammasome activation and IL-1 family processing by the inflammasome complex isimportant for the pathogenesis of metabolic disorders such as obesity, type-2 diabetes and atherosclerosis[39,41,50].It is known that free fatty acids such as palmitate in diet are important contributorsto cardiovascular problems. Excess fatty acids contribute to disease pathogenesis in part by leading to lipid accumulation, endoplamic reticulum stress and chronic inflammation[128-130]. A study by Wen, H., et al has shown that palmitate is an activator of NLRP3 inflammasome via increasing mitochondrial reactive oxygen species production and stopping autophagy activation in murine macrophages[18].

In this thesis study, I set forth to understand the impact of the bioactive lipokine,palmitoleate(PAO), on palmitate(PA)-induced inflammasome activation. Furthermore, I carried out mechanistic studies that could explain PAO's effect. Towards this

end I investigated how PAO modulate PA-induced mitochondrial reactive oxygen species (mtROS) in both LPS primed human macrophage cell line, THP1, and in primary bone marrow derived mouse macrophages. The outcome of my studies showed that PAO has a suppressive effect on the inflammasome at both the priming step and the second step, via reducing mtROS.The first mechanism that was investigated in relation to PAO's role in regulating inflammasome activation in involved the inactivation of AMPK via phosphorylation because AMPK plays an important role in mtROS production by controlling cellular autophagic response, which can turn over defective mitochondria and reduce mtROS levels in the cell. A seond mechanism that was investigated in this thesis in relation to PAO's impact on inflammasome activation involved the UPR- mediated control of inflammasome activity. Since PAO is known to regulate the UPR and ER stress and UPR, particularly the IRE1 arm, has been shown to play a role in inflammasome activation I also analyzed that the possible contributons of two UPR arms (namely IRE1 and PERK branches) on PA-induced inflammasome activation.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 General Laboratory Reagents

In this research, the reagents used were purchased from several different companies such as Fisher Scientific (NJ, US),AppliChem (Dermstadt, Germany), Sigma-Aldrich (St.Louis, MO, USA), Merck (NJ, USA), Amresco (Ohio, US). Ethanol, Methanol, Isopropanol, Bradford, Trizma Base and Sodium Chloride were got from Sigma-Aldrich (St.Louis, MO, USA). High Grade Pure Water was from HyClone (Rockford, USA). HEPES and Tween were bought from Amresco (Ohio, US). 40% Acrylamide Reagent was bought from Fisher BioReagents (Fisher Scientific, NJ, US). Ammonium persulfate was bought from Carlo Erba (Italy). ECL Prime Western Blot Detection Kit was purchased from Amersham Pharmacia Biotech Company (AMERSHAM, UK). Spectra Multicolor Broad Range Protein Ladder was bought from Thermo Scientific (Rockford, USA).Bovine Serum Albumin (BSA) was bought from Santa Cruz Biotechnology (Dallas, USA).

3.1.2 Cell Culture Materials and Reagents

Dulbecco's modified Eagle's Medium and Rosswell Park Memorial Institute Medium (RPMI) were obtained from Thermo Scientific HyClone (Rockford, USA). Fetal Bovine Serum (FBS),Trypsin-EDTA were obtained from GIBCO (Invitrogen, Carlsbad, CA, USA) while L-glutamine and Penicillin/Streptomycin solution were obtained from HyClone (Rockford, USA). The cell culture plates, scrapers, dishes and flasks were obtained from Greiner Bio One (Monroe, NC, USA) and Costar Corning Incorporated (NY, USA). Serological Pipettes were obtained from Sarstedt Inc (Newton, NC, USA). DMSO was obtained fromAppliChem (Dermstadt, Germany).

3.1.3 Spectrophotometry, NanoDrop, Polymerase Chain Reaction, Real Time-Polymerase Chain Reaction and Fluorescence Microscope

To determination of RNA amounts, NanoDrop obtained from Thermo Scientific (Wilmington, USA) was used. The concentrations of proteins were measured with spectrophotometer Beckman Du640 (Beckman Instruments Inc., Ca, USA). cDNA synthesis was done by using Techne TC-512.RT-PCR was done by using Light Cycler 480II Roche.

3.1.4 Antibodies

The antibodies used, their catalog number and their usage condition were given the list.

Antibody	**Catalog Number and Company**	**Dilution**	**Incubation Time**
Caspase-1(C20)	sc-515 Santa Cruz Biotechnology	1:1000 for p45 part 1:500 for p10 part	Overnight incubation at 4°C
Caspase-1(M20)	sc-514 Santa Cruz Biotechnology	1:1000 for p45 part 1:500 for p10 part	Overnight incubation at 4°C
Beta Actin	sc-47778 Santa Cruz Biotechnology	1:1000	1 hour at room temperature
Beta Tubulin	(H-235): sc-9104 Santa Cruz Biotechnology	1:1000	1 hour at Room Temperature
Phospho-PERK(Thr980)	3179S	1:1000	Overnight incubation

(16F8)	Cell Signaling		at 4°C
Anti-IRE1 (phospho S724) antibody	ab48187 Abcam	1:1000	1 hour at Room Temperature
Phospho-AMPKα (Thr172)	2531S Cell Signaling	1:1000	Overnight incubation at 4°C
MOMA-2	sc-59332 Santa Cruz Biotechnology	1:500	1 hour at Room Temperature
Alexa 488	ab150157 Abcam	1:200	20 minutes at Room Temperature

Table 3.1: The list of antibodies that were used during this research with their catalog no, working dilution and incubation conditions

3.2 SOLUTIONS AND MEDIA

3.2.1 Cell Culture Solutions

Cell Culture Medium: DMEM and RPMI medium, Conditional RPMI medium

Cell culture medium was prepared with 10 % Fetal Bovine Serum (FBS), 1 % L-Glutamine and it was stored at 4 ºC. For conditional RPMI medium, 15 % L- 929 conditional medium, 10 % Fetal Bovine Serum (FBS), 1 % L-Glutamine and 1 % Penicillin/Streptomycin were used and it was stored at 4 ºC.

0.25% Trypsin-EDTA (1X), Phenol Red, L-Glutamine (200 mM), Fetal Bovine Serum (FBS), 1X Phosphate Buffered Solution

They werepurchased from GIBCO (Invitrogen).

Albumin, from Bovine Serum (Fatty Acid Free)

It was purchased from SIGMA Life Science.

Palmitic Acid (palmitate)

It was purchased from Sigma-Aldrich (St.Louis, MO, USA). The main stock was prepared as 500 mM/mlby dissolving in absolute ethanol. Working solution was prepared by that enough amount of palmitate was dissolved in filtered 1 % BSA (Fatty acid free) containing RPMI. It was dissolved by vortexing and heating up to 55ºC for 30 minutes.

Palmitoleic Acid (palmitoleate)

It was purchased from Sigma- Aldrich (St.Louis, MO, USA).The main stock was prepared as 500 mM/ml in absolute ethanol and working solution was prepared by that enough amount of palmitoleate was dissolved in filtered 1 % BSA (Fatty acid free) containing RPMI.

Lipopolysaaccaride(LPS)

It was purchased from Sigma-Aldrich (St.Louis, MO, USA). It was prepared as 10 µg/ml aliquots and working solution was prepared bythat enough amount of LPS was dissolved in RPMI including 10% heat inactivated FBS and 1% L-glutamine.

3.2.2. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Immunoblotting Solutions

In this research, for Western blotting experiments, Mini PROTEAN Tetra Cell Western Blotting System (Bio Rad, CA, USA) was used. The gels used were prepared as 10% and 15 % resolving according to protein size and 5 % stacking Gels.

Phospho Lysis Buffer

To isolation of total protein from cell culture, Phospho lysis buffer was used. To preparation, 50 mM HEPES pH:7,9, 100 mM NaCl, 10 mM EDTA,10 mM NaF, 4 mM NaPP, 1% Triton, 2 mM Sodium orthovanadate (Na_3VO_4), 1mM phenylmethanesulfonylfluoride(PMSF), 1X phosphatase inhibitor cocktail 3(Sigma, P0044) and 1X (10µM/ ml) protease inhibitor cocktail were mixed.

10% and 15% Resolving Gels

For preparation of resolving gels, 40% Acrylamide mix, 1.5 M Tris-HCl (pH: 8.8), 10% SDS and 10% Ammonium persulfate, 0.08% TEMED were mixed and completed with enough amount volume of ddH_20.

5 % Stacking Gel

For preparation of stacking gel, 40% Acrylamide mix, 1.5 mM Tris-HCl (pH: 6.8), 10% SDS and 10% Ammonium persulfate, 0.1% TEMED were mixed and completed with suitable volume of ddH_2O.

10% Ammonium persulfate

2 g Ammonium persulfate was dissolved in 20 ml ddH_2O and it was stored at-20°C as aliquots.

1.5 M Tris-HCl (pH: 8.8)

54.45 g Tris Base (Trizma) was dissolved in 300 ml ddH_2O and pH was 8.8 adjusted by 1 N HCl. It was stored at 4 °C.

1.5 M Tris-HCl (pH: 6.8)

12 g Tris Base (Trizma) was dissolved with 100 ml ddH_2O and pH must be 6.8 adjusted with 1 N HCl.

10% SDS Solution

10 g SDS was dissolved in 100 ml ddH_2O.

10X Running Buffer

15 g/liter Tris Base, 144 g/liter Glycine and 300 ml 10% SDSwere dissolved in suitable volume of ddH_2O. Before usage, working solution was prepared as 1X.

10X Transfer Buffer

72 g/liter Glycine, 58 g/liter Tris Base, 2 ml 10% SDS were dissolved in suitable volume of ddH_2O. Before usage, working solution was prepared as1X by mixing 700 ml ddH_2O, 100 ml 10X Transfer Buffer, 200 ml Methanol.

10X Tris Buffered Saline (TBS)

1.5 M NaCl (87.76 g), 100 mM Trizma Base were dissolved in one liter ddH_2O and adjust pH 8.0 with 1N HCl**.**

1X-TBS-T

450 ml ddH_2O, 50 ml TBS, 500 µl Tweens were mixed.

10X Phosphate Buffered Saline (PBS)

80 g NaCl, 2 g KCl, 15.2 g $Na_2HPO_4.2H_2O$ were dissolved in 1 liter ddH2O and adjust pH to 6.8.

1X-PBS-T

450 ml ddH_2O, 50 ml PBS, 500 µl Tweens were mixed.

Blocking Solutions (5 % Bovine Serum Albumin in TBS-T and5 % Milk in TBS-T or PBS-T)

2.5 g Bovine Serum Albumin (Santa Cruz) was dissolved in 50 ml 1X TBS-T and 2.5 g milk powder was dissolved in 50 ml TBS-T or PBS-T.

3.2.3 Immunochemistry and MitoSOX Solutions

10X Triton

5 ml Triton X-100 was dissolved in 45ml dH_2O.

Blocking Buffer

3%BSA in 1X PBS was prepared by dissolving of 0, 3 g BSA in 1X PBS.

Permeabilization Buffer

0.3% TritonX-100 in 1XPBS was prepared.

Fixing Buffer

4% Formaldehyde in 1X PBS was prepared 40% Formaldehyde diluted to 4% by 1X PBS.

HBSS buffer

8000 mg/liter Sodium Chloride, 8000 mg/liter Potassium Chloride, 60 mg/liter Potassium Phosphate monobasic KH2PO4, 1000 mg/liter Glucose, 48 mg/liter Sodium Phosphate, dibasic Na2HPO4 anhydrate, 98 mg/liter Magnesium Sulfate, anhydrate MgSO4, 140 mg/liter Calcium Chloride, anhydrate, 350 mg/liter Sodium Bicarbonate were dissolved in dH_2O and autoclaved.

3.3 METHODS

3.3.1 Cell Culture

3.3.1.1 Cell Lines and Cell Culture Conditions of These Cells

For cell culture, DMEM and RPMI medium were used. In this research, THP-1 monocytes that were a human monocytic cell line, THP-1 macrophages, Primary Bone Marrow Derived Mouse Macrophages , IRE -/- mouse embryonic fibroblast cells (MEF), WT MEF, PERK -/- MEF, murine aneuploid fibrosarcoma cells (L929) were used. THP-1 monocytes, THP-1 macrophages were grown with RPMIcontained 10% heat inactivated Fetal Bovine Serum

(FBS) and 1% L-Glutamine while IRE -/- MEF, WT MEF, PERK -/- MEF, L929 were DMEMcontained 10% Fetal Bovine Serum (FBS) and 1X-L-Glutamine.BMDM cells were grown in RPMI that contained 10% Fetal Bovine Serum (FBS),15% conditioned medium from L929 cells, 1% Penicillin/Streptomycin and 1% 1X-L-Glutamine.All cells were grown in 37 °C incubators with 5% CO_2. They were controlled regularly to provide better grow conditions.

3.3.1.2 Passage of the Cell Lines

Firstly, all cells medium were removed and cells were washed with PBS. After that PBS was removed and 1 ml Trypsin-EDTA was added for 96 mm. The plates were incubated in incubator for 2-3 minutes. Then detached cells were collected with complete medium and suspend them in 15 ml or 50 ml falcons. The cells were reseeded with enough confluence to plates.

For THP-1 monocytes, the passage was different because THP-1 monocytes were suspension cell lines. Therefore, THP-1 monocytes were collected in 15 or 50 ml falcons and they were centrifuged at 500 rpm for 4 minutes. After that, supernatant was removed with not disrupting. Cells collected and were suspended with enough amount of RPMI. Then reseed the cells to plates. Before the usage, the mediums used were warmed at 37 °C in water bath.

3.3.1.3Freezing of Cultured Cells

The cells that had 70-80 % confluency in plates could be frozen by firstly removing their mediums. After that, they were washed PBS and Trypsin-EDTA was used to detach the cells. Then collect cells. If cells were suspension cells such as THP-1 monocyte cell lines, they were collected directly. After collecting cells, they were centrifuged for 7 minutes at 1000 rpm.

For freezing, the freezing medium was used contained 50 % FBS, 40 % DMEM and 10 % DMSO. The medium of the centrifuged cells were removed carefully and cells were resuspended with freezing medium prepared. The 1 ml of this suspension was put in cryovial

tube and they were stored at -80°C freezer. After one day later, some of frozen cells can be put in liquid nitrogen tank.

3.3.1.4Thawing of Cultured Cells

To thawing of the frozen cells, the frozen vials firstly were incubated in the 37°C water bath until they thawed. After that, they were suspended 1 ml pre-heated medium in cryovial and seeded to 25 cm^2 flask contained 5 ml medium and had filtered cap. For THP-1 cells, they were opened in 60 mm plates instead of 25 cm^2 flask. Then plates and flasks were incubated at 37°C incubator and after one day the medium was changed.

3.3.1.5 Preparation of Conditioned Medium from L929 Cells

$4{,}7x10^5$ cells were placed in a 75 cm^2 flask and 55 ml RPMI containing 10% heat inactivated FBS and 1% L-glutamine. Then these cells were grown for seven days inincubator with 5% CO2 at 37°C. After seven days, medium from the cells were collected and filtered with a 0.45-µm filter. This solution was frozen at -20°C.

3.3.1.6 Isolation of Bone Marrow Derived Macrophages

Firstly, femurs and tibiae were got from the 8 week old C57BL/6 mice by removing all muscle from bones. They were put in PBS until isolation. Then, the end of bones were cut and they were flushed with RPMI that contained 10% heat inactivated FBS and 1% Penicillin/Streptomycin solution using 21 G needle. After collection of these flushed cells in 50 ml Falcon tube, cells were spun at 1100 rpm for 5 minutes at 4°C.Then, they were filtered by cell strainer that were purchased from BD Bioscience (San Fransisco, USA) . Cells were spun again at 1100rpm for 5 min at 4°C. After spin, supernatant was removed and cells are resuspended with RPMI containing 10% heat inactivated FBS, 15% cell conditioned medium from L929 cells (they make M-CSF) and1% L-glutamine. Cells are seeded in 10 or 15 cm bacterial plates. These cells were cultured for a week for differentiation. At day 3, the cells were fed withRPMI containing 10% heat inactivated FBS, 15% cell conditioned medium from

L929 cells and1% L-glutamine. At day 6, the medium of cells were changed. If there was a lot of floating cells, they were spun down and resuspended with fresh medium and replaced on plates. After one week, only macrophage progenitors were sitting down on plastic plates. They were ready for experiments and they were able to use for 2-3 weeks.

3.3.1.7 Treatment of Cells

In this research, LPS, palmitate and palmitoleate were used to treat THP-1 and BMDM cells. One day before from treatment, the cells were seeded into plates. For treatment, palmitate and palmitoleate were given in 1% BSA (free fatty acids) RPMI medium and LPS was applied in RPMI medium contained 10% heat inactivated FBS and 1% L-glutamine. In these experiments, for negative controls, same amount of solvents according to treatment was given. For palmitate and palmitoleate treatment, negative controls were absolute ethanol and for LPS treatment, negative control was nuclease free water. The appropriate experimental conditions for treatment were given in the table below.

	Amount (Molarity and gram)	The solvent for treatment	The solvent for dissolving	Treatment duration	Chemicals used for negative control
LPS	50 ng/ml for THP-1 200 ng/ml for BMDM	Complete RPMI	Nuclease free water	3hours pretreatment before PA or PAO treatment	Nuclease free water
Palmitate	1000 µM/ml	RPMI with 1 % free fatty	Absolute Ethanol	9&16 hours for THP-1	Ethanol

		acid BSA		9 hours and 2 hours pretreatment before 22 hours no serum free medium treatment for BMDM	
Palmitate	200 µM/ml	RPMI with 1 % free fatty acid BSA	Absolute Ethanol	72 hours for WT MEF and IRE-/- &PERK-/- MEF	Ethanol
Palmitoleate	1000 µM/ml	RPMI with 1 % free fatty acid BSA	Absolute Ethanol	2 hours pretreatment and 24 or 16 hours treatment with PA	Ethanol

Table 3.2: The list of appropriate conditions of treatment used in this research with their solvent for dissolving and treatment time

3.3.2 Immunocytochemistry

To control of percentage of BMDM on total cells, immunocytochemistry method was used. One day before immunohistochemistry experiment, cells were seeded to wells contained sterile slides. After one day, medium was removed from the wells and cells were washed gently with 1X PBS twice. PBS was removed and cells were fixed with 4% formaldehyde for 10 minutes on ice. Then cells were washed with 1X PBS three times and for permebilization, they were treated with 0.3% Triton X in 1X PBS by incubating for 5 minutes at room temperature. After that, cells were blocked in 3% BSA in 1X PBS for 30 min at room temperature. MOMA-2 antibody in blocking solutions was applied for 1 hour at room temperature. After blocking, cells were washed with 1X PBS for 5 times by each wash for 1 minute on the shaker. Then cells were incubated secondary antibody such as Alexa488 or FITC for 20 minutes at room temperature. Again cells were washed for 5 times and one time washed with dH_2O. All liquid was removed and cells were stained with 50000X diluted DAPI from AppliChem (Dermstadt, Germany) for 30 seconds-1 minute. Then slides were mounted and placed in dark at room temperature for dry. The images were got with fluorescence microscope.

3.3.3 Quantification of Mitochondrial Reactive Oxygen Species with MitoSOX Red mitochondrial superoxide indicator

For the detection of mitochondrial reactive oxygen species(ROS), MitoSOX™ Red mitochondrial superoxide indicator that was purchased from Invitrogen (Grand Island,NY,USA) was used. Every vial of MitoSOX contains 50 μg of MitoSOX Red mitochondrial superoxide indicator and it was dissolved with 13 μL of DMSO to make5 mM MitoSOX stock solution. For quantification of mitochondrial ROS, one day before from treatment, cells were seeded into plates that contained sterilized slides. After the treatment according to experiment, the cell medium was removed and 300 μl of 5 μM MitoSOX

working solution was applied. Working solution was prepared with dilution of stock solution of MitoSOX withHBSS/Ca/Mg buffer before experiment. After putting the working solution onto cells that were incubated for 10 minutes at 37˚C, protected from light. Then cells were washed gently tree times with warm HBSS buffer. After that, for counterstaining, cells were fixated with 500 µl cold Acetone was applied onto cells and they were put at -20˚C.Then cells were washed with 1XPBS with three times and 300µl DAPI diluted as 50000 times was applied to cells for 30 seconds- 1 minutes. Finally, cells were washed with dH2O and mount with using FluorSave reagent from Millipore (Darmstadt, Germany) to imaging. Images were got as 40X by using fluorescence microscope and they were analyzed with ImageJ program.

3.3.4 Protein Extraction

3.3.4.1 Protein Precipitation from Cell Medium

For isolation of proteins from cell medium,methanol/chloroform precipitation method was used. Firstly, from treated cells 500 µl cell supernatant was collected, and 500 µl methanol and 125 µl chloroform were added to supernatant. Then, samples were vortexed vigorously until to seeing homogenous milky white suspension and samples were centrifuged at 13,000 × *g* for 5 minutes.After centrifuge, there was three phases: upper aqua/methanol part, a protein layer and lower chloroform part.The upper part was removed carefully by not removing protein phase. 500 µlmethanol was added to samples and samples were vortexed until protein layer break well. After that, the samples were centrifuged at 13,000 × *g* for 5 minutes. The entire methanol was removed from samples by sucking carefully.It was important that when removing methanol, the protein must not be disrupted. The proteins pellets were dissolved in 2X SDS loading dye and vortexed well. Then they were incubated at 95 °C for 5 minutes and then spun.

3.3.4.2 Protein Isolation from Cultured Cells

Firstly, phospholysis buffer mix prepared freshly was put into cells that frozen in liquid nitrogen. Then they were shaken gently and waited for 5 minutes in the dark. After that, cells were scraped with 1000 µl tip and collected in eppendorfs. They were centrifuged at 13000 rpm at 4°C for 12 minutes. The supernatants were collected and for measure the amount of protein, Bradford measurement was done. For this,1 µl protein sample was mixed with 49 µl nuclease free water and for blank, instead of protein sample, phospholysis buffer was used. After that, 450 µl Bradford Solution from SIGMA-ALDRICH (St.Louis, MO, USA), was added into prepared samples to measurement. The absorbance value of protein was measured at 595 nm and concentrations were calculatedaccording to BSA curve. Then the protein amounts were equaled and enough amounts of 5X SDS Loading Dye were added to samples. Then, samples were incubated atat 95°C for 5 minutes and they were spun.

3.3.5 Western Blotting

Before the running, samples were boiled at 95°C for 5 minutes. Then enough amount of protein (50 to 100µl) was loaded into Tris-glycine SDS-Polyacrylamide gel. To comparison of protein size, protein ladder was also loaded into gel. Gel was run in 1X running Buffer. The concentrations of gel were changed according to protein size. To looking at caspase-1 p10 part, protein was run on 15% gel with 100 V constantly. To look at caspase-1 p45 and B-actin or B-tubulin, 10% gels were used and running started with 80 V and after 30 minutes it was changed to 120 V. After running, the proteins in gel were transferred onto PVDF transfer membrane that was brought from Thermo Scientific. Firstly, transfer membrane was activated in methanol for 10 seconds and then it was washing in 1X transfer buffer. Then for wet transfer, the sandwich was prepared. Firstly, 3 sponges and 4 filter papers were soaked in 1X transfer buffer. Then these were put by this order: 2 sponges, 2 filter papers, gel, transfer membrane, 2 filter papers and 1 sponge. Then sandwich apparatus was closed and put in

transfer tank. For looking at caspase-1 p10 part, the proteins were transferred at 100 V for 90 minutes while for looking other proteins; transfer was done at 95 V for 120 minutes. The transfer was one at 4°C.After transfer, transferred membranes firstly washing with TBST-T for two minutes and then it was blocked with 5% PBS-T milk for caspase-1 and for other proteins with 5% TBS-T milk. After that, primary antibody in same solution with blocking is prepare with suitable dilution and put on plate that was surrounded with wet towels and incubated at 4°C overnight or 1 hour at room temperature. Then the membranes was washed with TBS-T (%0.5 tween) or PBS-T(%0.5 tween) according to blocking solution content for 3 times 5,15,5 minutes on shaker in order. After that,anti-mouse or anti-rabbit which were horseradish peroxidase (HRP) conjugated antibodies used as secondary antibody. The secondary antibody was prepared in same solution with blocking solution with suitable dilution. The membrane was incubated with secondary antibody for 1 hour at room temperature and then the membrane was washed with again TBS-T or PBS-T on shaker for 3 times as 5,15 and 5 minutes. After these processes, for detection, ECL-Prime (Amersham, UK) was prepared with 1:1 ratio of its solution A and solution B. Then, it was applied on to membrane and it was incubated at dark for 5 minutes. After that, the membrane was developed with X-ray films and Developer device.

3.3.6 Total RNA isolation from Cells

For RNA from cell lines were extracted by using Trisure Ambion Trizol Reagent from Invitrogen (Grand Island,NY,USA).For this, firstly, cells were suspended 1000µl Trisure and 200 µl Chloroform were added and then mixed them. After waiting for 3 minutes, they were spun at 13000 rpm for 17 minutes. Afterwards, the upper phase was collected carefully and 500 µl Isapropanol added and mixed for 15 seconds. After waiting them at room temperature for 10 minutes, they were spun at 13000 rpm for 12 minutes. The supernatant part was removed and 1 ml 75% ethanol was added to eppendorfs. They were spun at 8000 rpm for 8

minutes. Then again supernatant part was removed and 100% ethanol was added into eppendorfs. Afterwards, they were spun at 8000 rpm for 8 minutes. After centrifuge, all ethanol was removed and RNA pellets were dried for several minutes. Then they were dissolved by 10-25µl nuclease free water and stored at –80°C.

3.3.7 cDNA Synthesis

From RNA, first strand cDNAs was synthesized by using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Rockford, USA). Firstly, 0,550 ug RNA was mixed with 0, 5 µl The oligo $(dT)_{18}$ primer and to complete to volume as 6 µl , enough amount of nuclease free water was added. Then, they were incubated at 65^{o}C for 5 minutes. After incubation, 2 µl reaction buffer, 1 µl dNTP mix,0.5 µl RNAase inhibitor,0.5 µl Revert aid M-Mul V Reverse Transcriptase (200u/µl) were added to these samples and they weremixed gently. Finally, they were incubated 60 min at 42^{o}C followed by heat activation at 70^{o}C for 5 minutes.

3.3.8 Quantative RT-PCR for Expression Analysis for Gene Expression

3.3.8.1 Calculation of Efficiency of Primer Efficiency

To find the efficiency of primer, firstly, cDNA pool was prepared and it was diluted by serial dilution. The dilution factor was 5 and seven serial dilutions were done. After that, these samples were used for RT-PCR and results were used for calculation. Ct value to log of dilution factor graphs was drawn and the slope that was used to find our primer efficiency calculation. The efficiency calculation formula is E = 10^ (-1/slope). The primer sequences that were used in this research and their parameters were indicated on the table on below:

The name of Primer (For mouse)	Forward	Reverse	T_m	The Reference Article and Groups	The primer efficiencies

hIL1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	55,3-57,3	Lee YM, Fujikado N, Manaka H, Yasuda H, Iwakura Y. IL-1 plays an important role in the bone metabolism under physiological conditions. Int Immunol. 2010 Oct; 22(10):805-16.	1,88
hIL18	CAAGGAATTGTCTCCCAGTGC	CAGCCGCTTTAGCAGCCA	58-64	Ear, T., McDonald, P. P. *Autocrine role of endogenous interleukin-18 on inflammatory cytokine generation* by *human neutrophils*. FASEB J. 23, 194–203 (2009).	1,88
hBeta-Actin	GATGAGATTGGCATGGCTTT	CACCTTCACCGTTCCAGTTT	55,3-57,3	Susztak K, Ciccone E, McCue P, Sharma K, Bottinger EP. Multiple metabolic hits converge on CD36 as novel mediator of tubular epithelial apoptosis in diabetic nephropathy. *PLoS Med* 2005; 2: e45.	1,83
mIL1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	59,7-61,4	Cao, S.S., Song, B. & Kaufman, R.J. PKR protects colonic epithelium against colitis through the unfolded protein response and prosurvival signaling. Inflamm Bowel Dis	1,91
mIL18	TCTGACATGGCAGCCATTGT	CAGGCCTGACATCTTCTGC	57,3-59,8	Kawase Y, Hoshino T, Yokota K, Kuzuhara A, Nakamura M, Maeda Y,	1,91

		AA		Nishiwaki E, Zenmyo M, Hiraoka K, Aizawa H. Bone malformations in interleukin-18 transgenic mice. J Bone Miner Res 2003;18:975–83.	
mTNFalpha	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC	58-62	Stig S. Jakobsen, A. Larsen2, M. Stoltenberg, J.M. Bruun3, K. Soballe (2007). Effects of As-Cast and Wrought Cobalt-Chrome-Molybdenum and Titanium-Aluminium-Vanadium Alloys on Cytokine Gene Expression And Proteın Secretion In J774a.1 Macrophages.*European Cells and Materials* Vol. 14.pages 45-55.	2,06
mGAPDH	GTGAAGGTCGGTGTGAACG	GGTCGTTGATGGCAACAATCTC	60-66	Motohara T, Masuko S, Ishimoto T, Yae T, Onishi N, Muraguchi T, Hirao A, Matsuzaki Y, Tashiro H, Katabuchi H, Saya H, Nagano O. (2011) Transient depletion of p53 followed by transduction of c-Myc and K-Ras converts ovarian stem-like cells into tumor-initiating cells. Carcinogenesis. 32:1597-606, 2011.	2,0
hTNFalpha	AGCTGCCCCTCAGCTTGAA	CCCAGGGACCTCTCTAATCA	61-62.1	Chiu Sc, Yang NS. (2007). Inhibition or Tumor Necrosis factor-*a*through Selective Blockade of Pre-mRNA Splicing by Shikonin.	2,0

				Molecular Pahrmacology Vol. 71 no 6, 1640-1645.	
hGAPDH	CAATGACCCCTTCATT	TTGATTTTGGAGGGATCTCG	45.6-52.2	Lamont KR, Hasham MG, Dongia NM, Branca J, Chauaree M, Chase B, Breggia A, Hedlung J, Emery I, Cauallo F, Jasin M, Rüter J and Mills KD. (2013). Attenuating Homologous recombination stimulates and AID-induced antileukemic effect. *The Journal of Experimental Medicine.*Vol 210 NO.5 1021-1033.	1,98

Table 3.3) The list of qRT-PCR primers with their efficiency values and conditions

3.3.8.2 Quantification of Gene Expression Level by RT-PCR

For quantitative PCR analysis 6 µlSyber Green mix from Roche (Basel, Switzerland), 1µl forward primer, 1µlreverse primer were used. The volume as completed to 12µlby adding enough amount of cDNA and nuclease free water. The RT-PCR reaction that was used

contained initial denaturation step at 95^{o}C for10 minutes, 45 cycles of amplification reaction was done at 95^{o} 30 seconds, at 60^{o}C 30 seconds, at 72^{o}C 30 seconds with final extension at 72^{o}C 5 minutes. Finally, melting temperature step was followed at 95^{o}C 20 seconds ended with one more step for annealing and extension time. To calculation of expression level of the gene, Ct values of the target gene and reference gene such as GAPDH or B-actin were used. The formula that was used for calculation was (Primer efficiency)$^{-\Delta\Delta Ct}$ where ΔΔCt means ΔCt (target gene) - ΔCt (reference gene) and Ct means (threshold cycle).For statistical reason, all samples were analyzed as duplicate.

3.3.9 Enzyme-Linked Immunosorbent Assay

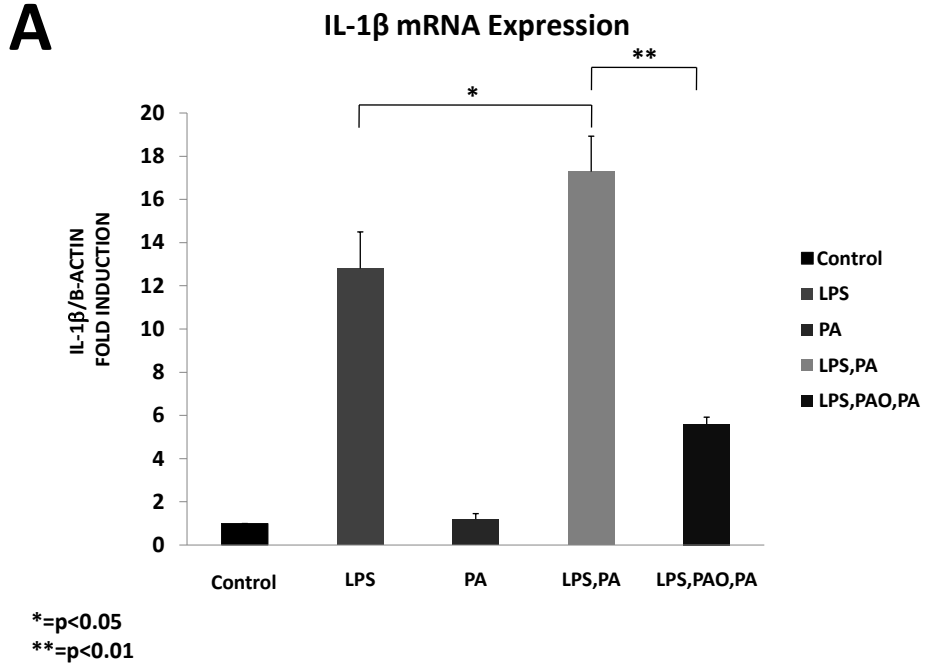
For quantitive analysis of amount of secreted IL-1β,Enzyme-Linked Immunosorbent Assay (ELISA) kit from Abcam(Cambridge, United Kingdom) was used. All reagents that were used were supplied with the kit. Before the starting experiment, cell supernatants were diluted as 2X or 3X. From 2000 pg/ml to 0 pg/ml concentration 1:3 ratio diluted 8 standards were prepared. Before the experiment, all materials and reagents were equilibrated to room temperature. Then 100 µl of samples and standards were applied to wells. For statistical reason, all sample applied as twice. Then they were incubated for 2,5 hours at room temperature by gently shaking. Then they were removed and wells were washed four times with 1X Wash solutions. All wash solution was removed and 100 µl 1X Biotinylated IL-1 beta detection antibody was applied to wells and incubated for 1 hour by gently shaking. Then all solutions were removed and wells were washed four times with 1X Wash solutions. Afterwards, 100 µl 1X HRP-Streptavidin solution was applied to wells and incubated for 45 minutes by gently shaking. Then again repeat washing step and then 100 µl TMB one-step substrate reagent was added to well and incubate 30 minutes in the dark by gently shaking. Finally 50µl stop solution was added and immediately read at 450 nm. All steps were done at

room temperature. The calculation was done after forming standard curve by using standard concentration and their absorbance.

4. RESULTS

4.1 The effect of palmitoleate on palmitate-induced inflammasome activation in human monocytic cell line, THP-1

The in vitro activation of NLRP3 inflammasome requires two separate signals. The first signal is mediated throught the TLR receptors upon engaging lipopolysaccaride (LPS) and leads to mRNA expression ofIL-1β and IL-18. The second signal is needed to activate caspase-1 and the assembly of NLRP3 inflammasome complex[22]. In this study, I first, analyzed the effect of palmitate-induced IL-1β and IL-18 mRNA expression and how this is modified by palmitoleate pre-treatmentin human THP-1 monocytic cell line. The results demonstrated that palmitate (together with LPS) leads to an increase in the mRNA level of IL-1βand this increase could beblocked by palmitoleate pre-treatment (Figure 4.1 A). On the other hand, only LPS or palmitate together with LPS treatment did not increase pro-IL-18 mRNA level.(Figure 4.1 B). We did not detect changes in the mature form of IL-18 as this is constitutively production of IL-18 in monocytes and macrophages[131]. Therefore, IL-18 analysis must be done in protein level.These results indicate that palmitoleate prevents palmitate-induced priming of the inflammasome activity.



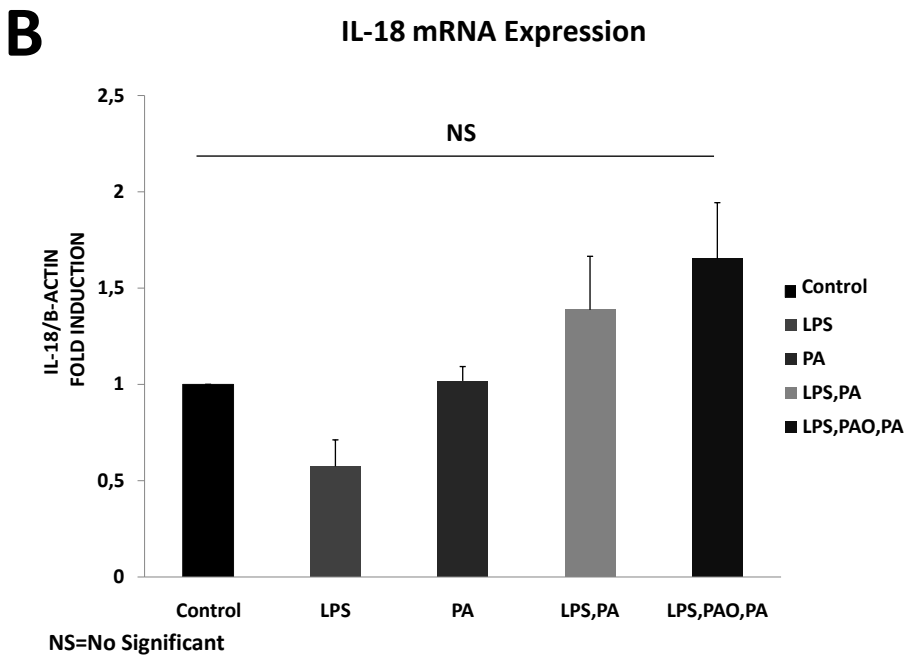


Figure 4.1: The effect of palmitoleate on palmitate-induced IL-1β and IL-18 mRNA expression levels in the human monoytic cell line, THP-1.

THP-1 monocytes ($2x10^6$) were treated with 50 ng/ml LPS for 3 hours, 1000 µM/ml palmitoleate for 2 hours and finally 1000 µM/ml palmitate for 9 hours. Total RNA was isolated from cells and mRNA level of IL-1β (A) and IL-18 (B)were analyzed by qRT-PCR. (n=3 Student's t-test was performed: *=$p<0.05$ and **=$p<0.01$). (Control= 1% BSA+ ethanol, LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

4.2 The effect of palmitoleate on palmitate-induced inflammasome activationin primary Bone Marrow Derived Mouse Macrophages

After observing an in the mRNA expression of IL-1β by palmitate and prevention by palmitoleate in human monocytes, a similar was done in primary bone marrow derived mouse macrophages (BMDM). BMDM cells were isolated from 8 week old C57BL/6 mice and after seven day differentiation protocol; they percent differentiation into macrophages was determined by immunostaining with a macrophage-specific antibody, anti-mouse+macrophage antibody (anti-MOMA2). As seen in Figure 4.2, the differentiation protocol led to more than 90% differentiated macrophages, implying this protocol provides an appropriate number of macrophages suitable for downstream experiments.

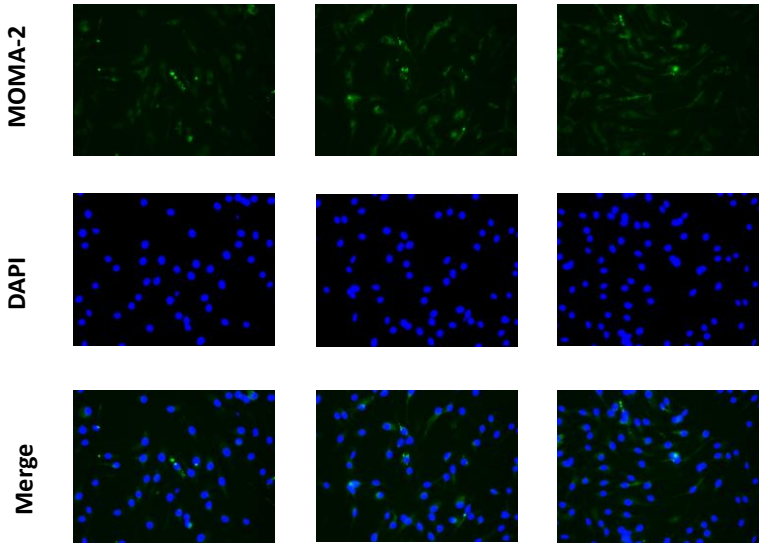
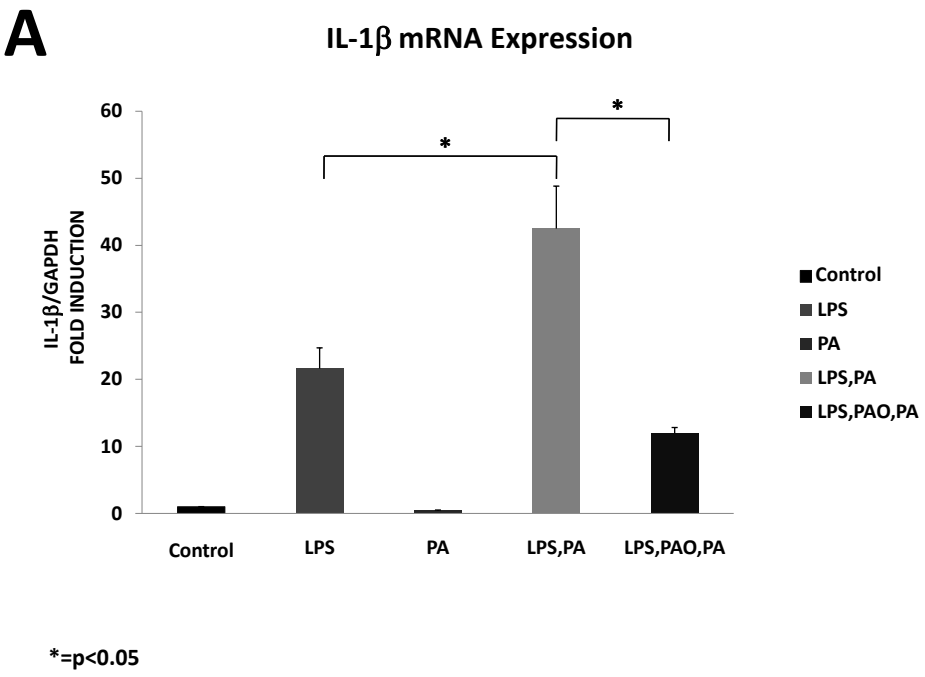


Figure 4.2: The Investigation of the efficiency of BMDM differentiation protocol.

Bone marrow-derived and differentiatedcells were stained with MOMA-2 antibody and then anti-rabbit Alexa-488 secondary antibody to observe the differentiated macrophages under immunofluorescent microscope. Then differentiated cells (green signal) were counted and the number was divided to total cell number (DAPI nuclear staining, blue signal) to determine the efficiency of differentiation (percent differentiation was more than 90%).

As seen in Figure 4.3Apalmitate with LPS treatment further raised the mRNA levels of IL-1βand this increase wassignificantly blocked with palmitoleate treatment in primary BMDM. However, neither LPS treatment nor palmitate with LPS treatment increased themRNA level of IL-18 in BMDM (Figure 4.3 B). Once again this is due tothe fact that a constitutive production of IL-18 is reported for monocytes and macrophages.



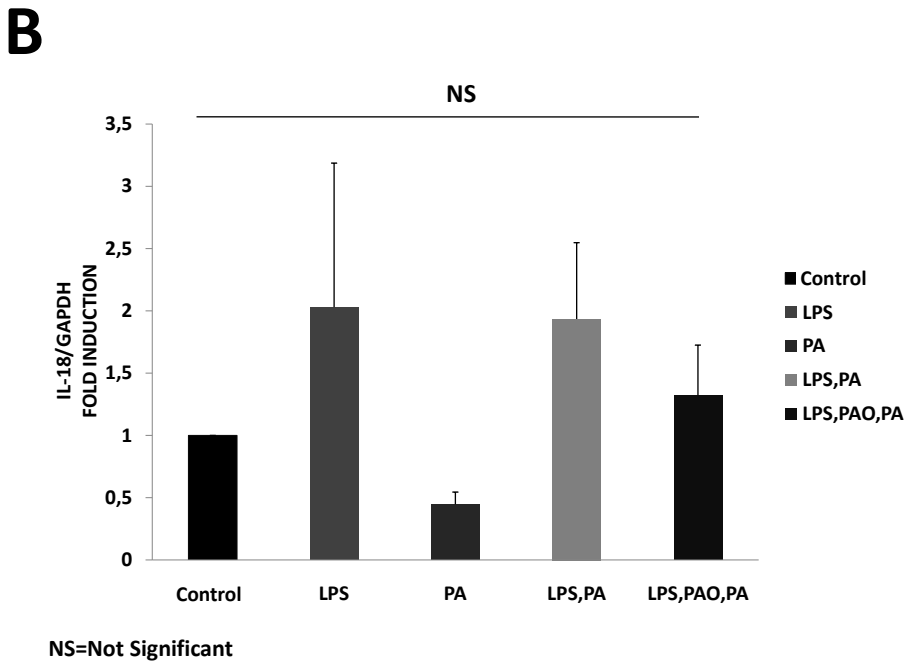


Figure 4.3:The effect of palmitoleate on palmitate-induced mRNA expression of IL-1β and IL-18 in primarybone marrow derived mouse macrophages.

Primary BMDM ($1,5x10^6$) were treated with 200 ng/ml LPS for 3 hours, 1000 µM/ml palmitoleate for 2 hours and finally 1000 µM/ml palmitate for 9 hours. Total RNA was isolated from cells and mRNA levels of IL-1β (A) and IL-18 (B) were analyzed by qRT-PCR (n=3; Statistic: Student's t-test: *=p<0.05).(Control=1% BSA+ ethanol, LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

Moreover, a study by Wen et al demonstrated that palmitate is a secondary signal for NLRP3 inflammasome and it resulted in activation of caspase-1 and secretion of IL-1β and IL-18 in murine macrophages[18]. So I next investigated how palmitoleic acid modifies the second signal mediated by palmitate. To analyze caspase-1 activity, the cell supernatant and the cell lysate were collected and cleaved caspase-1 levels were analyzed by Western blot. Figure 4.4 shows that palmitoleate decreased the level of active caspase-1 form that is induced by palmitate and LPS together. As seen in the same figure, only LPS or only palmitate are not sufficient to activate caspase-1 in BMDM.

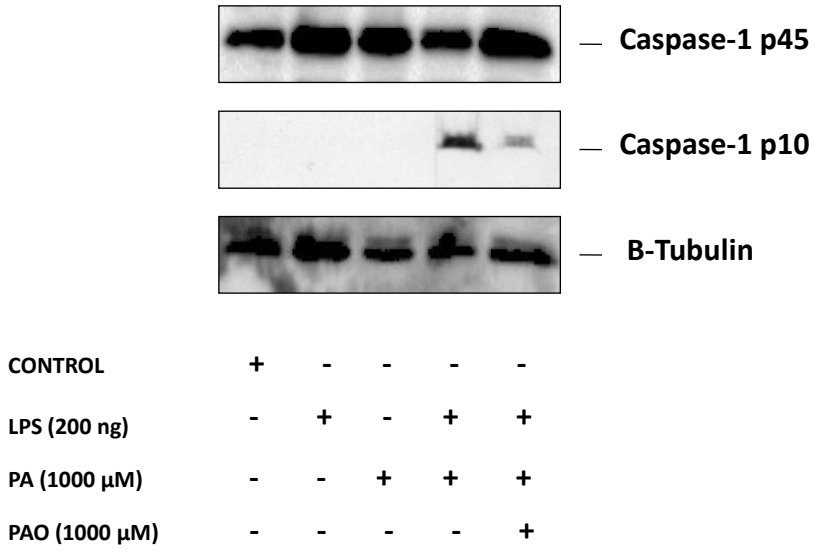


Figure 4.4:Palmitoleate prevents caspase-1 activation by palmitate in primarybone marrow derived mouse macrophages.

Primary BMDM were treated with 200 ng/ml LPS for 3 hours followed by 1000 μM/ml palmitoleate or with 1000 μM/ml palmitate and palmitoleate together for 2 hours. After this, the cells were placed in a medium without serum and incubated for 22 hours. Cell lysates and cell supernatant were collected for Western Blot analysis of caspase-1 species.(Control=1% BSA+ ethanol, LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

Furthermore, since active caspase-1 leads to maturation and secretion of IL-1β, the supernatants were analyzed forIL-1β by the enzyme-linked immunosorbent assay (ELISA)As seen in Figure 4.5 indeed, palmitoleate treatment lead to significantly reduction in palmitate-induced IL-1β in secretion in primary BMDM. Collectively, these results confirm the observations initially made in human THP1 monocytic cell line regarding to prevention of inflammasome activation by palmitoleic acid in primary macrophages.

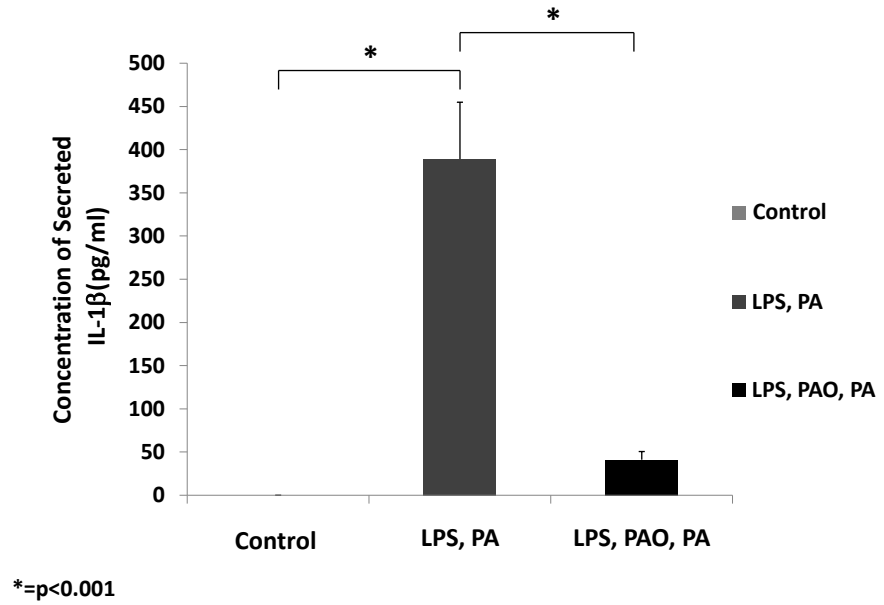


Figure 4.5:Palmitoleate prevents palmitate-induced maturation and secretion ofIL-1β in primary bone marrow derived mouse macrophages.

Primary BMDM were primed with 200 ng/ml LPS for 3 hours and then treated with 1000 µM/ml palmitoleate or 1000 µM/ml palmitate and palmitoleate together for 2 hours. After this, the cells were changed into serum free medium and incubated an additional 22 hours. Then, cell supernatants were collected for ELISA analyses for IL-1β (n=4; Student's t-test was performed: *=p<0.001). (Control=1% BSA+ ethanol, LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

4.3 The Effect of palmitoleate on palmitate-induced mRNA expression of Tumor Necrosis Factor -αin human monocytic cell line, THP-1

With the activation of the inflammasome, mature forms of IL-1β and IL-18 are secreted from macrophages. These pro-inflammatory factors are recognized by their respective receptors

and inducethe production of secondary pro-inflammatory factors such as Tumor NecrosisFactor -α (TNF-α)[31]. In this part of the study, the effect of palmitoleate on palmitate-induced mRNA expression of TNF-α was investigated. The treatment conditions were same withIL-1β andIn this part of the study, the effect of palmitoleate on palmitate-induced mRNA expression of TNF-α was investigated. The treatment conditions were same withIL-1β and IL-18 expression analysis experiments. As seen in figure 4.6 palmitoleate also significantly decreased palmitate-induced TNF-α expression in human monocytes. These findings are in consistency with the palmitoleate's ability to block inflammasome activation by palmitate.

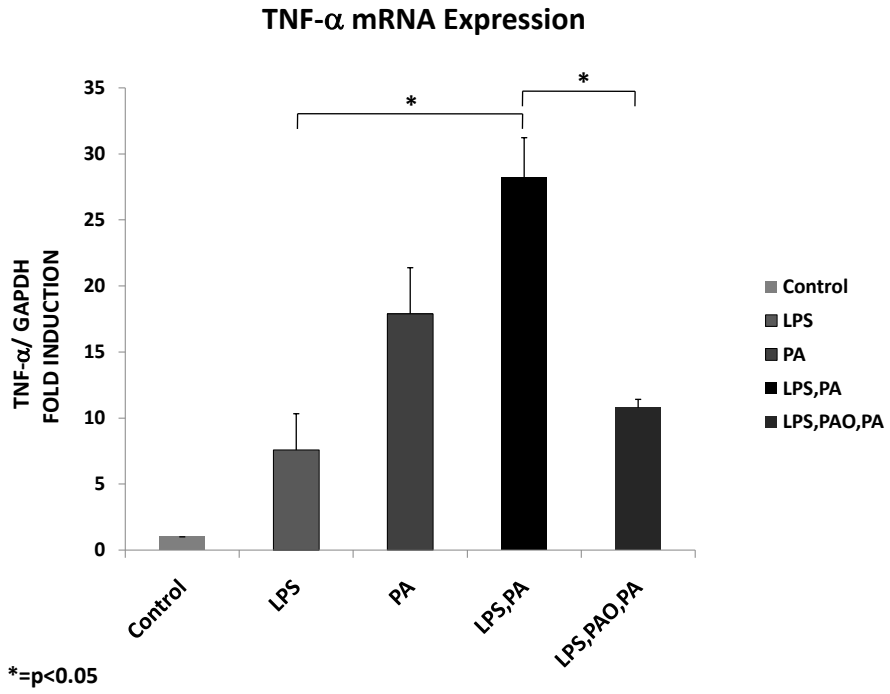


Figure 4.6: **The effect of palmitoleate on palmitate-induced mRNA expression of TNF-αin human monocytic cell line, THP-1**.

THP-1 monocytes ($2x10^6$) were treated with 50 ng/ml LPS for 3 hours, 1000 µm/ml palmitoleate for 2 hours and finally followed by 1000 µm/ml palmitate for 9 hours. Total RNA was isolated from cells and mRNA levels of IL-1β and IL-18 were analyzed by qRT-PCR (n=3; Student's t-test: *=p<0.05).(Control=1% BSA+ ethanol, LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

4.4 The effect of palmitoleate on palmitate-induced mRNA expression of TNF-αin primarybone marrow derived mouse macrophages

After observing that palmitoleate modulates palmitate-induced TNF-α expression in human monocytes, its effect on palmitate-induced TNF-α expression was also examined in primary BMDM. Palmitoleate significantly eliminated palmitate-induced TNF-α expression in primary bone marrow derivedmacrophages (Fig.4.7). These findings are consistent with observations made in the human monocytic cell line, THP1.

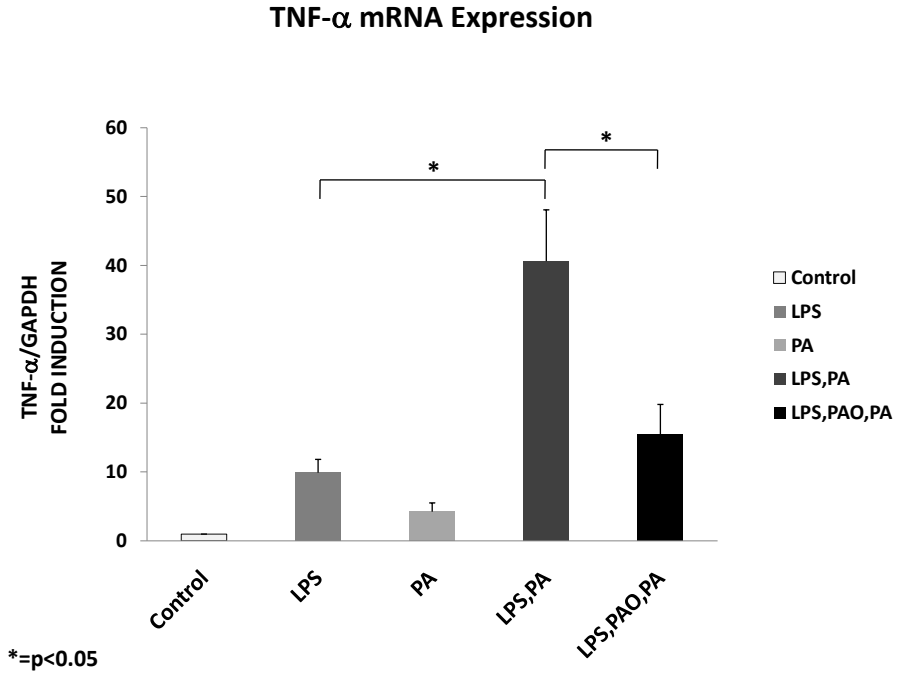


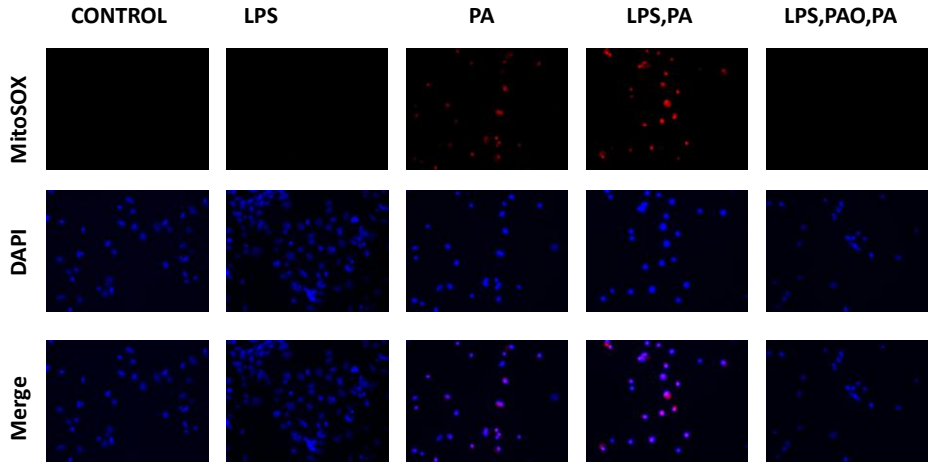
Figure 4.7:The effect of palmitoleate on palmitate-induced mRNA Expression of TNF-αin primarybone marrow derived mouse macrophages.

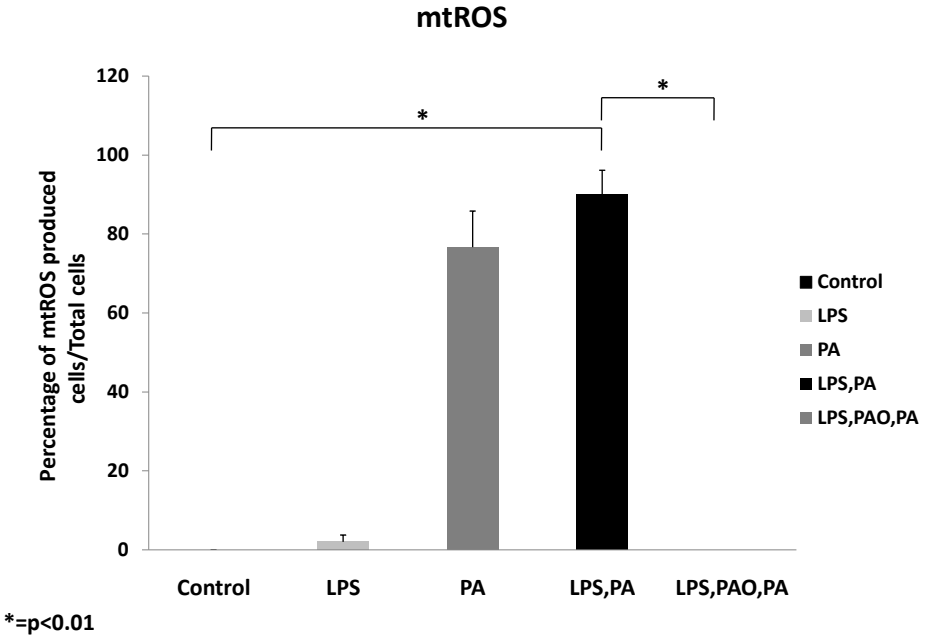
Primary BMDM ($1,5x10^6$) were treated with 200 ng/ml LPS for 3 hours, 1000 µm/ml palmitoleate for 2 hours and finally 1000 µm/ml palmitate for 9 hours. Total RNA was isolated from cells and mRNA level of TNF-α was analyzed by qRT-PCR (n=3, Student's t-test: *=p<0.05). (Control=1% BSA+ ethanol, LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

4.5 The effect of palmitoleate on palmitate-induced mitochondrial reactive oxygen species by palmitoleate in human macrophage cell line, THP-1.

The second hit for NLRP3 inflammasome is mainly provided by reactive oxygen species (ROS), ion flux and lysosomal destabilization[5]. An earlier study showed that palmitate causes inflammasome activation by increasing the production of mitochondrial reactive oxygen species (mtROS)[18]. Mitochondria are the main source of ROS production and studies in

skeletal muscle cells indicated that exposure to high level of palmitate results in damage of the mitochondria and modification of its structure[132]. Since earlier data in my thesis study indicated that palmitoleate blocks palmitate-induced activation of inflammasome, I sought to investigate the mechanism by which palmitoleate acts. Hence, I investigated the effect of palmitoleate on palmitate stimulated production ofmtROS. For this purpose, ROS were analyzed by immunohistochemistry using a kit specific for mtROS from Invitrogen, the MitoSOX assay. Using this form of analysis the percent ratio of the cells that produced mtROS to total cells was calculated. As seen in figure 4.8, palmitoleate lead to marked reduction in mtROS induced by palmitate, indicating prevention of mitochondrial damage and ROS production by palmitate is the mechanism of action employed by palmitolate in cells.





*=p<0.01

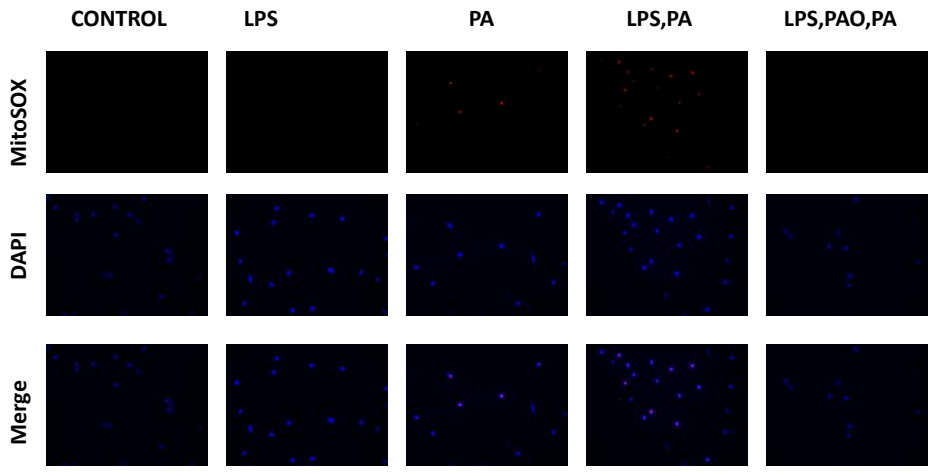
Figure 4.8: The elimination of palmitate-induced mtROS production by palmitoleate in THP1 macrophages.

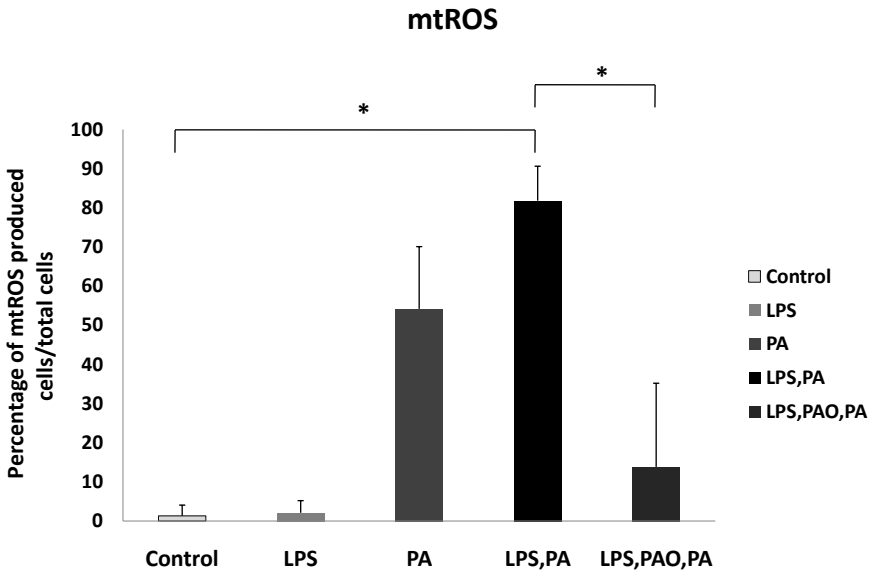
THP1 monocytes were differentiated with 100 ng/ml PMA for 1 day and they remained in 10% RPMI medium for 2 more days. Differentiated THP1 macrophages were primed with 50 ng/ml LPS for 3 hours and then they were pre-treated 1000 µM/ml palmitoleate for 2 hours followed with 1000 µM/ml palmitate treatement or no treatment for an additional16 hours. To analyze mtROS, MitoSox immunofluorescent staining assay was applied to living cells. Cells were fixed with cold acetone and counterstained with DAPI for nucleus. The immunofluorescent image analysis was done by using ImageJ program. (n=3; Student's t-test *=$p<0.01$). (Control=1% BSA+ethanol,

LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

4.6 The effect of palmitoleate on palmitate-induced mitochondrial reactive oxygen species in primary bone marrow derived mouse macrophages

Next the effect of palmitoleate on palmitate stimulated mtROS production was analyzed in primary BMDM. As seen in Fig. 4.9, palmitoleate also reduced palmitate-induced mtROS production in the MitoSox assay in primary BMDM. These results confirm the observations regardingpalmitoleate's inhibitory effect on palmitate-induced mtROS production also occurs in primary macrophages.





*=p<0.01

Figure 4.9:Palmitoleate preventspalmitate-induced mtROS production in primary bone marrow derived mouse macrophages.

BMDM were primed with 200 ng/ml LPS for 3 hours and they were treated 1000 µM/ml palmitoleate for 2 hours and then followed with 1000 µM/ml palmitate and palmitoleate together for 24 hours. To analyze mtROS, MitoSox immunofluorescent staining assay was applied to living cells. Cells were

fixed in cold acetone and the nucleus was counterstained with DAPI. The analysis of the immunofluorescent images was done using ImageJ program (n=3; Student's t-test: *=p<0.01).(Control=1% BSA+ethanol, LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

4.7 The effect of palmitoleate on palmitate-induced Inactivation of 5' AMP-activated protein kinase

It is known that palmitate induces inflammasome activation through increasing the production of reactive oxygen species mitochondria. In an earlier study it was also shown that palmitate leads to a reduction in 5' AMP-activated protein kinasephosphorylation, inactivating AMPK and reducting mitophagy, thereby leading to enhanced mtROS levels in the cells[18]. AMPK can be activated upon phosphorylation at Thr172 site[133]. In order to understand further how palmitoleate reduces mtROS production induced by palmitated, I next investigated palmitoleate's effect on AMPK phosphorylation in primary BMDM. As seen in Fig. 4.10palmitate indeed decreased the phosporylation of AMPK in LPS-primed primary BMDM while palmitoleate treatment prevented this. In other words, palmitoleate treatment regained the phosphorylation of AMPK. These results indicate that palmitoleate by preserving AMPK phosphorylation can reduce mtROS levels in cells. Further studides are needed to confirm whether PAO preserves mitophagy in palmitate-treated cells. Also, it needs to be investigated whether this is a direct effect of palmitoleate on AMPK phosphorylation or whether AMPK phosphorylation is modulated indirectly palmitoleates' effect on the UPR branches.

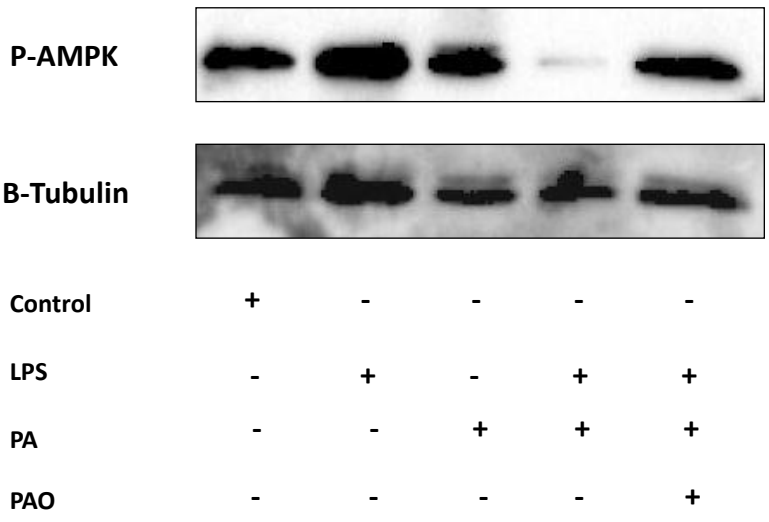


Fig.4.10:The effect of palmitoleate on palmitate-induced reduction in the phosphorylation of AMPK.

Primary BMDM were treated with 200 ng/ml LPS for 3 hours and 1000 μM/ml palmitoleate for 2 hours. After that, they were treated with 1000 μM/ml palmitate and palmitoleate together for 2 hours. Then cells were changed into medium without serum and incubated for 22 hours. Cell lysates were collected and analyzed by Western Blot using anti- p-AMPKor anti-B-tubulin antibodies.(Control=1% BSA+ethanol, LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

4.8 The effect of PERK and IRE1 branches of the Unfolded Protein Response on palmitate-induced mitochondrial reactive oxygen species production in mouse embryonic fibroblasts

Recent studies showed that palmitate is an inducer of UPR activation in different cell lines such as pancreatic β cells, hepatocytes and macrophages[134-136]. Moreover, studies show that activation of UPR pathways has role in regulation of ROS production[95-99].To illustrate that, it was indicated that in PERK lack cells show more ROS production when comparing wild type cells after they are stimulated with tunicamycin, a well known ER stress inducer[99]. However, another study suggested that the lack of PERK causes rearrangement of ER-mitochondria and change calcium signaling[137].It is known that at sites of ER-mitochondrial contact, excess ER calcium release causes sustained excessive amount of calcium influx to mitochondria and causes mitochondrial damage such as mtROS production[138,139]. Furthermore, it is known that palmitate causes calcium depletion in ER and distrupt ER homeostasis[140]. Recent study shows that palmitate associated distruption of calcium caused redistribution of calcium from ER to mitochondria and causes mtROS production in hepatic cells[141]. I hypothesized that palmitoleate's ability to prevent mtROS may be an indirect effect mediated by an endoplasmic reticulum – mitochondria cross talk. In my experiments I asked whetherUPR, in particular, the PERK and IRE1 arms, have an impact on palmitate-induced mtROS. For this investigation, I used wild type (WT), PERK-deficient ($PERK^{-/-}$) or IRE1-deficient ($IRE1^{-/-}$) mouse embryonic fibroblasts(MEFs) treated with palmitate. Using the MitoSOX assay mtROS production was detected with immunofluorescent microscopy. The percentage of the ratio of cells that produced mtROS to total cells was calculated. I observed that after palmitate treatment, PERK-deficient MEFs indicated more mtROS production while IRE1-deficient MEFs indicated less mtROS production when comparing WT MEFs. For looking PERK-deficient MEFs , they indicated 30% higher mtROS production than WT MEFs while IRE-

deficient MEFs indicated 10% lower mtROS than WT MEFs (Fig. 4.11). These data suggested that PERK can have protective role for inflammasome activation due to deficiency of PERK enhanced mtROS production which is second hit for inflammasome activation. On the other hand, IRE1 can be contributor effect for inflammasome activation because its deficiency decreased mtROS production.

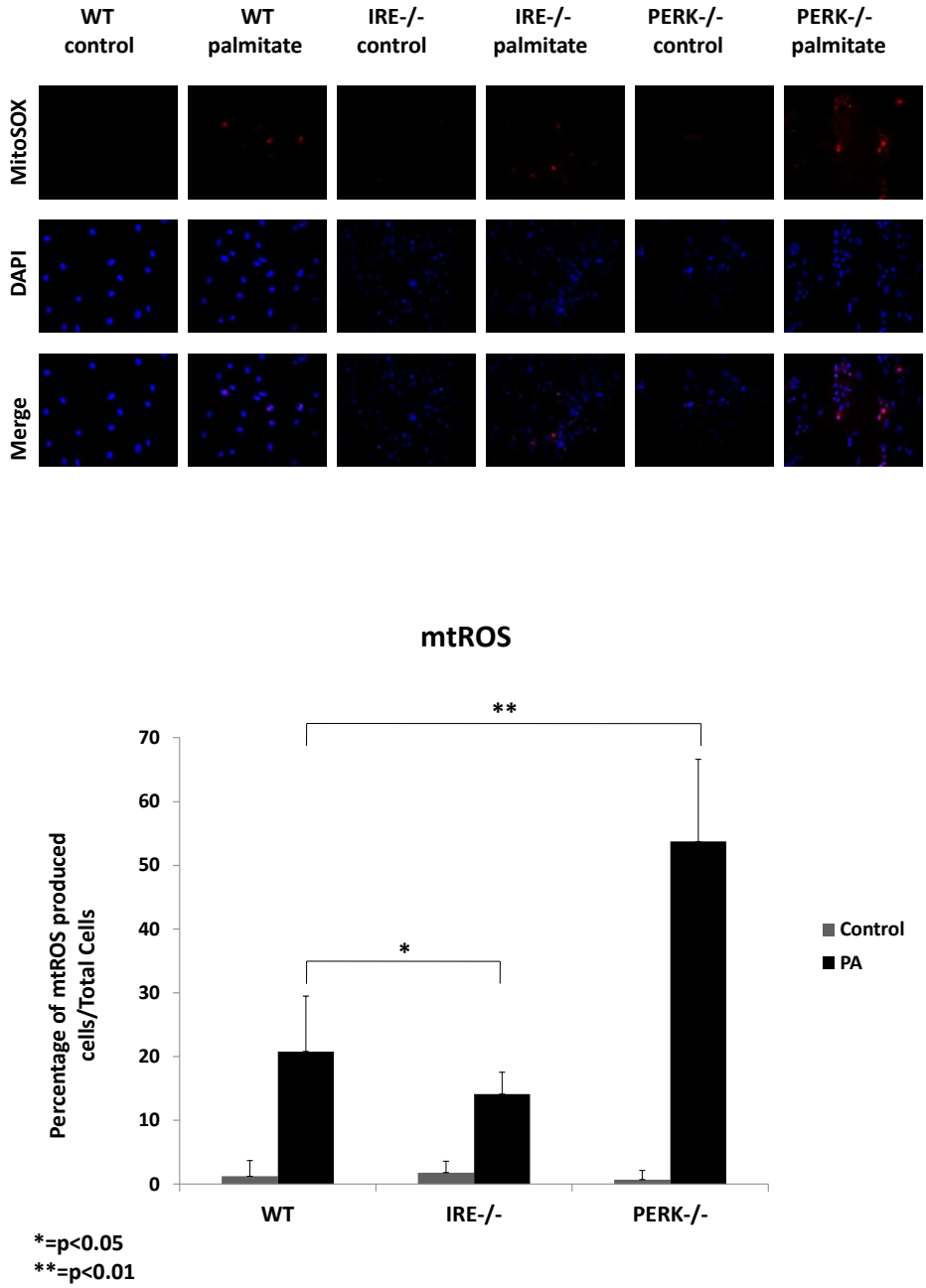


Figure 4.11:ThePERK and IRE1 arms modulate palmitate-induced mtROS production in mouse embryonic fibroblasts

WT, PERK$^{-/-}$, IRE$^{-/-}$ MEFs were treated with 200 µm/ml palmitate for 72 hours. To analyze mtROS, immunofluoresecent staining with MitoSox assay was applied to living cells. Then cells were fixed in cold acetone and nucleuses were counterstained with DAPI. The immunofluorescent image analysis was done by using ImageJ program. (n=3, Student's t-test: *=p<0.01).(Control=1% BSA+ethanol, LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

The impact of palmitoleate on palmitate-induced activation of the two UPR arms was analyzed. As seen in Fig. 4.12palmitoleate treatment caused a marked decrease in phosphorylation level of IRE1 while it did not affect PERK phosphorylation. These results indicate that under conditions that induced the inflammasome with palmitate and LPS treatement, palmitoleate prevents activation of only the IRE1 branch of the UPR. These results suggest that palmitoleate may indirectly act on mitochondrial ROS production and inflammasome activation indirectly by modifying IRE1 activation. Further studies are needed to define the detailed mechanism of these lipids on IRE1 regulation.

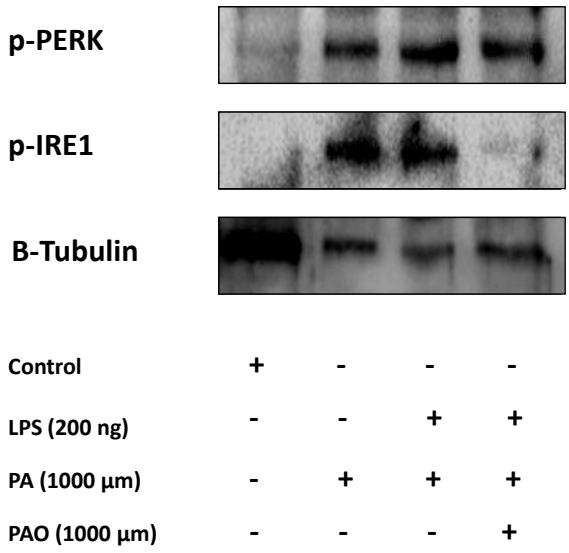


Fig. 4.12:Themodulatory effect of palmitoleate on palmitate-induced activation of the Unfolded Protein Response arms regulated by PERK and IRE1.

Primary BMDM were treated with 200 ng/ml LPS for 3 hours, 1000 µm/ml palmitoleate for 2 hours and finally 1000 µm/ml palmitate and palmitoleate together for 2 hours. Then cells were changed into meduium without serum and incubated for 22 hours. Cell lysates were collected and analyzed by

Western Blot using anti-p-PERK, anti-p-IRE1 andanti-B-tubulin.(Control=1% BSA+ethanol, LPS=Lipopolysaccaride, PA=Palmitate, PAO= Palmitoleate).

5. DISCUSSION AND CONCLUSION

Previous studies indicated that saturated free fatty acids such as palmitate can cause cardiovascular disease and metabolic disease by inducing endoplasmic reticulum stress, chronic inflammation and lipid accumulation [129-131]. One of the main consequences of palmitate-induced stress is the activation of NLRP3 inflammasome. Palmitate's effect on inflammasome is due to its ability to reduce autophagy and increase mitochondrial reactive oxygen species in murine macrophages[18].

The major focus of my thesis study is on the modulation of saturated fatty acid-induced inflammasome activation by a bioactive lipokine, palmitoleate. The effect of palmitoleate on palmitate-induced inflammasome activation was investigated at both the priming step and inflammasome complex formation stage. The results of my studies showed that palmitoleate can suppress palmitate-induced IL-1β expression in human monocytes and primary BMDM. These results suggest that palmitolate can modulate palmitate-stimulated priming step of inflammasome activation.

Following the stimulation of IL-1β and IL-18 expression, these factors can be processed into mature forms by the inflammasome and secreted from cells. These mature and secreted pro-inflammatory factors cause further stimulation of cells through recognition by their respective receptors. This leads to the production of secondary pro-inflammatory factors such asTNF-α[31]. In this study, the effect of palmitoleate on palmitate induced mRNA expression of TNF-α was investigated. The outcome of these experiments showed that palmitolate leads to areduction in palmitate-stimulated TNF-α. Hence, the decrease in IL-1β can be related to the decrease in this secondary factor.

From the previos studies it is known that palmitoleate prevents saturated free fatty acid-induced ER stress[124]. Moreover it also regulates metabolic homeostasis by enhancing insulin sensitivity and suppressinghepatostetosis[120-122]. On the other hand, it is known that the inflammasome is activated when it meets with danger signals such as metabolic overload of saturated free fatty acids or cholesterol. Activated inflammasome complex causes maturation and secretion of proinflammatory factors such as IL-1β and IL-18 by converting pro-caspase-1 to active form[5]. Here, I observed that palmitoleate treatment reduced palmitate-induced caspase-1 cleavage in LPS-primed primary BMDMs. PAO also significantly decreased palmitate-stimulated increase in IL-1β secretion in LPS-primed primary BMDMs. All of these results suggest that palmitoleate is a potential inhibitor for palmitate-induced inflammasome activation via decreasing caspase-1 activation and IL-1β secretion.

The inflammasome activation by palmitate is known to be mediated by the generation of mtROS[18]. mtROS is one of the main activators of inflammasome[5]. Here, I observed that palmitoleate decreased palmitate-stimulated mtROS production significantly. This suggests that palmitoleate's protective effect on palmitate-induced inflammasome activation could be related to the reduction in mtROS production.

AMPK is an important enzyme for cellular energy metabolism regulation that is activated via a phosphorylation at threonine-172 part[142]. It is known that palmitate causes decreased phosphorylation of AMPK and this results in a decrease in autophagy. As a result the elimination of dysfunctional mitochondria is compromised, leading to increased levels of mtROS[18]. The results of the study showed that palmitoleate can block palmitate-induced

reduction in the phosphorylation level of AMPK. This result indicates that the inhibitory effect of palmitoleate on palmitate-induced mtROS production is related to palmitoleate's ability to reactivate AMPK.In addition to them, it is known that liver kinase B1 (LKB1) is upstream part of AMPK activation. LKB1 phosphorylates and activates AMPK[18]. Therefore, to understand the possible role of AMPK activity regulation by palmitoleate, the effect of PAO on LKB1 regulation should be investigated.

The outcome of our studies indicate that palmitate, which at high exposure to the cells is a well known activator of UPR pathway,increasemtROS production in macrophages[136,18]. It is shown that palmitate caused calcium release from ER and excess amount calcium influx to mitochondriaand causes mitochondrial damage such as mtROS production[138-140]. In addition to them, recent studies give clues about possible role of UPR on mtROS production[95-99]. To illustrate for PERK branch of UPR, two studies suggested controversial results about the role of PERK on production of mtROS. One study suggests that PERK deficiency cause more ROS production when cells are stimulated with tunicamycin when comparing wild type cells while other study indicates that in PERK deficient cells , the contaction of ER-mitochondira and calcium signal between them are rearranged and ROS production increase in wild type when comparing PERK deficient cells. These controversial results can be related with different treatment conditions in studies and also the role of UPR in prosurvival and proapoptotic pathways[99,137].These findings lead to question whether there is a role for any of the UPR arms on the regulation of mitochondrial ROS generation. In this thesis, I was able to analyze the impact of PERK and IRE1 on formation of mtROS under lipotoxicity. Future studies should also include an investigation of the ATF6 branch in relation to mtROS generation. Here, I examined the role of PERK and IRE1 on mtROS production by using WT, $PERK^{-/-}$, or $IRE^{-/-}$MEF cells. Upon treatment with palmitate the release of mtROS was investigated by immunoflurorescent staining specific for mtROS (MitoSox assay from Invitrogen). I observed that while PERK had a suppressive role in palmitate-induced mtROS production, IRE1 promoted it. These data suggested that PERK and IRE1 have important but opposite rolesin the regulation of mtROS in lipotoxicity. In addition, mtROS is a main contributor for the second signal in inflammasome activation, so PERK and IRE1 can possibly have a modulatory effect on inflammasome activation via altering mtROS generation.Furthermore, palmitoleate's impact on inflammasome inhibition may be related to its ability to first block UPR activation and subsequent mtROS generation, the second hit for

inflammasome activation. However, palmitate's impact on UPR was studied in conditions that are not appropriate for inflammasome activation. Therefore, I analyzed the effect of palmitoleate on palmitate-stimulated activation of IRE1 and PERK under cellular conditions that also provide the two activating signals required for the inflammasome. I observed that palmitoleate reduced palmitate-induced phosphroylation of IRE1, but did not affect PERK phosphroylation. These results showed that under the conditions that activate the inflammasome (LPS and palmitate treatement together) UPR branches are both activated but palmitoleate only suppresses the IRE1 branch. These findings imply that palmitoleate may selectively modulate the IRE1 branch to regulate the inflammasome activation under lipotoxic stress. Future studies are needed to demonstrate whether palmitoleate's actions on mtROS are selectively regulated by the IRE1 branch. Futhermore, these findings spark an interest in identifying the direct target of IRE1 in regulating mtROS and inflammasome activation. These may be either protein or non-coding RNA targets. While there is no known protein target of IRE1, earlier studies have shown several miRNAs are regulated by the endoribonuclease activity of IRE1 in relation to inflammasome activation through mechanisms involving Thioredoxin-interacting protein (TXNIP)[103].

In conclusion, the results of my thesis studies clearly demonstrate a role for palmitoleate in modifying saturated fatty acid-induced mtROS generation and subsequent inflammasome activation. These studies demonstrate that upstream of mtROS production palmitoleate modifies both AMPK activity via altering its phosphorylation status and the IRE1 branch of the UPR under conditions that induce inflammasome activation. These exciting findings warrant future studies to investigate both the target of IRE1 and the mechanism of AMPK phosphorylation that is controlled by palmitoleate. One possibility is that AMPK phosphorylation is regulated by IRE1 itself, a hypothesis worth testing. Other possibilites are that IRE1 has a novel protein substrate that controls mitochondrial ROS metabolism or mitophagy. It's also plausible that similar to earlier mechanisms relating IRE1 to inflammasome activation, the unkown player downstream of IRE1 may be a non-coding RNA such as miRNA. Understanding the molecular details of lipid-regulation of inflammasome activity will provide new therapeutic avenues in the treatment of chronic metabolic and inflammatory diseases such as atherosclerosis, diabetes and obesity.

6. FUTURE PERSPECTIVE

In this thesis study, I investigated that modulatory effect of palmitoleate, a bioactive lipokine on saturated fatty acid induced inflammasome activation.I observed that palmitoleate eliminated saturated fatty acid-induced inflammasome activation by eliminating mtROS generation. Moreover my results indicated that palmitoleate modifies both AMPK activity through regaining phosphorylation and activation and IRE1 activity under inflammasome conditions. These data suggest that palmitoleate modulatory effect is through AMPK activity. Furthermore the regulation of IRE1 activity by palmitoleate suggests that IRE1 can be important upstream part of palmitate-induced inflammasome activation. These results give clues about modulatory effect of palmitoleate on palmitate-induced inflammasome activation and its possible mechanism of this modulation but further studies are needed to understand the mechanism of palmitoleate modification and the possible role of UPR branchs in inflammasome activation.

To further confirmation of the effect of palmitoleate on palmitate-induced inflammasome activation, other proinflammatory factor that is IL-18 maturation and secretion should be investigated. Moreover, to understand the mechanism of how palmitoleate affect on palmitate-

induced priming, the NF-κB activation should be investigated. In addition to them, to further investigation of the mechanism of palmitoleate modulation of palmitate-induced decrease in autophagy and mtROS, the effect of palmitolate on mitochondria structure and mitophagy formation should be analyzed by using imaging technics such as electron microscope.

My findings in this study cause another important question. It was observed that palmitoleate results in modulation of both AMPK activity and IRE1 activity under conditions which causes inflammasome formation. For this reason, firstly, whether palmitoleate effect is directly or indirectly on AMPK activity should be investigated. To achieve this aim, by using compound C that is specific AMPK inhibitor can be used. The modulatory effect of palmitoleate on palmitate-induced inflammasome activation should be analyzed after suppression of AMPK activity to answer this question.Also, how IRE1 arm of UPR modulates AMPK activity should be answered to further understand the possible mechanism of palmitoleate modulatory effect on palmitate-induced inflammasome activation. In addition to them, by using specific siRNA and inhibitor against to IRE1or IRE1knock out cells, the regulation of palmitate-induced IL-1β, IL-18 maturation and secretion, caspase-1 activation by IRE1 should be investigated. Moreover, the possible role of PERK and ATF6 on inflammasome activation should be analyzed. Also the modification of AMPK activity by these two branches of UPR should be investigated.

In the long term, as a future experiment, the possible substrate of IRE1 and IRE1 regulated noncoding RNA should be investigated because the inflmammasome activation also can be regulated with these downstream of IRE1 branch. For these investigations, to analyzing the possible substrate of IRE1, immunoprecipitation of IRE1 and then mass spectrometry analysis should be done while to investigation of possible IRE1 regulated noncoding RNA, RNA sequencing should be done. Understanding of possible molecular mechanism of lipid-induced inflammasome activation is important to suggest new target for developing new therapeutic approach to treatment of inflammasome related disorders such as atherosclerosis, diabetes and obesity.

Finally, after these in vitro studies, in vivo studies by using model organisms are required to investigate the possible effect of palmitoleate on inflammasome related metabolic disorders.

Therefore, the next step can be to identify the therapeutic potential of palmitoleate in vivo for metabolic disorders.

7.REFERENCES:

1) Takeuchi O, and Akira S. (2010).Pattern recognition receptors and inflammation.*Cell* 140, 805–820.

2)Martinon F, Mayor A, Tschopp J. (2009).The inflammasomes: guardians of the body. *Annu. Rev. Immunol.*, 27 (2009), pp. 229–265

3) Martinon F, Burns K, Tschopp J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10 (2): 417–26.

4) Latz E. (2010) The inflammasomes: Mechanisms of activation and function. *Curr Opin Immunol* 22:28–33.

5) Schroder K, and Tschopp J. (2010). The inflammasomes.*Cell* 140: 821-832.

6) Mariathasan S, Newton K, Monack D, Vucic D, French D, Lee W, Roose-Girma M, Erickson S, Dixit V. (2004). Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430 (6996): 213–8.

7) Cookson BT, Brennan MA. (2001). Pro-inflammatory programmed cell death. *TrendsMicrobiol* 9:113–114.

8) Dagenais M, Skeldon A, Saleh M. (2012). The inflammasome: in memory of Dr. Jurg Tschopp. *Cell DeathDiffer* 19: 5–12, 2012.

9)Boyden ED and Dietrich WF. (2006). Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet.* 38(2):240-4.

10)Martinon F, Petrilli V, Mayor A, Tardiel A, Tcshopp J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature.* 440(7081):237-41.

11)Miao EA, Alpuche-Aranda CM, Dors M, Clark AE, Bader MW, Miller SI, Aderem A. (2006). Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol.* 7(6):569-75.

12)Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. (2009). AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA.*Nature*. 458(7237):509-13.

13) Faustin B, Lartigue L, Bruey JM, Luciano F, Sergienko E,Bailly-Maitre B, Volkmann N, Hanein D, Rouiller I, Reed JC. (2007). Reconstituted NALP1 inflammasomereveals two-step mechanism of caspase-1 activation. *Mol. Cell* :713–24

14)Bruey JM, Bruey-Sedano N, Luciano F, Zhai D, Balpai R, Xu C, Kress Cl, Bailly-Maitre B, Li X, Osterman A, Matsuzama S, Terskikh AV, Faustin B, Reed JC. (2007). Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1. Cell 129 (1):45-56.

15)Sutterwala FS, Flavell RA. (2009) NLRC4/IPAF: a CARD carrying member of the NLR family. *Clin Immunol* 2009, 130:2-6.

16)Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440(7081): 228–232.

17)Rajamäki K, Lappalainen J, Oörni K, Välimäki E, Matikainen S, Kovanen PT, Eklund KK. (2010)Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. *PLoS ONE* 5, e11765 (2010).

18)Wen, H., D. Gris, Y. Lei, S. Jha, L. Zhang, M. T. Huang, W. J. Brickey, and J. P. Ting. (2011). Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nat. Immunol.* 12:408-415.

19) P´etrilli V, Papin S, Dostert C, Mayor A, Tschopp J. (2007). Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ.* 14:1583–89.

20)Tschopp J, Schroder K. (2010). NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat Rev Immunol 10(3): 210–215.

21) DeYoung KL, Ray ME, Su YA, Anzick SL, Johnstone RW, Trapani JA, Meltzer PS, Trent JM. (1997). Cloning a novel member of the human interferon-inducible gene family associated with control of tumorigenicity in a model of human melanoma. *Oncogene* 15:453–57.

22) Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E.(2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol.*2009; 183:787–791.

23)Kumar H, Kawai T, Akira S (2011) Pathogen Recognition by Innate Immune System. *International Reviews of Immunology* 30:16-34.

24)Newman ZL, Leppia SH, Moayeri M.(2009).CA-074Me protection against anthrax lethal toxin.*Infect. Immun.* 2009. 77:4327-4336.

25)Sato T, Machida T, Takahashi S, Iyama, S, Sato Y, Kuribayashi K, Takada K, Oku T, Kawano Y, Okamoto T, Takimoto R, Matsunaga T, Takayama T, Takahashi M, Kato J, Niitsu Y. (2004). Fas-mediated apoptosome formation is dependent on reactive oxygen species derived from mitochondrial permeability transition in Jurkat cells. *J. Immunol.* 2004. 173:285-296.

26) Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. (2008) Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 2008. 320:674-677.

27) Latz E.(2010) Nox-free inflammasome activation. Blood 2010. 116:1393-1394.

28)Zhou R, Yazdi AS, Menu P, Tschopp J.(2010) A role for mitochondria in NLRP3 inflammasome activation. *Nature* 2010. 469:211-225.

29)Sheedy FJ, Grebe A, Rayner KJ, Kalantari P, Ramkhelawon B, Carpenter SB, Becker CE, Ediriweera HN, Mullick AE, Golenbock DT, Stuart LM, Latz E, Fitzgerald KA, Moore KJ. (2013).CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation.*Nature Immunol.* 14, 812–820 (2013).

30) Podrez EA, Poliakov E, Shen Z, Zhang R, Deng Y, Sun M, Finton PJ, Shan L, Gugiu B, Fox PL, Hoff HF, Salomon RG, Hazen SL. (2002). Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. *J. Biol. Chem.* 277 (41): 38503–16.

31) Baillie AG, Coburn CT, Abumrad NA. (1996). Reversible binding of long-chain fatty acids to purified FAT, the adipose CD36 homolog. *J. Membr. Biol.* 153 (1): 75–81.

32) Tandon NN, Kralisz U, Jamieson GA. (1989). Identification of glycoprotein IV (CD36) as a primary receptor for platelet-collagen adhesion. *J. Biol. Chem.* 264 (13): 7576–83.

33)Rać ME, Safranow K, Poncyljusz W. (2007). Molecular basis of human CD36 gene mutations.*Mol. Med.*13 (5-6): 288-96.

34)Hotamisligil GS.(2006). Inflammation and metabolic disorders.*Nature* 444:860–67

35) Feve B, Bastard JP. (2009). The role of interleukins in insulin resistance and type 2 diabetes mellitus. *Nat. Rev. Endocrinol.* 5:305–11

36) Dinarello CA. (1998) Interleukin-1 beta, interleukin-18, and the interleukin-1 beta convertingenzyme.*Ann N Y Acad Sci* 1998; 856: 1–11.

37) Mandrup-Poulsen T, Pickersgill L, Donath MY. (2010). Blockade of interleukin 1 in type 1 diabetes mellitus.*Nat. Rev. Endocrinol.* 6:158–66.

38) Dinarello CA, Donath MY, Mandrup-Poulsen T.(2010) Role of IL-1β in type 2 diabetes. *Curr.Opin.Endocrinol.Diabetes Obes.*17, 314–321 (2010).

39) Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, Ravussin E, Stephens JM, Dixit VD.Vandanmagsar, B. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nature Med.* 17, 179–188 (2011)

40) Seino S and Study Group of Comprehensive Analysis of Genetic Factors in Diabetes Mellitus.(2001) S20G mutation of the amylin gene is associated with type II diabetes in Japanese. Study Group of Comprehensive Analysis of Genetic Factors in Diabetes Mellitus.*Diabetologia* 44, 906–909 (2001).

41) Stienstra R, van Diepen JA, Tack CJ, Zaki MH, van de Veerdonk FL, Perera D, Neale GA, Hooiveld GJ, Hijmans A, Vroegrijk I, van den Berg S, Romijn J, Rensen PC, Joosten

LA, Netea MG, Kanneganti TD.(2011) Inflammasome is a central player in the induction of obesity and insulin resistance. *Proc Natl Acad Sci* USA 2011; 108: 15324.

42) Hansson GK. (2005). Inflammation, atherosclerosis, and coronary artery disease.*N. Engl. J. Med.*352:1685–95

43)Yin Y, Pastrana JL, Li X, Huang X, Mallilankaraman K, Choi ET, Madesh M, Wang H, Yang XF. (2013). Inflammasomes: Sensors of metabolic stresses for vascular inflammation. *Front Biosci* 2013;18:638-49.

44) Galea J, Armstrong J, Gadsdon P, Holden H, Francis SE, Holt CM. (1996).Interleukin-1 beta in coronaryarteries of patients with ischemic heart disease.Arterioscler Thromb Vasc Biol. 1996; 16:1000–1006.

45) Mallat Z, Corbaz A, Scoazec A, Besnard S, Leseche G, Chvatchko Y, Tedgui A. (2001). Expression ofinterleukin-18 in human atherosclerotic plaques and relation to plaque instability. Circulation.2001; 104:1598–1603.

46) Kirii H, Niwa T, Yamada Y, Wada H, Saito K, Iwakura Y, Asano M, Moriwaki H, Seishima M. (2003). Lack of interleukin-1 beta decreases the severity of atherosclerosis in ApoE-deficient mice.*Arterioscler Thromb Vasc Biol.* 2003; 23:656–660.

47) Elhage R, Jawien J, Rudling M, Ljunggren HG, Takeda K, Akira S, Bayard F, Hansson GK. (2003). Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice. *Cardiovasc Res.* 2003; 59:234–240.

48) Menu P, Pellegrin M, Aubert JF, Bouzourene K, Tardivel A, Mazzolai L, Tschopp J.(2011). Atherosclerosis in ApoE-deficient mice progresses independently of the NLRP3inflammasome. *Cell Death Dis.* 2, e137 (2011).

49) Kolodgie FD, Narula J, Burke AP, Haider N, Farb A, Hui-Liang Y, Smialek J, Virmani R. (2000). Localization of apoptotic macrophages at the site of plaque rupture in sudden coronary death. *Am J Pathol.*2000; 157:1259–1268.

50) Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nuñez G, Schnurr M, Espevik T,Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung V, Latz E.(2010). NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464, 1357–1361 (2010).

51)Weber C, Noels H. (2011). Atherosclerosis: current pathogenesis and therapeutic options. Nat Med. 2011;17:1410–1422.

52) Kwon GP, Schroeder JL, Amar MJ, Remaley AT, Balaban RS.(2008).Contribution of macromolecular structure to the retention of low-density lipoprotein at arterial branch points.*Circulation* 117, 2919–2927 (2008).

53) Hansson GK, Hermansson A.(2011).The immune system in atherosclerosis.*Nat. Immunol.* 12, 204–212 (2011).

54) Mallat Z, Taleb S, Ait-Oufella H, Tedgui A. (2009). The role of adaptive T cell immunity in atherosclerosis. *J. Lipid Res.* 50:S364-S369.

55) Yvan-Charvet L, Pagler T, Gautier EL, Avaygen S, Siry RL, Han S, Welch CL, Wang N, Randolph GJ, Snoeck HW, and Tail AR.(2010). ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation.*Science* 328,1689-1693.

56) Kunjathoor VV, Febbraio M, Podrez EA, Moore KJ, Andresson L, Koehn S, Rhee JS, Silverstein R, Hoff H, Frreman MW. (2002). Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipd loading in macrophagres. *J.Biol. Chem.* 277,49982-49988.

57)Moore KJ, Kunjathoor VV, Koehn SL, Manning JJ, Tseng AA, Silver JM, McKee M, Frreman MW.(2005). Loss of receptor-mediated lipid uptake via scavenger receptor A or CD36 pathways does not ameliorate athersclerosis in hyperlipidemic mice. *J. Clin. Invest. 115*, 2192-2201.

58) Randolph GJ.(2008) Emigration of monocyte-derived cells to lymph nodes during resolution of inflammation and its failure in atherosclerosis.*Curr.Opin.Lipidol.*19, 462–468 (2008).

59) Ylä-Herttuala S, Bentzon JF, Daemen M, Falk E, Garcia-Garcia HM, Herrmann J, Hoefer I, Jukema JW, Krams R, Kwak BR, Marx N, Naruszewicz M, Newby A, Pasterkamp G, Serruys PW, Waltenberger J, Weber C, Tokgözoglu L. (2011). Stabilisation ofatherosclerotic plaques.Position Paper of the European Society of Cardiology (ESC).Working Group of Atherosclerosis and Vascular Biology.*Thromb.Haemost.*106, 1–19 (2011).

60) Tabas I. (2010). Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol*. 2010;10:36–46.

61) Chevet E, Cameron PH, Pelletier MF, Thomas DY, Bergeron JJM. (2001) The endoplasmic reticulum: integration of protein folding, quality control, signaling and degradation.*Current Opinion in Structural Biology*, vol. 11, no. 1, pp. 120–124, 2001.

62) English AR, Zurek N, Voeltz GK.(2009). Peripheral ER structure and function.*Curr.Opin. Cell Biol.* 21, 596–602.

63) Espenshade PJ, Hughes AL. (2007). Regulation of sterol synthesis in eukaryotes.*Annual Review of Genetics*, vol. 41,pp. 401–427, 2007.

64) Verkhratsky A.(2005). Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. *Physiological Reviews*, vol. 85, no. 1, pp. 201–279, 2005.

65) Kaufman RJ. (2002). Orchestrating the unfolded protein response in health and disease. *J Clin Invest* 110: 1389–1398.

66)Hetz C. (2012). The unfolded protein response: controlling cell fatedecisions under ER stress and beyond. *Nat. Rev. Mol. Cell Biol.*13 89–102.

67)Ron D, Walter P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response.*Nature Reviews MolecularCell Biology*, vol. 8, no. 7, pp. 519–529, 2007.

68) Walter P, Ron D.(2011). The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 334, 1081–1086.

69) Shamu CE, Walter P.(1996). Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus.*EMBO J.* 15, 3028–3039.

70) Tirasophon W, Lee K, Callaghan B, Welihinda A, Kaufman RJ.(2000). The endoribo-nuclease activity of mammalian IRE1 autoregulates its mRNA and is required for the unfolded protein response. *Genes Dev.* 14, 2725–2736.

71) Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. (2000). Cou-pling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1.*Science* 287, 664–666.

72) Kim R, Emi M, Tanabe K, Murakami S. (2006). Role of the unfolded protein response in cell death.*Apoptosis* 11, 5–13.

73) Zhao P, Xiao X, Kim AS, Leite MF, Xu J, Zhu X, Ren J, Li J, (2008). c-Jun inhibits thapsigargin-induced ER stress through up-regulation of DSCR1/Adapt78. *Exp. Biol. Med.* (Maywood) 233, 1289–1300.

74)Nguyen DT, Kebache S, Fazel A, Wong HN, Jenna S, Emadali A, Lee EH, Bergeron JJM, Kaufman Rj, Larose L, Chevet E. (2004). Nck-dependent activation of extracellular signal

regulated kinase-1 and regulation of cell survival during endoplasmic reticulum stress. *Mol Biol Cell* 15: 4248-4260.

75) Shi Y, Vattem KM, Sood R, An J, Liang J, Stramm L, Wek RC.(1998). Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control.*Mol. Cell. Biol.* 18, 7499–7509.

76) Harding HP, Novoa I, Zhang Y, Zeng H, Wek, R, Schapira, M, Ron, D.(2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* 6, 1099–1108.

77) Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. (2003). Nrf 2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol. Cell. Biol.* 23, 7198–7209.

78) Shen J, Prywes R. (2004). Dependence of site-2 protease cleavage of ATF6 on prior site-1 protease digestion is determined by the size of the luminal domain of ATF6. *J. Biol. Chem.* 279, 43046–43051.

79) Ma Y, Brewer JW, Diehl JA, Hendershot LM.(2002). Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. *J. Mol. Biol.* 318, 1351–1365.

80) Yamamoto K, Sato T, Matsui T, Sato M, Okada T, Yoshida H, Harada A, Mori K.(2007). Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. *Dev. Cell* 13, 365–376.

81) Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K.(2001). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 107, 881–891.

82) Hetz C, Chevet E, Harding HP. (2013).Targeting the unfolded protein response in disease.*Nat. Rev. Drug Discov.,* 12 (2013), pp. 703–719.

83) Ma Y, Brewer JW, Diehl JA, and Hendershot LM. (2002). Two distinctstress signaling pathways converge upon the CHOP promoter duringthe mammalian unfolded protein response. *J Mol Biol* 318:1351–1365, 2002.

84) Malhotra, J.D.; Kaufman, R.J.(2007). Endoplasmic reticulum stress and oxidative stress: A vicious cycle or a double-edged sword?*Antioxid.Redox Signal.*2007, *9*, 2277–2293.

85) Novoa I, Zeng H, Harding HP, and Ron D. (2001).Feedback inhibitionof the unfolded protein response by GADD34-mediated dephosphorylationof eIF2alpha.*J Cell Biol* 153: 1011–1022, 2001.

86) Yamaguchi H, and Wang HG. (2004). CHOP is involved in endoplasmicreticulum stress-induced apoptosis by enhancing DR5 expressionin human carcinoma cells. *J Biol Chem* 279: 45495–45502, 2004.

87)McCullough KD, Martindale JL, Klotz LO, Aw TY, and HolbrookNJ.(2001) Gadd153 sensitizes cells to endoplasmic reticulumstress by down-regulating Bcl2 and perturbing the cellular redoxstate. *Mol Cell Biol* 21: 1249–1259, 2001.

88) Morishima N, Nakanishi K, Tsuchiya K, Shibata T, and Seiwa E.(2004).Translocation of Bim to the endoplasmic reticulum (ER) mediatesER stress signaling for activation of caspase-12 during ERstress-induced apoptosis. *J Biol Chem* 279: 50375–50381, 2004.

89) Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, InoueK, Hori S, Kakizuka A, and Ichijo H.(2002). ASK1 is essential forendoplasmic reticulum stress-induced neuronal cell death triggeredby expanded polyglutamine repeats. *Genes Dev* 16: 1345–1355, 2002.

90)Cheung HH, Lynn Kelly N, Liston P, and Korneluk RG. (2006). Involvementof caspase-2 and caspase-9 in endoplasmic reticulumstress-induced apoptosis: a role for the IAPs. *Exp Cell Res* 312:2347–2357, 2006.

91)Dahmer MK.(2005). Caspases-2, -3, and -7 are involved in thapsigargininducedapoptosis of SH-SY5Y neuroblastoma cells. *J NeurosciRes* 80: 576–583, 2005.

92)Di Sano F, Ferraro E, Tufi R, Achsel T, Piacentini M, and CecconiF.(2006). Endoplasmic reticulum stress induces apoptosis by anapoptosome-dependent but caspase 12-independent mechanism.*J Biol Chem* 281: 2693–2700, 2006.

93) Morishima N, Nakanishi K, Takenouchi H, Shibata T, and YasuhikoY. (2002).An endoplasmic reticulum stress-specific caspase cascadein apoptosis.Cytochrome c-independent activation of caspase-9 by caspase-12.*J Biol Chem* 277: 34287–34294, 2002.

94) Raha S, Robinson BH.(2000). Mitochondria, oxygen free radicals, disease and ageing. *Trends Biochem.* Sci2000;25:502–508.

95) Tu BP, Weissman JS.(2004). Oxidative protein folding in eukaryotes: mechanisms and consequences. *J. Cell Biol* 2004;164:341–346.

96)Zhang K,Kaufman RJ. (2008).From endoplasmic-reticulum stress to the inflammatory response. *Nature*, 454 (2008), pp. 455–462.

97) Cuozzo JW, Kaiser CA. (1999).Competition between glutathione and protein thiols for disulphide-bondformation.*Nature Cell Biol* 1999;1:130–135.135.

98) Zhang DD. (2006). Mechanistic studies of the Nrf2-Keap1 signaling pathway. *Drug Metab. Rev* 2006;38:769–789.

99) Cullinan SB, Diehl JA.(2004). PERK-dependent activation of Nrf2 contributes to redox homeostasis andcell survival following endoplasmic reticulum stress. *J. Biol. Chem* 2004;279:20108–20117.

100) Strowig T, Henao-Mejia J, Elinav E and Flavell R.(2012). Inflammasomes in health and disease. *Nature* 2012; 481: 278–286.

101) Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. (2010). Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 2010; 11: 136–140.

102) Nishiyama A, Matsui M, Iwata S, Hirota K, Masutani H, Nakamura H, Takagi Y, Sono H, Gon Y, Yodoi J. (1999).Identification of thioredoxin-binding protein2/vitamin D(3) up-regulated protein 1 as a negative regulator of a thioredoxin function and expression. *J BiolChem* 1999; 274: 21645 21650.

103) Lerner AG, Uption J-P, Praveen PVK, Ghosh R, Nakagawa Y, Igbaria A, Shen S, Nguyen V, Backes BJ, Heiman M., Heintz N,Greengard P, Hui S, Tang Q, Trusina A, Oakes SA, Papa FR. (2012). IRE1a inducesthioredoxin-interacting protein to activate the NLRP3 inflammasome andpromote programmed cell death under irremediable ER stress. *Cell Metab*.

16, this issue, 250–264.

104)Oslowski, C. M., Hara, T., O'Sullivan-Murphy, B., Kanekura, K., Lu, S., Hara, M., Ishigaki, S., Zhu, L. J., Hayashi, E., Hui, S. T., Greiner, D., Kaufman, R. J., Bortell, R., and Urano, F. (2012) Thioredoxin-interacting protein mediates ER stress-induced beta cell death through initiation of the inflammasome. *Cell metabolism* 16, 265-273.

105)Kim S, Joe Y, Jeong SO, Zheng M, Back SH, Park Ws, Wryter S, Chung HT., (2013). Endoplasmic reticulum stress is sufficient for the induction of IL1B production via activation of the NF-kb and inflammasome pathways. *Innate Immunity*[Epub ahead of print].

106) Kitamura M. (2009) Biphasic, bidirectional regulation of NF-kappaBby endoplasmic reticulum stress.*Antioxid Redox Signal* 2009;11: 2353–2364.

107) Menu P, Mayor A, Zhou R, Tardivel A, Ichijo H, Mori K, Tschopp J. (2012). ER stress activates the NLRP3inflammasome via an UPR-independent pathway. Cell Death Dis2012; 3: e261.

108)Nakamura T, Furuhashi M, Li P, Cao H, Tuncman G, Sonenberg N, Gorgun CZ, Hotamisligil GS. (2010). Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasisCell, 140 (2010), pp. 338–348.

109)Lu B, Nakamura T, Inouye K, Li J, Tang Y, Lundbäck P,Valdes-Ferrer SI, Olofsson PS, Kalb T, Roth J, Zou Y, Erlandsson-Harris H, Yang H, Ting JP, Wang H, Andersson U, Antoine DJ, Chavan SS, Hotamisligil GS, Tracey KJ.(2012). Novel role of PKR in inflammasome activation and HMGB1 release. Nature 2012;488:670-4.

110) Kris-Etherton PM .(1999). AHA Science Advisory.Monounsaturatedfatty acids and risk of cardiovascular disease.*American Heart Association*.Nutrition Committee. Circulation 100(11):1253–1258.

111) Parthasarathy S, Khoo JC, Miller E, Barnett J, Witztum JL, Steinberg D. (1990). Low density lipoprotein rich in oleic acid is protected against oxidative modification: implications for dietary prevention of atherosclerosis. *Proc Natl Acad Sci U S A.* 1990 May;87(10):3894–3898.

112)Gillingham LG, Harris-Janz S, Jones PJ. (2011). Dietary monounsaturated fatty acids are protective against metabolic syndrome and cardiovascular disease risk factors. Lipids 46: 209–228, 2011.

113) Vessby B. (2000).Dietary fat and insulin action in humans. *Br J Nutr* 83(Suppl 1):S91–S96.

114) Karpe F, Dickmann JR, Frayn KN. (2011). Fatty acids, obesity, and insulin resistance: time for a reevaluation. Diabetes 2011;60:2441–2449.

115)Ros E. (2003).Dietary cis-monounsaturated fatty acids andmetabolic control in type 2 diabetes.*Am J Clin Nutr* 78(3Suppl):617S–625S.

116) Kien CL, Bunn JY, Ugrasbul F. (2005). Increasing dietary palmiticacid decreases fat oxidation and daily energy expenditure.*Am J Clin Nutr* 82(2):320–326.

117) Martín-Peláez1, S.; Covas, M.I.; Fitó, M.; Kušar, A.; Pravst, I. (2013). Health effects of olive oilpolyphenols: Recent advances and possibilities for the use of health claims. *Mol. Nutr. Food Res*.2013, *57*, 760–771.

118) Maguire LS, O'Sullivan SM, Galvin K, O'Connor TP, O'Brien NM. (2004). Fatty acid profile, tocopherol, squalene and phytosterol content of walnuts,almonds, peanuts, hazelnuts and the macadamia nut. *Int J Food Sci Nutr*2004, 5:171-178.

119) Ozogul Y, Ozogul F, Cicek E, Polat A, Kuley E. (2008). Fat content and fatty acidcompositions of 34 marine water fish species from the MediterraneanSea. *Int J Food Sci Nutr* 2008, 29:1-12.

120) Cao H, Gerhold K, Mayers JR, Wiest MM, Watkins SM, Hotamisligil GS. (2008). Identification of a lipokine, a lipid hormone linking adipose tissue tosystemic metabolism.*Cell* 2008, 134:933-944.

121)Tsuchiya Y, Hatakeyama H, Emoto N, Wagatsuma F, Matsushita S, Kanzaki M. (2010) Palmitate-induced down-regulation of sortilin and impaired GLUT4trafficking in C2C12 myotubes. *J Biol Chem* 2010;285:34371–81.

122) Bajaj M, Suraamornkul S, Romanelli A, Cline GW, Mandarino LJ, Shulman GI, DeFronzo RA. (2005). Effect of a sustained reduction in plasma free fatty acid concentration onintramuscular long-chain fatty acyl-CoAs and insulin action in type 2 diabetic patients. *Diabetes.* 2005;54:3148–53.

123)Stefan N, Kantartzis K, Celebi N, Staiger H, Machann J, Schick F, Cegan A, Elcnerova M, Schleicher E, Fritsche A, Häring HU.(2010).Circulating palmitoleate strongly and independently predicts insulinsensitivity in humans. *Diabetes Care* 2010;33:405–7.

124) Kuda O, Stankova B, Tvrzicka E, Hensler M, Jelenik T, Rossmeisl M,Flachs P, Kopecky J. (2009). Prominent role of liver in elevated plasma palmitoleate levels in response torosiglitazone in mice fed high-fat diet. *J Physiol Pharmacol* 2009;60:135–40.

125) Akazawa Y, Cazanave S, Mott JL, Elmi N, Bronk SF, Kohno S, Charlton MR, Gores GJ. (2010). Palmitoleateattenuates palmitate-induced Bim and PUMA up-regulation and hepatocytelipoapoptosis. *J Hepatol* 2010;52:586–93.

126)Kadotani A, Tsuchiya Y, Hatakeyama H, Katagiri H, Kanzaki M. (2009). Different impacts of saturated and unsaturated free fatty acids on COX-2 expression inC2C12 myotubes. *Am J Physiol Endocrinol Metab* 2009;297. E1291–E303.

127)Diakogiannaki E, Morgan NG. (2008). Differential regulation of the ER stress response by long-chain fatty acids in the pancreatic beta-cell. *Biochem Soc Trans*2008;36:959–62.

128) Weigert C, Brodbeck K, Staiger H, Kausch C, Machicao F, Häring HU, Schleicher ED. (2004). Palmitate, but not unsaturatedfatty acids, induces the expression of interleukin-6 in human myotubesthrough proteasome-dependent activation of nuclear factor-kappaB. *J Biol Chem* 2004;279:23942–52.

129) Prieur X, Roszer T, Ricote M. (2010). Lipotoxicity in macrophages: evidence from diseases associated with the metabolic syndrome. *Biochim Biophys Acta* 2010;1801:327e37.

130) Chapman MJ, Sposito AC. (2008). Hypertension and dyslipidaemia in obesity and insulinresistance: pathophysiology, impact on atherosclerotic disease andpharmacotherapy. *Pharmacol Ther* 2008;117:354e73.

131) Puren AJ, Fantuzzi G, Dinarello CA. (1999). Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in human blood mononuclear cells and mouse spleen cells. *Proc Natl Acad Sci U S A*96(5)*:*2256-61.

132) Schrauwen P, Hesselink MK. (2004).Oxidative capacity, lipotoxicity, andmitochondrial damage in type 2 diabetes.*Diabetes* 2004, 53:1412-1417.

133)Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG. (1996). Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J. Biol. Chem.* 271 (44): 27879–27887.

134) Karaskov E, Scott C, Zhang L, Teodoro T, Ravazzola M, VolchukA. (2006). Chronic palmitate but not oleate exposure induces endoplasmicreticulum stress, which may contribute to INS-1 pancreatic beta-cellapoptosis. *Endocrinology* 2006;147:3398–3407

135)Ricchi M, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, Fantoni LI, Marra F, Bertolotti M, Banni S, Lonardo A, Carulli N, Loria P. (2009). Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes.*J Gastroenterol Hepatol* 2009, 24:830-840.

136)Erbay E, Babaev VR., Mayers JR, Makowski L, Charles KN, Snitow M.E, Fazio S, Wiest MM, WatkinsSM., Linton MF, Hotamisligil GS.(2009). Reducing endoplasmic reticulum stress through a macrophage lipid chaperone alleviates atherosclerosis. *Nat. Med*. 15, 1383–1391.

137)Verfaillie T, Rubio Romero N, GARG A, Bultynck G, Rizzuto R, Siviero R, Decuypere J, Piette J, Linehan C, Gupta S, Samali A, Agostinis P. (2012). PERK is required at the ER-mitochondrial contact sites to convey apoptosis after ROS-based ER stress. *Cell death and differentiation, 19* (11), 1880-91.

138) Csordas G, Hajnoczky G. (2009). SR/ER-mitochondrial local communication:calcium and ROS. *Biochim.Biophys. Acta*, 1787 (2009), PP. 1352-1362.

139) Horng T. (2014).Calcium signaling and mitochondrial destabilization in the triggering of the NLRP3 inflammasome.*Trends in Immunology,* 35 (6), 253-261.

140)Borradaile NM, Han X, Harp JD, Gale SE, Ory DS, Schaffer JE.(2006). Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death.*Journal of Lipid Research* 47(12):2726-2737.

141) Egnatchik RA, Leamy AK, Jacobson DA, Shiota M, Young JD. (2014).ER calcium release promotes mitochondrial dysfunction and hepatic cell lipotoxicity in response to palmitate overload. *Molecular Metabolism*3 (2014) 544-553.