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Mitochondrial dynamics in response to fatty acids in human macrophages

Thesis

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1. Zusammenfassung

Chronische Entzündungsvorgänge im Fettgewebe begleiten die Entwicklung von Adipositas und dessen Folgekrankheiten wie Diabetes und Atherosklerose. Bei der Induktion von Entzündungen im Fettgewebe spielen Makrophagen eine Schlüsselrolle. Die Mechanismen ihrer Aktivierung sind jedoch noch nicht umfassend geklärt. Hypertrophe Adipozyten geben freie Fettsäuren in den extrazellulären Raum ab. Gesättigten Fettsäuren wie Palmitat stimulieren C-Jun-N-terminale Kinase (JNK) und Nuclear Factor kappa B (NF-κB) Signalwege und die Produktion entzündungsfördernden Zytokine wie IL-6, IL-8, IL-1β und TNFα in Makrophagen. Die Aktivierung entzündliche Wege durch Palmitat erfolgt durch Toll-ähnliche Rezeptoren (TLR) 2 und 4, sowie die Stressreaktionen des endoplasmatischen Retikulums (ER) und die erhöhte Diacylgycerin- (DAGs) und Ceramid-Produktion. Gesättigte Fettsäuren nehmen außerdem Einfluss auf den oxidativen Stoffwechsel der Zellen. So reduziert die mitochondriale Fettsäureoxidation ER-Stress und die Expression entzündlicher Zytokine in Palmitat-behandelten Makrophagen. Außerdem unterstützen reaktive Sauerstoffspezies (ROS) in den Mitochondrien die von Palmitat herbeigeführte Produktion entzündungsfördernder Zytokine. Kürzlich wurden mitochondriale Funktionen in Zusammenhang mit ihrer Morphologie betrachtet. Mitochondrien nehmen unterschiedliche Formen an, welche vom Zelltyp und den Stoffwechselanforderungen abhängen. Mitochondriale Fragmentierung wurde in β-Zellen und Myozyten als Reaktion auf Überernährung in Form ein hohen Glukose- und Fettsäurezufuhr festgestellt. Fragmentierte Mitochondrien stehen mit Funktionsstörungen, erhöhten Werten von ROS und Zelltod in Verbindung. Ziel dieser Studie ist es, die Rolle der mitochondrialen Fragmentierung in Palmitat-induzierten Entzündungen in menschlichen Makrophagen zu erforschen. In unseren Versuchen induzierten Fettsäuren unabhängig von ihrer Sättigung die mitochondriale Fragmentierung. Darüber hinaus führte eine Mischung aus langkettigen, gesättigten und ungesättigten Fettsäuren sowie die Lipolyse von triglyceridreichen Fettproteinen zu mitochondrialen Fragmentierung. Die Ausprägung der Fragmentierung in Palmitat-behandelten Makrophagen war abhängig von Dauer und Konzentration der Behandlung. Die Mitochondrien haben sich wieder in einem Netzwerk verbunden, sobald Palmitat aus dem Zellkultur-Medium entfernt wurde. Die Hemmung der Aktivität des dem Dynamin verwandten Protein 1(DRP1), welches die Spaltung der

Mitochondrien antreibt, reduzierte die mitochondriale Fragmentierung. Palmitat förderte die DRP1-Oligomerisierung. Weiterhin zeigten unsere Ergebnisse die Veränderungen der Membranlipidzusammensetzung in Mitochondrien als eine mögliche Ursache der mitochondrialen Fragmentierung. Die Hemmung der Palmitat-induzierten Fragmentierung von Mitochondrien erhöhte die mitochondriale ROS Produktion, die c-Jun-Phosphorylierung und die Expression von entzündungsfördernden Zytokinen. Zusammengenommen, zeigen unsere Ergebnisse, dass mitochondriale Fragmentierung ein Schutzmechanismus ist, welcher Palmitat-induzierte Entzündungsreaktionen abschwächt. Weitergehende Versuche sind nötig, um die Rolle eine mitochondrialen Fragmentierung bei den mit Adipositas in Verbindung stehenden Krankheiten in vivo zu untersuchen.

2. Summary

Obesity is considered as a type of chronic inflammation. It enhances the risk of developing cardiovascular disease, diabetes, and some cancers. The key players in the induction of inflammation in adipose tissue are macrophages. However the mechanism of macrophage activation in obese fat tissue is still not fully understood. Elevated level of saturated fatty acids in adipose tissue promotes inflammation and insulin resistance. Exposure of macrophages to saturated fatty acids stimulates pro-inflammatory c-Jun N-terminal kinase (JNK), nuclear factor kappa B (NF-κB) signaling, and production of pro-inflammatory cytokines, such as IL-6, IL-8, IL-1β, and TNFα. Palmitate is a major saturated free fatty acid released by adipocytes. It activates inflammatory pathways through Toll-like receptors (TLR) 2 and 4, provokes endoplasmic reticulum (ER) stress and increases levels of diacylglycerols (DAGs) and ceramides. Saturated fatty acids also affect cellular oxidative metabolism. Thus, mitochondrial fatty acid oxidation reduces ER-stress and expression of inflammatory cytokines in palmitate-treated macrophages. On the other hand mitochondrial reactive oxygen species (ROS) promote palmitate-mediated pro-inflammatory cytokine production. Recently, mitochondrial functions were linked to their morphology. Mitochondrial fission has been reported in β-cells and myocytes in response to high levels of glucose and free fatty acids, and was associated with disruption of mitochondrial functions, increased ROS level, and cell death. The aim of this study was to investigate the role of mitochondrial fragmentation in palmitate-induced inflammation in human macrophages. In our settings fatty acids, independently of their saturation, affected mitochondrial morphology. Mixtures of long chain saturated and unsaturated fatty acids as well as triglyceride-rich lipoprotein lipolysis products promoted mitochondrial fission. Mitochondrial fragmentation in palmitate-treated macrophages revealed a time- and concentration-dependent character, and was reversible upon palmitate removal. This observation, together with unaltered levels of mitochondrial protein and DNA content, and intact mitochondrial respiration, suggested that mitochondria were not damaged and were functionally active. Mechanistically, palmitate-induced mitochondrial fragmentation was not regulated by ER stress or loss of mitochondrial membrane potential. However, inhibition of palmitate incorporation into mitochondrial membrane phospholipids decreased mitochondrial fragmentation. Other approach to prevent mitochondrial fission was the inhibition of dynamin-related protein 1 (DRP1) activity,

which drives mitochondrial fission by forming ring- like structures around mitochondria and constricting mitochondrial membranes. Palmitate altered mitochondrial membrane lipid composition and promoted DRP1-oligomerization. The inhibition of palmitate-induced mitochondrial fragmentation enhanced mitochondrial ROS production, c-Jun phosphorylation, and upregulated expression of pro-inflammatory cytokines. Taken together, these results suggest that mitochondrial fragmentation is a protective mechanism attenuating palmitate-induced inflammatory responses. Future experiments will be required to investigate the role of mitochondrial fragmentation in obesity-associated diseases in vivo.

3. Abbrevations

AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
ATP	adenosine triphosphate
ATP5	ATP synthase subunit 5
CoA	coenzyme A
DAG	diacylglycerol
DRP1	dynamin related protein-1
ER	endoplasmic reticulum
FAO	fatty acid beta-oxidation
Fig.	figure
FIS1	fission protein-1
h	hour
JC-1	tetrachloro-tetraethyl benzimidazol carbocyanine iodide
JNK	c-Jun N-terminal kinase
IL	interleukin
LA	linoleate
LPA	lysophosphatidic acid
Mdivi-1	mitochondrial division inhibitor-1
MFF	mitochondrial fission factor
MFN	mitofusin
МΦ	macrophages
NF-κB	nuclear factor kappa B
OA	oleate
OCR	oxygen consumption rate
OPA1	optic atrophy protein-1
PA	palmitate
PaO	palmitoleate

PC	phosphatidylcholine
PE	phosphatidylethanolamine
PhA	phosphatidic acid
ROS	reactive oxygen species
SFA	saturated fatty acids
TGRL	triglyceride rich lipoproteins
TLR	toll-like receptors
TMRE	tetramethylrhodamine ethyl ester perchlorate
TNFα	tumor necrosis factor alpha
UCP	uncoupling proteins
UQCRC2	cytochrome b-c1 complex subunit 2
Δψm	mitochondrial membrane potential

4. Comprehensive Summary

4.1. Introduction

Macrophages are a type of white blood cell that recognizes, engulfs, and eliminates pathogens and apoptotic cells. The major function of macrophages is the maintenance of tissue homeostasis. This definition includes many different aspects of macrophages activities: elimination of invading pathogens, external particles and cell debris, wound healing, tissue remodeling, and immune regulation. To accomplish the broad diversity of their functions macrophages sensitively react to stimulus and are highly plastic cells (1). There are several classifications of macrophages according to their functions and localization. Tissue-resident and monocyte-derived macrophages can be defined as two subpopulations. Tissue-resident macrophages are mostly involved in tissue homeostasis in the steady state, they scavenge apoptotic cells and monitor invasion of pathogens. They are one of the first responders to infections. In case of infection resident macrophages get activated and more monocytes are recruited from the blood stream to the infection site. In the damaged tissue monocytes rapidly differentiate into macrophages and activate the immune response (2). The other classification of macrophages is based on functions upon the activation. There are two major subsets in this classification: M1 and M2 macrophages. The M1 and M2 definition was formulated in analogy to the Th1/Th2 polarization concept. Classically activated macrophages are called M1 type and they promote Th1 responses. This phenotype is induced by interferon gamma (IFNγ) and lipopolysaccharide or by several cytokines (e.g., TNFα and granulocyte-macrophage colony-stimulating factor). M1 macrophages activate inflammatory responses, have high antimicrobial activity and capacity to present antigen. M1 cells produce ROS, reactive nitrogen intermediates and pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6. Alternatively activated macrophages are called M2. They are activated by IL-4 and IL-13. M2 macrophages participate in Th2 responses, promote killing and encapsulation of parasites, and tissue repair (3).

Several types of tissue macrophages have specific names and functions such as Langerhans cells in the skin, Kupffer cells in the liver, and cardiac macrophages in the heart (4). Recently, a new subtype of macrophages was defined – adipose tissue macrophages. An increased amount of macrophages in adipose tissue correlates with inflammation and metabolic syndrome. In obesity macrophages are the essential

effector cells, they orchestrate metabolic inflammation in adipose tissue and induce insulin resistance. In healthy animals the phenotype of adipose tissue macrophage is similar to M2 macrophages. These macrophages in non-obese individuals were proposed to be involved in maintaining adipose tissue homeostasis and suppress inflammation through secretion of anti-inflammatory cytokines. However, enlarging white adipose tissue releases cytokines to recruit monocytes and stimulate M1 polarization (5). The altered balance between M2 and M1 macrophages is the first step in the reorganization of adipose tissue microstructure upon obesity. M1 macrophages form “crown-like structures” around dead adipocytes. Macrophages phagocytize dead adipocytes and become lipid engorged; lipid accumulation initiates pro-inflammatory cytokine expression, which promotes insulin resistance in the surrounding adipocytes (6). Pro-inflammatory cytokines also promote peripheral insulin resistance during infection to decrease nutrient storage. This metabolic adaptation is necessary for an effective defense against bacterial and viral pathogens, because activated immune cells preferentially utilize glycolysis to fuel their functions in host defense. In principle the initial decrease of insulin sensitivity can be an adaptation to limit nutrient storage. However, in obese adipose tissue this adaptive strategy becomes inefficient and harmful for the organism (4). Several pathways are involved in the induction of M1 phenotype in obese adipose tissue. Macrophages are not stimulated by classical M1 activators, such as IFNγ or any bacterial products. Macrophages are mainly exposed to free fatty acids released from adipocytes. In general, many lipids are considered as toxic for different cell types. Saturated fatty acids (SFAs) can induce inflammation by different pathways that could eventually cross-talk. SFAs activate TLR4 and TLR2 and trigger the unfolded protein response. Both of these pathways stimulate the expression of pro-inflammatory genes via JNK activation. Fatty acids can be incorporated into pools of phospholipids in cellular membranes, catabolized by beta-oxidation in mitochondria, or stored in form of triacylglycerols in lipid droplets. The role of mitochondria in SFA-induced inflammation is poorly investigated. A previous study from our laboratory demonstrated that β-oxidation reduces ER-stress in palmitate-treated macrophages (7). Another publication showed that hypoxia-induced mitochondrial ROS production enhances palmitate-induced inflammatory responses in macrophages (8).

Mitochondrial morphology varies from a complex network to a pool of small and disconnected organelles. Mitochondrial dynamics appears to influence all aspects of

mitochondrial function, including respiration, calcium buffering, ROS production, and apoptosis. In healthy cells mitochondrial fusion and fission are well-balanced. This balance is controlled by the actions of several GTP-binding proteins. DRP1 mediates mitochondrial fission by wrapping around constricted parts of mitochondria (9). Because DRP1 lacks a clear hydrophobic transmembrane domain, it is thought to bind mitochondrial outer membrane via resident receptor proteins: fission protein 1, mitochondrial fission factor (MFF), and mitochondrial dynamics protein of 49 and 51 kDa (10). Several post-translational modifications of DRP1 have been reported to regulate its translocation. Mitochondrial fusion is mediated by some other dynamin-related proteins in the outer and inner membrane. The first step of fusion involves physical tethering of two mitochondria via interactions between mitofusins (MFN) 1 and 2 on the outer membranes of two organelles. After outer membrane fusion, a related dynamin family GTPase, optic atrophy 1 (OPA1) promotes inner membrane fusion. Both activation of pro-fission proteins or inhibition of fusion proteins result in mitochondrial fragmentation. Mitochondrial fragmentation occurs in response to excess nutrition to reduce the respiratory capacity of cells (11). Furthermore, high levels of glucose and free fatty acids result in mitochondrial fission and damage (12). In addition, fragmented mitochondria are associated with elevated ROS production, inflammation, and insulin resistance (13, 14). On the other hand, mitochondrial fragmentation in β-cells is necessary for glucose-stimulated insulin secretion and promotes ATP production (15). Mitochondrial fission in response to fat overload can also be an adaptive mechanism as fragmented mitochondria have a high respiration efficiency to enhance the β-oxidation of fatty acids, thereby preventing lipotoxicity (16). In this study we investigated the effects of fatty acids on mitochondrial morphology in macrophages and its role in inflammatory responses.

4.2. Results and Discussion

In macrophages exposure to palmitate stimulates secretion of pro-inflammatory cytokines through Toll-like receptors, by activation of ER stress and accumulation of palmitate-derived lipids, such as ceramides. Mitochondria are involved in fatty acid metabolism and contribute to inflammatory responses. Although mitochondrial morphology regulates many mitochondrial functions, including oxidative phosphorylation, mitophagy, or apoptosis, the functional role of mitochondrial dynamics in palmitate-induced inflammation has never been studied before. I

observed that in monocyte-derived human macrophages mitochondria are highly interconnected and display a network in the cytosol. Treatment with palmitate caused a significant shortening of mitochondria and disruption of the network. The length of individual mitochondrial branches was traced per cell using ImageJ software, and mitochondrial form factor, averaging mitochondria length and the degree of branching, was quantified. To establish an experimental setting for studying palmitate-induced mitochondrial fragmentation I first analyzed different concentrations and time points of palmitate treatment. The results showed that mitochondrial fragmentation upon palmitate treatment occurred in a time- and concentration-dependent manner. In further experiments cells were exposed to 500 µM palmitate for 6 h, unless mentioned otherwise. Mitochondrial fragmentation is thought to be harmful for the cell, as it is associated with ROS production, mitochondrial damage and apoptosis (10). However, upon treatment with palmitate mitochondria in macrophages were not damaged, and I did not detect any reduction of mitochondrial protein and DNA level. Mitochondrial clustering in the perinuclear region is linked to mitochondrial dysfunction and cell death (17, 18), but it was not detectable in our model. Moreover, palmitate did not disrupt mitochondrial cristae structure and mitochondria were fused back to the network after palmitate was removed from the cell culture medium. To better understand the effect of palmitate on mitochondrial functions, I investigated mitochondrial oxygen consumption and ATP production. Palmitate did not alter oxygen consumption and ATP production in macrophages. Thus, the data indicated that mitochondria did not undergo mitophagy and remained functionally active. Previous studies demonstrated palmitate induced mitochondrial fission in muscle cells. However, mitochondrial fragmentation in myocytes upon palmitate stimulation (13, 19) was associated with increased oxidative stress, mitochondrial depolarization and loss of ATP production. In contrast, our data suggested that mitochondrial fragmentation did not lead to mitochondrial dysfunction and cell death.

To study mitochondrial fragmentation in response to fatty acids under more physiological conditions I treated macrophages with a mixture of palmitate and unsaturated fatty acids, such as oleate and linoleate. Moreover, I stimulated macrophages with human postprandial triglyceride-rich lipoproteins (TGRL). Incubations with the combination of fatty acids or TGRL disrupted the macrophage

mitochondrial networks after 6 h to a similar extent as palmitate. As an *ex vivo* model I assessed mitochondrial morphology in peritoneal macrophages from apolipoprotein E knockout (ApoE-/-) mice. After the isolation from peritoneal lavage mitochondrial form factor in macrophages from knockout mice was significantly lower as compared to wild type macrophages. However, mitochondria were in the network after the incubation in cell culture medium overnight. Mitochondrial form factor had the same value in ApoE-/- and wild type macrophages upon 24 h of culture. Similar effect was observed for palmitate-induced mitochondrial fragmentation in human macrophages: incubation in fresh medium restored mitochondrial morphology after palmitate treatment. Together, these results suggested that mitochondrial morphology is regulated by free fatty acids.

In further experiments I focused on palmitate-induced fragmentation to get insights to the role of mitochondrial dynamics in macrophage inflammatory responses to SFAs. In healthy cells mitochondrial dynamics is a well-balanced process. Both fission and fusion are controlled by the actions of several GTP-binding proteins. The inhibition of fusion as well as activation of fission unbalances mitochondrial dynamics, resulting in mitochondrial fragmentation. Upon dissipation of mitochondrial membrane potential (Δψm) OPA1 gets cleaved and loses the ability to tether inner mitochondrial membranes (20). Since long-chain fatty acids are known to induce loss of Δψm (21) I asked if palmitate treatment affects Δψm. Mitochondrial membrane potential decreased significantly after palmitate treatment, but it was recovered in an hour, and the cleavage of OPA1 was not detectable. Furthermore, the levels of proteins responsible for mitochondrial outer membrane fusion, such as MFN1 and MFN2, were not altered. Based on these results I concluded that palmitate does not affect the fusion machinery. Our further findings suggested that DRP1 regulates palmitate-induced mitochondrial fragmentation. DRP1 activity was blocked by siRNA knockdown, overexpression of a dominant-negative mutant, or pre-treatment with a DRP1 inhibitor Mdivi-1. These pharmacological and genetic inhibitions of DRP1 reduced mitochondrial fragmentation upon palmitate treatment. However, DRP1 mitochondrial translocation (9) was not involved in the activation of mitochondrial fragmentation by palmitate. Palmitate treatment promoted neither the expression, nor mitochondrial accumulation of DRP1. Confocal microscopy confirmed that DRP1 has been already localized on mitochondria in macrophages. The Pearson's correlation coefficient for co-localization of DRP1 and MFF did not significantly increase after the

exposure to palmitate. Although several publications showed that DRP1 phosphorylation and translocation drives mitochondrial fragmentation induced by palmitate in myocytes and endothelial cells (13, 22), DRP1 phosphorylation was low and remained unaltered in our settings. One of the critical steps in DRP1-mediated fission is DRP1 oligomerization and formation of division apparatus (23). Palmitate promoted DRP1 oligomerization, which may underlie mitochondrial fragmentation. My observations are in agreement with findings in β-cells and epithelial cells (24). In epithelial cells it was proposed that DRP1 is in dynamic equilibrium on mitochondria and that fission is driven by its oligomerization (23). In the described model actin filaments target productive oligomerization to fission sites. I postulate that membrane lipid composition regulates the formation of DRP1 oligomers. Mitochondrial membrane lipid composition is shown to regulate mitochondrial shape [26]. Although DRP1 does not have a known lipid-binding domain there is evidence that DRP1 activation can be influenced by membrane lipid composition. In vitro models demonstrated that DRP1 interacts with cardiolipin on liposomes (25, 26), and another recent publication showed that phosphatidic acid in mitochondrial membranes can control DRP1-mediated fission (27). Free fatty acids from the extracellular space are taken up by cells and rapidly converted into acyl-CoA, followed by fatty acid oxidation or incorporation into phospholipids, triglycerides, and their biosynthetic intermediates. The exposure to palmitate resulted in the upregulation of palmitate-containing lysophosphatidic acid (LPA), phosphatidic acid (PhA), diacylglycerol (DAG), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in mitochondrial membranes. To clarify whether conversion of palmitate to palmitoyl-CoA is crucial for mitochondrial fragmentation I inhibited the activity of long chain fatty acyl-CoA synthetases by triacsin C. The pre-treatment with triacsin C attenuated the effect of palmitate on mitochondrial morphology. In addition, the treatment with palmitoyl-CoA induced mitochondrial fragmentation similarly to the effect of palmitate. The mitochondrial fragmentation upon palmitoyl-CoA treatment was also regulated by DRP1 oligomerization. Several intermediates of phospholipid biosynthesis are known to impact mitochondrial dynamics, with PhA showing pro-fusion, and LPA and DAG pro-fission properties (28). Next I asked how the modulation of lipid synthesis pathways affects mitochondrial fragmentation. To inhibit the synthesis of DAG from phosphatidic acid I incubated cells with propranolol, a chemical inhibitor of phosphatidic acid phosphatase Lipin. Pre-treatment with propranolol significantly

decreased palmitate-induced mitochondrial fragmentation and enhanced accumulation of LPA and PhA while decreasing amounts of palmitoyl-containing PE and PC. Propranolol is commonly used as β-adrenoreceptor antagonist. To validate the effect of β-adrenoreceptor blocking on mitochondrial morphology macrophages were treated with sotalol, a potent β-adrenoreceptor inhibitor. Incubation with sotalol followed by palmitate treatment did not attenuate mitochondrial fragmentation. To prove the specificity of propranolol on Lipin activity siRNA knockdown of LPIN1 and 2 was performed in macrophages. Silencing of LPIN1 and 2 reduced mitochondrial fragmentation after treatment with palmitate. These results confirmed that PhA/DAG ratio regulates mitochondrial morphology in response to fatty acids. Further investigation of exact lipid or combination of lipid species that drive mitochondrial fragmentation seemed very challenging because lipid biosynthesis contains many reaction steps which are fine adjusted in cells. Accumulation of one lipid species leads to alterations in the balanced system of lipid metabolism. Nevertheless palmitate-induced mitochondrial fragmentation can be attenuated using DRP1 activity inhibition and prevention of palmitate incorporation into cellular lipids. Applying these approaches to keep mitochondria interconnected upon palmitate treatment I studied the impact of mitochondrial shape on palmitate-induced inflammatory responses. Our previous results indicated that mitochondrial ROS production contributes to inflammation in response to SFAs under hypoxia (8). Although palmitate does not affect mitochondrial ROS production under normoxic conditions in macrophages, preventing mitochondrial fragmentation in control macrophages increased fluorescence intensity of MitoSOX Red, which is a mitochondrial superoxide-sensitive fluorescent dye. Next, mitochondrial ROS formation was measured when palmitate-induced mitochondrial fragmentation was inhibited either by blocking DRP1 activity or reducing palmitate incorporation into cellular lipids. Both approaches notably increased mitochondrial ROS production. These results suggested that if mitochondria lose their ability to disconnect upon palmitate treatment they produce significant amounts of ROS. In several studies nutritional overload induced either by fatty acids or high glucose was associated with mitochondrial fragmentation, ROS production, mitochondrial damage, and apoptosis (10, 12, 29, 30). In contrast, in our model mitochondria did not produce ROS and were not irreversibly damaged by palmitate. Mitochondrial ROS production is associated with changes of Δψm (31, 32). In our system palmitate promoted loss of Δψm, which was transient and recovered by

the time mitochondria became fragmented. However, pre-treatment with Mdivi-1 prevented Δψm drop, mitochondrial fragmentation, and stimulated ROS production. I claim that inhibition of fragmentation increases Δψm and leads to mitochondrial ROS formation. Our previous observations suggested that the JNK cascade is sensitive to mitochondrial ROS during palmitate-induced inflammation (8). Therefore, I checked whether the reduction of mitochondrial fragmentation increases phosphorylation of JNK target c-Jun. The level of phosphorylated c-Jun was higher after preventing mitochondrial fragmentation using Mdivi-1, propranolol, or overexpression of the dominant-negative DRP1 K38A mutant. Analyzing the expression and secretion of pro-inflammatory cytokines I observed that inhibition of mitochondrial fragmentation enhanced expression of IL-6, IL-8, and TNFα mRNAs and IL-8 protein secretion (Figure 1). Interestingly, depletion of Lipin2 in macrophages enhanced the expression of pro-inflammatory cytokines mediated by saturated fatty acids (33). In that study authors have not assessed mitochondrial morphology. Induction of mitochondrial fragmentation in hepatocytes by knockdown of MFN1 protected against insulin resistance induced by a high-fat diet (16). Recently, published data demonstrate that mitochondrial fragmentation in β-cells stimulates ATP generation and it is necessary for glucose-stimulated insulin secretion (15). I espouse an idea that fragmented mitochondria oxidize more fatty acids and reduce accumulation of toxic lipids and their intermediates. Thus, fatty acid-induced mitochondrial fragmentation is a preventive mechanism to mitigate SFA-induced inflammation.

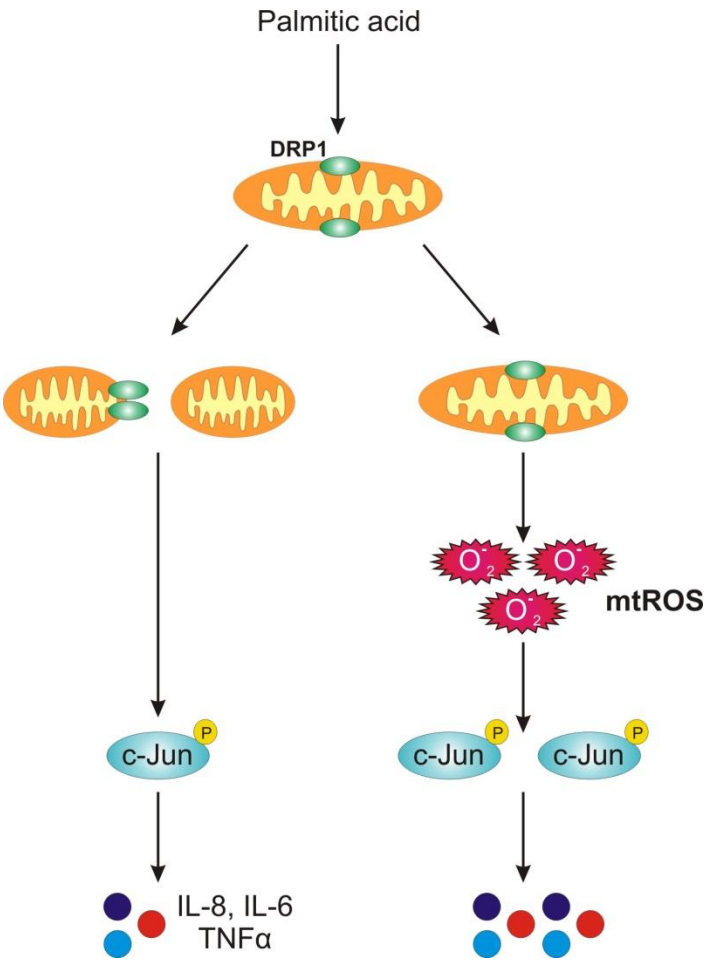

Figure 1: Protective role of mitochondrial fragmentation in palmitate-induced inflammatory responses.

4.3. Conclusion

This study aimed to investigate the mechanism and the roles of palmitate-induced mitochondrial fragmentation in primary human macrophages. Fatty acids, such as palmitate, cause fragmentation of mitochondria, which display an interconnected network diffusely distributed throughout the cytosol in untreated macrophages. Mechanistically, mitochondrial fragmentation required DRP1 and was regulated by incorporation of palmitate into mitochondrial phospholipids and their precursors. The alteration in mitochondrial lipid composition provoked DRP1 oligomerization and mitochondrial fission. This work provides evidence that mitochondrial proteins and DNA are not damaged by palmitate, and mitochondria retain their functionality. Functionally, inhibition of mitochondrial fragmentation increased ROS production, JNK signaling, and pro-inflammatory cytokine expression in the context of palmitate-

induced inflammation. Collectively, my results indicate that mitochondrial fragmentation is a protective mechanism, which prevents ROS production and decreases inflammatory responses in human macrophages. In future experiments the physiological relevance of mitochondrial dynamics should be addressed more in detail. Since some anti-diabetic drugs target mitochondrial functions, the morphology of mitochondria should be considered upon their application.

5. Publications

1. **Zezina E**, Snodgrass RG, Schreiber Y, Zukunft S, Schürmann C, Heringdorf DMZ, Geisslinger G, Fleming I, Brandes RP, Brüne B, Namgaladze D. Mitochondrial fragmentation in human macrophages attenuates palmitate-induced inflammatory responses. Biochim Biophys Acta. 2018 Feb 2;1863(4):433-446.
2. Snodgrass RG, Boß M, **Zezina E**, Weigert A, Dehne N, Fleming I, Brüne B, Namgaladze D. Hypoxia Potentiates Palmitate-induced Pro-inflammatory Activation of Primary Human Macrophages. J Biol Chem. 2016 Jan 1;291(1):413-24.

6. Publication

Mitochondrial fragmentation in human macrophages attenuates palmitate-induced inflammatory responses

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Abbreviations used in this article: ad-virDRP1K38A dominant-negative DRP1; AICAR 5-aminoimidazole-4-carboxamide ribonucleotide; ATP5 ATP synthase subunit 5; DAG diacylglycerol; DRP1 dynamin related protein 1; ER endoplasmic reticulum; FAO fatty acid beta-oxidation; JC-1 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazol carbocyanine iodide; JNK c-Jun N-terminal kinase; LA linoleate; LPA lysophosphatidic acid; Mdivi-1 mitochondrial division inhibitor-1; MFF mitochondrial fission factor; MFN mitofusin; OA oleate; OCR cellular oxygen consumption rate; OPA1 optic atrophy protein 1; PA palmitate; PaO palmitoleate; PC phosphatidylcholine; PE phosphatidylethanolamine; PhA phosphatidic acid; ROS reactive oxygen species; TGRL triglyceride rich lipoproteins; TLR toll-like receptors; TMRE tetramethylrhodamine ethyl ester perchlorate; UCP uncoupling proteins;UQCRC2 cytochrome b-c1 complex subunit 2; Δψm mitochondrial membrane potential.

Highlights

- Fatty acids promote mitochondrial fragmentation in human macrophages
- Palmitate-induced mitochondrial fragmentation is DRP1-dependent
- Mitochondrial fragmentation attenuates inflammatory responses

Abstract

Macrophages in adipose tissue contribute to inflammation and the development of insulin resistance in obesity. Exposure of macrophages to saturated fatty acids alters cell metabolism and activates pro-inflammatory signaling. How fatty acids influence macrophage mitochondrial dynamics is unclear. We investigated the mechanism of palmitate-induced mitochondrial fragmentation and its impact on inflammatory responses in primary human macrophages. Fatty acids, such as palmitate, caused mitochondrial fragmentation in human macrophages. Increased mitochondrial fragmentation was also observed in peritoneal macrophages from hyperlipidemic apolipoprotein E knockout mice. Fatty acid-induced mitochondrial fragmentation was independent of the fatty acid chain saturation and required dynamin-related protein 1 (DRP1). Mechanistically, mitochondrial fragmentation was regulated by incorporation of palmitate into mitochondrial phospholipids and their precursors. Palmitate-induced endoplasmic reticulum stress and loss of mitochondrial membrane potential did not contribute to mitochondrial fragmentation. Macrophages treated with palmitate maintained intact

mitochondrial respiration and ATP levels. Pharmacological or genetic inhibition of DRP1 enhanced palmitate-induced mitochondrial ROS production, c-Jun phosphorylation, and inflammatory cytokine expression. Our results indicate that mitochondrial fragmentation is a protective mechanism attenuating inflammatory responses induced by palmitate in human macrophages.

Inroduction

Nutritional overload accompanied by excessive lipid accumulation provokes pathologies such as metabolic syndrome, type 2 diabetes, and resulting cardiovascular complications. Chronic inflammation in adipose tissue is crucial to the development of obesity-related metabolic dysfunctions [1, 2]. Saturated fatty acids released by adipocytes, such as palmitate, activate inflammatory signaling in macrophages to stimulate the synthesis and secretion of pro-inflammatory cytokines [3, 4] thus, promoting adipose tissue inflammation.

The complexity of the cellular response to palmitate is not fully understood. Palmitate activates inflammatory pathways through Toll-like receptors (TLR) 2 and 4 [3, 5]. Intracellularly, fatty acids are incorporated in membrane phospholipids, triglycerides, and their biosynthetic intermediates. Increased saturation of phospholipid chains in endoplasmic reticulum (ER) membranes has been proposed to activate the ER-stress response [6, 7]. In addition, fatty acid overload increases levels of diacylglycerols (DAGs) and ceramides, contributing to inflammation [8, 9]. By these mechanisms the exposure of macrophages to saturated fatty acids activates c-Jun N-terminal kinase (JNK) and nuclear factor κB signaling, resulting in the expression of pro-inflammatory cytokines.

Mitochondria are central metabolic organelles. In addition to their ATP-producing and biosynthetic roles, mitochondria are involved in cellular lipid metabolism, Ca2+ homeostasis, and inflammatory responses [10]. Mitochondrial fatty acid β-oxidation (FAO) is a key process in fatty acid catabolism. We previously reported that FAO attenuates inflammatory and ER-stress responses in palmitate-treated macrophages [11]. Mitochondria also contribute to pro-inflammatory cytokine production through the generation of reactive oxygen species (ROS) [12]. Although palmitate does not promote mitochondrial ROS formation itself, hypoxia-induced mitochondrial ROS enhance the expression and secretion of pro-inflammatory cytokines in palmitate-treated macrophages [13].

Mitochondria are highly dynamic organelles with the ability to fuse into complex networks or disperse into separate small and roundish organelles. Mitochondrial elongation is mediated by mitofusin (MFN) 1 and 2, and optic atrophy 1 (OPA1) protein. While MFN1 and MFN2 are responsible for fusion of the outer mitochondrial membrane, OPA1 is located in the inner mitochondrial membrane [14]. Dynamin-related protein 1 (DRP1) is a central regulator of mitochondrial fission. The mechanism of DRP1 activation is thought to involve its translocation from cytosol to the outer mitochondrial membrane. This is mediated by several adaptor proteins such as mitochondrial fission factor (MFF) and fission protein 1. Oligomerization of DRP1 in ring-like structures and consequent GTP hydrolysis causes membrane constriction, resulting in mitochondrial division [15]. DRP1 activity can be regulated by phosphorylation and other post-translational modifications of DRP1 and its receptors [16]. Mitochondrial morphology regulates many mitochondrial functions, such as oxidative phosphorylation, mitophagy, or apoptosis [17-19].

Mitochondrial fragmentation occurs in response to excess nutrition to reduce the respiratory capacity of cells [20]. Thus, high levels of glucose and free fatty acids result in mitochondrial fission and damage [21]. In addition, fragmented mitochondria are associated with elevated ROS production, inflammation, and insulin resistance [22, 23]. In contrast, mitochondrial fragmentation in β-cells stimulates ATP production and is required for glucose-stimulated insulin secretion [24]. In hepatocytes mitochondrial fragmentation protects against insulin resistance, induced by a high-fat diet [25]. Furthermore, mitochondrial fission assists in the removal of damaged mitochondria by mitophagy [26]. These findings suggest that mitochondrial fragmentation may also have protective roles in cellular pathophysiology.

In this study we investigated the effects of fatty acids on mitochondrial morphology in macrophages and its role in inflammatory responses. We provide evidence that mitochondrial fission in response to fat overload can be an adaptive mechanism attenuating ROS formation and inflammatory reactions elicited by saturated fatty acids.

Material and methods

Cell culture

Human peripheral blood monocytes were isolated from commercially obtained buffy coats from anonymous donors (DRK-Blutspendedienst Baden-Württemberg-Hessen, Institut für Transfusionsmedizin und Immunhämatologie, Frankfurt, Germany) as previously described [13]. The ethics committee of Goethe-University waived the necessity of written informed consent when using the buffy coats from anonymized blood donors. J774 and THP1 cell lines were purchased from ATCC. Mouse care and experiments involving mice were approved by and followed the guidelines of the Hessian animal care and use committee. ApoE knockout mice were purchased from Taconic M&B A/S (Ejby, Denmark, strain B6.129P2-Apoetm1Unc N6) and bred at the local animal care facility under standard conditions with 12/12 hour dark/light cycle and free access to rodent chow diet (Altromin 1324) and water. Animals were sacrificed at an age between 10 and 12 weeks. Primary mouse peritoneal macrophages were collected by peritoneal lavage, seeded on chambered coverslides (ibidi GmbH), and washed to remove non-adherent cells. When indicated, cells were treated with 10 µM orlistat (Cayman), 500 µM AICAR (Enzo), 5 µM genipin (Cayman), 5 µM triacsin C (Enzo), 100 µM propranolol (Sigma-Aldrich), 50 µM sotalol (Sigma-Aldrich), 50 µM Mdivi1 (Enzo), 100 µM GSK 137647, 100 µM AH 7614 (Tocris) for 1 h prior to incubations with palmitate. Cells were stimulated with 1 µg/ml tunicamycin (Sigma-Aldrich) and 100 nM thapsigargin (Enzo) for 4 h, or with 100 µM palmitoyl-CoA (Larodan) for 6 h.

Preparation of fatty acids and triglyceride-rich lipoproteins

Palmitate, oleate, linoleate (Sigma-Aldrich), and palmitoleate (Cayman) were prepared by diluting 100 mM stock solution in 70% ethanol/0.1 M NaOH into 10% fatty acid-free, low-endotoxin bovine serum albumin (BSA) solution (Sigma-Aldrich A-8806, adjusted to pH 7.4) to obtain a molar ratio between a fatty acid and BSA 6:1. BSA was used in control incubations. Triglyceride-rich lipoproteins were kindly provided by Prof. Jörg Heeren, Universitätsklinikum Hamburg-Eppendorf, Germany.

Oxygen consumption rate analysis

The cellular oxygen consumption rate (OCR) was analyzed using a Seahorse 96 extracellular flux analyzer (Agilent). Macrophages were plated in Seahorse 96-well cell culture plates at 3.5-5x104 cells/well one day before the assay and equilibrated for 1 h in Krebs Henseleit buffer (111 mM NaCl, 4.7 mM KCl, 1.25

mM CaCl2, 2 mM MgSO4, 1.2 mM NaH2PO4) supplemented with 11 mM L-glucose and 2 mM L-glutamine. Cells were treated with 2.5 µM oligomycin (Sigma-Aldrich), 1 µM CCCP (Sigma-Aldrich), 1 µg/ml antimycin (Sigma-Aldrich) and 1 µM rotenone (Sigma-Aldrich) as indicated.

ATP determination

Macrophages were treated with 500 µM palmitate for 6 and 24 h, and cells were harvested in boiling water. ATP concentrations were measured by an ATP Determination Kit (A22066) (Molecular Probes).

RNA extraction and quantitative real-time PCR

Total RNA from 1x106 cells was isolated using peqGOLD RNAPure reagent (PeqLab Biotechnology) according to manufacturer´s protocol and transcribed using the Maxima first-strand cDNA synthesis kit (Thermo Scientific). Quantitative real-time PCR assays were performed with the iQ Custom SYBR Green Supermix (Bio-Rad) using the CFX96 system (Bio-Rad). Primer sequences are available upon request.

Western blot analysis

Cell pellets were harvested in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 0.5% NP-40, 1 mM PMSF, protease inhibitor cocktail). Protein lysates were sonicated, centrifuged at 12000xg for 10 min at 4°C, the supernatants were heat-denatured in 5x Laemmli sample buffer and separated on polyacrylamide gels, followed by transfer onto nitrocellulose membranes. Primary antibodies directed against MFN2 (#GTX102055, Acris antibodies), MFN1 (AF7880, R&D), core protein 2 of bovine ubiquinol-cytochrome c reductase (UQCRC2, complex III), bovine ATP synthase α/β subunits (ATP5, complex V) (rabbit polyclonal) (provided by Dr. I. Wittig), DRP1 (#8570), phospho-DRP1 (S637, #6319), phospho-DRP1 (S616, #34555), phospho-c-Jun (Ser-73, #3270, all from Cell Signaling), OPA (612606, BD Biosiences), actin (A-2066, Sigma-Aldrich), nucleolin (sc-13057, Santa Cruz Biotechnology) were used followed by IRDye 680 or IRDye 800-coupled secondary antibodies (LICOR Biosciences). Blots were visualized and quantified using the Odyssey imaging system (Licor Biosciences). To analyze DRP-1 oligomerization, the boiling step was omitted and 50 µg of samples were subjected to non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (without dithiothreitol in the loading buffer) on 6.5% polyacrylamide gels. For mitochondrial fractionation,

macrophages were rinsed with PBS then harvested in mitochondria buffer (250 mM sucrose, 1 mM EDTA, 20 mM Tris-HCl, protease and phosphatase inhibitor cocktails, pH 7.5). Cell suspensions were passed through a 25G needle 15 times followed by centrifugation at 1000 g for 10 min. The supernatants were centrifuged at 10000xg for 15 min. The pellet and supernatant represented the mitochondrial and cytosolic fractions, respectively.

Cytokine secretion analysis

Macrophage supernatants were analyzed for IL-8 using a cytometric bead array (BD Biosciences) according to manufacturer´s protocol. Samples were measured using a LSRII/Fortessa flow cytometer (BD Biosciences).

RNA interference

Knockdown of DRP1 and UCP2 were performed by siRNA (siGENOME human SMARTpool, Thermo Scientific) at 50 nM and Hyperfect transfection reagent (Qiagen) according to the manufacturer‘s recommendations. Cells were treated 72 h post-transfection.

Adenoviral transduction of macrophages

For adenoviral transduction, macrophages were incubated with control adenovirus (Ad-Track-GFP, Addgene) or adenovirus coding a dominant-negative DRP1 mutant (pcDNA3-DRP1K38A, Addgene) 48 h before treatments.

Microscopy

Human macrophages were seeded onto 8-well chambered coverslides (ibidi GmbH) and stained with 0.5 µM MitoTracker Green (Molecular Probes) for 15 min at 37°C before live imaging or fixation. Mouse peritoneal macrophages were seeded on chambered coverslips after the isolation from peritoneal lavage. Non-adherent cells were removed by washing upon 2 h of incubation. Macrophages were stained with 0.5 µM MitoTracker Green for 15 min prior to microscopy. For immunofluorescence cells were fixed by incubating with 4% paraformaldehyde (Sigma) for 20 min at room temperature, permeabilized with 0.1% TritonX-100 (Sigma) for 30 min at room temperature and incubated with primary antibodies in 5% BSA overnight at 4°C. Cells were incubated with secondary antibodies (Thermo Scientific) for two hours at room temperature. All fluorescence imaging was performed using a Plan-Apochromat 63×/1.4 oil objective on a Zeiss LSM 510 confocal microscope driven by Zen 2009 software (Carl Zeiss). Scoring of mitochondrial network morphology was performed by ImageJ software. Form

factor (FF) and aspect ratio were used to determine mitochondrial shape [27]. FF [(perimeter2)/(4π•surface area)] reflects combined measure of length and degree of branching, aspect ratio [(major axis)/(minor axis)] is the ratio between the major and minor axis of the ellipse equivalent to the mitochondria. Both parameters are independent of image magnification and have a minimal value of 1. Aspect ratio and form factor were decreased upon palmitate treatment in human primary macrophages. We chose FF as a representative criterion for mitochondrial morphology for further assays. All quantifications were done in more than 50 cells per experiment and n≥3 of experiments.

Measurements of mitochondrial membrane potential

The mitochondrial membrane potential was determined using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazol carbocyanine iodide (JC-1 BioVision) and tetramethylrhodamine ethyl ester perchlorate (TMRE, Thermo Scientific). Cells were stained with 5 µM JC-1 for 30 minutes at 37 °C after the treatment with palmitate, or 10 nM TMRE for 15 min at 37 °C before palmitate treatment. Fluorescence was measured by a LSRII/Fortessa flow cytometer (BD Biosciences). For fluorescence microscopy, cells were stained with TMRE and with MitoTracker Green (Molecular Probes) as described above and imaged by Zeiss LSM 510 (Carl Zeiss).

Mitochondrial ROS detection

Mitochondrial ROS generation in macrophages was detected by MitoSOX (Life Technologies). Macrophages were incubated with 5 µM MitoSOX for 30 min at 37 °C. Fluorescence was analyzed using a LSRII/Fortessa flow cytometer (BD Biosciences). For fluorescence microscopy, cells were stained with MitoSOX and with MitoTracker Green (Molecular Probes) as described above and imaged by Zeiss LSM 510 (Carl Zeiss).

Analysis of intracellular triglycerides

Cell pellets were lysed in PBS containing 1% (vol./vol.) Triton X-100. Determination of triglycerides in lysates was performed using a commercial kit from Roche Diagnostics according to manufacturer instructions.

Lipid analysis by LC-MS/MS

Lipid extraction and quantification was performed as described [28] with some modifications to focus on the extraction of DAGs and phospholipids using a two-phase liquid-liquid extraction method. Lipids from mitochondrial fraction were

analyzed using an Agilent 1290 Infinity UHPLC system coupled to a QTrap 5500 mass spectrometer (Sciex). Chromatographic separation of lysophosphatidic and phosphatidic acids was performed using a Luna C18 (2) Mercury column (20 x 2 mm Phenomenex) with a same material pre-column. Quantification was performed with MultiQuant software version 3.02 (Sciex) using the internal standard method. Liquid chromatography separation of lipids for DAG and phospholipids detection was performed using a Kinetex C18 column (1.7 µm, 100x2.1 mm, Phenomenex) at 40°C. Analyst 1.6.2 and MultiQuant 3.0 (both from Sciex), were used for data acquisition and analysis, respectively.

Statistical analysis

Graphical data are presented as mean ± S.E. of at least three independent experiments. Data were analyzed by one-way ANOVA test (GraphPad Prism). Differences were considered statistically significant at $p < 0.05$.

Results

Fatty acids induce mitochondrial fragmentation in macrophages

In primary human macrophages mitochondria exist as an interconnected network distributed diffusely throughout the cytosol. Treatment with palmitate disrupted the mitochondrial network in primary human cells (Fig. 1A), THP1 human, and J774 mouse macrophage cell lines (Suppl. Fig. 1A, B). To determine whether fatty acid saturation is crucial for mitochondrial fragmentation we further treated macrophages with unsaturated fatty acids palmitoleate, oleate and linoleate. Treatment with unsaturated fatty acids induced mitochondrial fragmentation to a similar extent as palmitate, as shown by a reduction of a form factor, a quantitative parameter describing the extension of mitochondrial networks (Fig. 1B). These results suggest that fatty acids elicit mitochondrial fragmentation in macrophages independently of their chain length and degree of saturation. Since fatty acids circulate in body fluids as mixtures of different saturated and unsaturated species, we incubated macrophages with a combination of palmitate and unsaturated fatty acids, e.g. oleate and linoleate. The combination of palmitate and an unsaturated fatty acid promoted mitochondrial fragmentation to a similar extent as palmitate alone (Fig. 1C).

To study more physiological conditions we exposed macrophages to human postprandial triglyceride-rich lipoproteins (TGRL). TGRL can undergo hydrolysis by lipoprotein lipase on the macrophage surface, releasing free fatty acids.

Incubations with TGRL for 6 h disrupted the macrophage mitochondrial network (Fig. 1D), while inhibition of lipoprotein lipase activity using orlistat prevented TGRL-induced cellular triglyceride accumulation (Suppl. Fig. 2) and abolished mitochondrial fragmentation following TGRL-treatment.

To question how lipid overload impacts macrophage mitochondrial morphology in vivo we imaged mitochondria in freshly isolated peritoneal macrophages from wild type or apolipoprotein E knockout (ApoE-/-) mice. Hyperlipidemia in ApoE-/- mice caused lipid accumulation in macrophages. Therefore these cells can be considered as in vivo model of macrophages exposed to lipids. The analysis of mitochondrial morphology in peritoneal macrophages showed a significant reduction of form factor in macrophages from ApoE-/- mice (Fig. 1E, F), validating our cell culture findings in vivo.

Palmitate does not disturb mitochondrial functions

Since palmitate is known to induce inflammatory responses in macrophages, we focused on palmitate-induced fragmentation to better understand the role of mitochondrial dynamics in inflammatory responses to saturated fatty acids. Palmitate induced mitochondrial fragmentation in a time- and concentration-dependent manner. Concentrations as low as 100 µM palmitate significantly attenuated the mitochondrial form factor (Fig. 2A). Mitochondrial networks began to segregate after 1 h with incubations of 500 µM palmitate, an effect that was more prominent after 3 h. By 6 h almost all the mitochondria demonstrated a shift to a short and round morphology (Fig. 2B). In further experiments cells were exposed to 500 µM palmitate for 6 h, unless mentioned otherwise.

Next, we asked whether palmitate-induced mitochondrial fragmentation reflects mitochondrial damage, as has been reported for several cell types [21, 29]. Microscopic analyses revealed that, although mitochondria become more punctuate following palmitate treatment (see Fig. 1A), they did not cluster in the perinuclear region as previously reported [30, 31]. Transmission electron microscopy of palmitate-treated macrophages showed that the inner structure of mitochondrial cristae remained intact after exposure to palmitate (data not shown). Furthermore, following the removal of palmitate from macrophage media, mitochondria re-fused into a network. A second exposure to palmitate again resulted in mitochondrial fragmentation (Fig. 2C). These findings suggest that palmitate acutely alters mitochondrial morphology. To ascertain the integrity of

mitochondria following fatty acid treatment we quantified mitochondrial proteins and mtDNA in macrophages exposed to palmitate up to 24 h. Levels of the mitochondrial protein cytochrome b-c1 complex subunit 2 (UQCRC2) and ATP synthase subunit 5 (ATP5) remained unaltered (Fig. 2D), and the levels of mtDNA following a 24 hour palmitate treatment were unaffected (Fig. 2E).

To examine whether palmitate impacts on mitochondrial respiration Seahorse extracellular flux assays were performed. Cells were incubated with palmitate for 6 hours before the oxygen consumption rate (OCR) was measured in a basal state and following additions of oligomycin and CCCP to determine ATP-linked and maximal respiration, respectively. Palmitate affected neither basal nor maximal OCR (Fig. 2F). Cellular levels of ATP also remained unaltered following palmitate treatment (Fig. 2G). Collectively, these results suggest that palmitate-induced mitochondrial fragmentation does not alter mitochondrial function and does not promote the loss of mitochondria.

ER-stress does not regulate mitochondrial fragmentation

It has been reported that ER-mitochondrial contacts are involved in the regulation of mitochondrial morphology [32]. To clarify the role of ER-stress in palmitate-induced mitochondrial fragmentation we examined the expression of established ER-stress markers CHOP and GRP78 in lipid-treated macrophages. Palmitate, but not oleate or linoleate, activated the ER-stress response as assessed by expression of CHOP and GRP78 mRNA (Fig. 3A, B). Moreover, palmitate-induced ER-stress was inhibited by co-incubation with unsaturated fatty acids (Fig. 3A, B). Although unsaturated fatty acids blocked palmitate-induced ER-stress, they failed to inhibit palmitate-mediated mitochondrial fragmentation (Fig. 1C), suggesting that these two responses are independent of each other.

To confirm the above findings we next inhibited palmitate-induced ER-stress by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) [33] and assessed mitochondrial fragmentation. Although pre-treatment with AICAR decreased CHOP and GRP78 expression (Fig. 3C, D) it did not affect palmitate-induced mitochondrial fragmentation (Fig. 3E). Furthermore, pharmacological induction of ER-stress by thapsigargin and tunicamycin did not alter mitochondrial morphology in macrophages (Fig. 3F). These results indicate that ER-stress is not involved in palmitate-induced mitochondrial fragmentation.

Palmitate decreases mitochondrial membrane potential

Mitochondrial membrane potential (Δψm) is a sensitive indicator for the energetic state of mitochondria. It has been shown that a loss of mitochondrial membrane potential has a negative impact on mitochondrial fusion [34]. Inhibition of fusion results in mitochondrial fragmentation. As long-chain fatty acids are known to activate uncoupled respiration and a loss of Δψm we questioned whether palmitate-treatment impacts mitochondrial membrane potential. To examine how palmitate affects Δψm in macrophages OCR was measured before and after acute treatments with palmitate using Seahorse extracellular flux assays. In the assay ATPase activity was inhibited by oligomycin, which reduced OCR. Under these conditions, mitochondrial oxygen consumption is possible due to uncoupled respiration. Palmitate increased mitochondrial oxygen consumption upon inhibition of ATPase activity comparably to a CCCP protonophore positive control (Fig. 4A). This suggests that palmitate activates a proton leak across the mitochondrial inner membrane.

Uncoupled respiration is associated with a loss of Δψm. To assess Δψm in macrophages we used the Δψm-sensitive fluorescent dyes JC-1 and TMRE. Measurements of Δψm with JC-1 showed that palmitate decreased Δψm following a 1-h treatment, but there was no significant difference after 6 h (Fig. 4B). To confirm findings with JC-1, Δψm was monitored at shorter time points using live cell imaging with TMRE. Live cell microscopy showed that palmitate-treatment rapidly decreased Δψm, which returned to baseline levels after 6 h (Fig. 4C). Both methods confirm that mitochondria have normal Δψm after 6 h of incubation with palmitate, while mitochondria are fragmented at this time point.

Fatty acids promote uncoupled respiration by activating uncoupling proteins (UCP) in mitochondrial membrane, of which UCP2 is present in macrophages. To reduce the effect of palmitate on UCP2-mediated proton leak we pretreated macrophages with genipin, a chemical inhibitor of UCP2, and performed knockdown experiments using UCP2 siRNA (Suppl. Fig. 3A). Although inhibition of UCP2 activity prevented palmitate-induced loss of Δψm, as evaluated by TMRE microscopy, it failed to reduce mitochondrial fragmentation (Fig. 4D, E).

One of the well-described mechanisms linking Δψm loss to mitochondrial fragmentation suggests that decreased Δψm enhances the cleavage of OPA1 thus, reducing mitochondrial fusion. However, palmitate did not promote OPA1 cleavage and accumulation of its short form as compared to CCCP-treatment

(Fig. 5C). Taken together, these data demonstrate that palmitate-induced loss of Δψm is transient and does not promote mitochondrial fission.

Palmitate-induced mitochondrial fragmentation is DRP1 dependent

Several proteins regulate mitochondrial dynamics. We examined whether degradation of pro-fusion proteins promotes palmitate-induced mitochondrial fragmentation. Palmitate neither reduced the amounts of MFN1 or MFN2 (Fig. 5A, B), nor triggered the cleavage of OPA1 (Fig. 5C). The cytoplasmic dynamin-related GTPase DRP1 is a key executor of mitochondrial fission. DRP1 is activated by phosphorylation at Ser616 and inhibited by phosphorylation at Ser637. To investigate the role of DRP1 we assessed levels of its phosphorylation upon palmitate treatment. Palmitate did not affect the phosphorylation of Ser616 or Ser637 (Fig. 5C). Moreover, palmitate did not enhance DRP1 association with mitochondrial fractions by Western analysis (Fig. 5D). Confocal microscopy confirmed that DRP1 was already localized on mitochondria in macrophages (Fig. 5E). The Pearson's correlation coefficient for DRP1 and MFF increased from 0.5±0.2 in non-treated cells to 0.6±0.2 after exposure to palmitate. As the difference is not significant, it can be concluded that palmitate does not promote translocation of DRP1 to mitochondria.

DRP1 oligomerization at the mitochondrial surface is a crucial step in the formation of a division apparatus [35]. Remarkably, palmitate induced DRP1 oligomerization (Fig. 5F). To clarify whether palmitate-induced mitochondrial fragmentation is DRP1-dependent, mitochondrial morphology in macrophages was measured upon inhibition of DRP1 activity. Pre-treatment with the DRP1 inhibitor Mdivi-1 reduced palmitate-induced mitochondrial fragmentation (Fig. 5G). To confirm the results we performed a knockdown of DRP1 with siRNA (Suppl. Fig. 3B) and adenoviral overexpression of a dominant-negative DRP1 (ad-virDRP1K38A). Both of these approaches attenuated palmitate-induced reductions of mitochondrial form factor (Fig. 5G). Conclusively, DRP1 is involved in mitochondrial fragmentation upon palmitate treatment.

Alterations of mitochondrial lipid composition accompany mitochondrial fragmentation induced by palmitate

Free fatty acids from the extracellular space are rapidly taken up by cells and converted into acyl-CoA, a pre-requisite for fatty acid oxidative metabolism or incorporation into phospholipids, triglycerides and their biosynthetic intermediates.

Palmitate-treatment increased levels of palmitate-containing lysophosphatidic acid (LPA), phosphatidic acid (PhA), DAG, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in mitochondrial membranes (Fig. 6A). To elucidate whether conversion of palmitate to palmitoyl-CoA is crucial for mitochondrial fragmentation we treated macrophages with palmitoyl-CoA or inhibited the activity of long chain fatty acyl-CoA synthetases by triacsin C. Palmitoyl-CoA caused mitochondrial fragmentation comparable to the effect of palmitate, whereas pre-treating macrophages with triacsin C significantly attenuated palmitate-induced changes in mitochondrial morphology (Fig. 6B). To test whether palmitoyl-CoA-induced mitochondrial fragmentation is DRP1-dependent, we assessed mitochondrial morphology upon palmitoyl-CoA treatment in DRP1-deficient cells. Our results show that mitochondrial fragmentation is reduced in macrophages pre-treated with DRP1 siRNA after incubation with palmitoyl-CoA (Suppl. Fig. 4A). In addition, palmitoyl-CoA caused DRP1 oligomerization similar to palmitate (Suppl. Fig. 4B).

Several intermediates of triglyceride- and phospholipid biosynthesis were shown to affect mitochondrial dynamics, with PhA showing pro-fusion, and LPA and DAG pro-fission properties [36]. To assess how perturbations in these biosynthetic pathways affect mitochondrial responses to palmitate we inhibited the DAG synthesis from PhA by propranolol. Pretreatment with propranolol, a chemical inhibitor of lipin, significantly decreased palmitate-induced mitochondrial fragmentation (Fig. 6B) and DRP1 oligomerization (Suppl. Fig. 4C). We confirmed that propranolol enhanced levels of LPA and PhA, but decreased palmitoyl-containing PE and PC (Fig. 6C-F). To validate pharmacological inhibition of lipin, we silenced the expression of major lipin isoforms, LPIN1 and LPIN2, in macrophages by siRNA (Suppl. Fig. 5A). As shown in Suppl. Fig. 5B, silencing of both LPIN1 and LPIN2 attenuated palmitate-induced mitochondrial fission. Since propranolol is also a well-known β-adrenergic receptor antagonist, we treated macrophages with another potent β-adrenoceptor antagonist – sotalol. Pre-treating cells with sotalol prior to palmitate treatment did not reduce mitochondrial fragmentation (Suppl. Fig. 5C), confirming that propranolol action is unrelated to its β-adrenergic antagonistic function.

Mitochondrial fragmentation reduces inflammatory responses

Although fatty acid-induced inflammatory pathways are well known, the role of mitochondria in this process is unclear. Our previous work suggested that mitochondrial ROS production contributed to elevated inflammatory responses of human macrophages to palmitate under hypoxia [13]. To investigate whether mitochondrial fragmentation regulates ROS production we analyzed mitochondrial ROS by microscopic visualization of MitoSOX and MitoTracker Green co-stained macrophages and by quantitative FACS analysis of MitoSOX fluorescence intensity. Consistent with our previous observations [13], palmitate on its own did not increase MitoSOX fluorescence (Fig. 7A, B). However, inhibiting DRP1 activity or reducing palmitate incorporation into cellular lipids using Mdivi1 or propranolol, respectively, significantly increased the MitoSOX signal, while decreasing the extent of mitochondrial fragmentation (Fig. 7A, B). These results indicate that mitochondrial fragmentation attenuates mitochondrial ROS generation in palmitate-treated macrophages. An elevated mitochondrial membrane potential is known to favor ROS production [37]. To question how inhibition of mitochondrial fragmentation affects mitochondrial membrane potential we measured JC-1 fluorescence in cells pretreated with Mdivi1. Mdivi1 reduced palmitate-induced loss of $\Delta\psi m$ (Fig. 7C), suggesting that this may be a reason for increased ROS production.

Mitochondrial ROS enhanced JNK signaling in the context of palmitate-induced inflammation [13]. Therefore, we questioned how mitochondrial dynamics affects phosphorylation of the JNK substrate c-Jun following palmitate treatment. Palmitate-treatment induced c-Jun phosphorylation, which was significantly increased upon pre-treatments with Mdivi-1, or propranolol (Fig. 7D). Similarly, infecting cells with a dominant-negative DRP1 K38A adenovirus elevated c-Jun phosphorylation in response to palmitate (Fig. 7E). Thus, mitochondrial fragmentation restrains the activation of pro-inflammatory JNK signaling by palmitate.

Finally, we analyzed how mitochondrial fragmentation influences mRNA expression and secretion of pro-inflammatory cytokines in palmitate-treated macrophages. Attenuating mitochondrial fragmentation using Mdivi-1 or propranolol enhanced mRNA expression of IL-6, IL-8, and TNF□ after palmitate-treatments (Fig. 7F-H). Infection of cells with a dominant-negative DRP1 K38A adenovirus also increased IL-8 and TNF□ mRNA expression in response to

palmitate (Fig. 7I, J). In accordance with mRNA expression data, pre-treatments with Mdivi-1 or propranolol elevated IL-8 secretion upon exposure to palmitate (Fig. 7K). Collectively, these data indicate that mitochondrial fragmentation serves to attenuate inflammatory responses towards palmitate.

Discussion

Saturated fatty acid-induced inflammation in macrophages contributes to obesity-associated insulin resistance. The role of mitochondrial dynamics in inflammatory pathways is unclear and has never been investigated in primary macrophages following exposure to fatty acids. The salient finding of our study is the profound fatty acid-triggered fragmentation of macrophage mitochondrial networks. Notably, fatty acids induced fragmentation independently of their degree of saturation. This contrasts with observations in skeletal muscle, where saturated fatty acids promoted mitochondrial fission, whereas unsaturated fatty acids had no effect [22]. In this and other studies of nutritional overload induced either by fatty acids or high glucose, mitochondrial fragmentation was associated with ROS production, mitochondrial damage, and apoptosis [21, 38, 39]. However, in our model mitochondria are not irreversibly damaged by palmitate, as we neither detected reduced mitochondrial protein or DNA content nor functional impairment of mitochondria as evidenced by intact respiration and mitochondrial ATP generation in palmitate-treated cells. The ability of both saturated and unsaturated fatty acids to induce mitochondrial fragmentation suggests that this process is unrelated to lipid stress and lipotoxicity associated with increased cellular levels of saturated fats. Rather, our data support the notion that fragmented mitochondrial networks may attenuate the damage induced by saturated fatty acids through reduced mitochondrial ROS generation and inflammatory responses, as discussed below.

Our results suggest that palmitate does not affect the fusion machinery. The protein levels of MFN1, MFN2, and OPA1 are not changed. Although mitochondrial membrane potential decreased significantly after palmitate treatment, cells recovered without any detectable cleavage of OPA1. This contrasts with the observations in brown adipocytes, where palmitate combined with norepinephrine induced OPA1 cleavage, associated with stimulation of uncoupled respiration [40]. Apparently, the degree of uncoupled respiration

induced by fatty acids in macrophages is not of sufficient strength and duration to induce substantial OPA1 cleavage.

Our findings support the key role of DRP1 in palmitate-induced mitochondrial fragmentation. Inhibition of DRP1 activity by siRNA, overexpression of a dominant-negative mutant, or pre-treatment with Mdivi-1 attenuated mitochondrial fragmentation. However, we found no evidence of the involvement of the major mechanisms known to regulate DRP1 activation, including Ser616 and Ser637 phosphorylation [41], or DRP1 mitochondrial translocation [15]. DRP1 is constitutively associated with mitochondria in untreated macrophages, and palmitate does not increase mitochondrial or total levels of DRP1. Thus, we postulate that fatty acids promote mitochondrial fragmentation by activating DRP1, which is already present at the mitochondrial membranes. This is supported by our findings that palmitate promoted DRP1 oligomerization, which is a critical step in DRP1-mediated fission [40]. Although several publications suggested DRP1 phosphorylation and mitochondrial translocation drives mitochondrial fragmentation induced by palmitate [22, 42], observations in □-cells are in agreement with our data [43].

What mechanisms may underlie fatty acid-triggered activation of mitochondrial DRP1? We observed profound alterations of palmitate-containing phospholipids and their precursors in mitochondrial membranes upon palmitate treatment of macrophages. Attenuating palmitate-induced mitochondrial fragmentation by the inhibitor of long chain fatty acyl-CoA synthetases triacsin C supports the notion that fatty acid-triggered changes of mitochondrial lipid composition are crucial for altering mitochondrial dynamics in favor of fission. Mitochondrial membrane lipid composition is known to regulate mitochondrial networking [36]. We suggest that phospholipids or their precursors formed from metabolized fatty acids alter mitochondrial membrane structure in favor of DRP1-dependent mitochondrial fission. Although DRP1 does not have a known lipid-binding domain, there is evidence that its activation can be influenced by membrane lipid composition. In vitro models demonstrated that DRP1 interacts with cardiolipin [44, 45], and a recent publication showed that DRP1 binds PhA, which keeps DRP1 in an inactive state thus, favoring mitochondrial fusion [46]. Increasing mitochondrial content of PE also supports fusion [47]. In contrast, LPA and DAGs may promote fission [36]. Our observations that a lipin knockdown, and the lipin inhibitor

propranolol attenuated palmitate-induced mitochondrial fragmentation suggest that decreasing the PhA/DAG ratio drives pro-fission effects of fatty acids. However, since any experimental perturbation of lipid biosynthetic pathways affects mitochondrial content of many different lipid species capable of altering mitochondrial dynamics, it is experimentally very challenging to exactly pinpoint the species responsible for mitochondrial fragmentation in response to fatty acids. Although fatty acids can induce additional signaling pathways triggered by e.g. stimulation of G protein coupled receptors, such as GPR120, we found no evidence that GPR120 affects fatty acid-induced mitochondrial fragmentation. Neither a GPR120 agonist, nor a GPR120 antagonist affected mitochondrial morphology in the absence or presence of the GPR120-ligating fatty acids linoleate and oleate (Suppl. Fig. 6). These results prove that the activation of GPR120 does not affect mitochondrial dynamics. However, activation of other receptors cannot be excluded from plausible mechanisms of fatty acid-induced mitochondrial fission.

Decreasing mitochondrial fragmentation by attenuating DRP1 or lipin we propose an anti-inflammatory effect of mitochondrial fragmentation in a model of saturated fatty acid-induced inflammation. Our previous study indicated that mitochondrial ROS production enhanced palmitate-stimulated inflammation by increasing the activity of the JNK signaling cascade [13]. In the current work we observed that inhibition of mitochondrial fission in macrophages upon palmitate treatment promoted ROS generation. Thus, in our experimental system mitochondrial fragmentation apparently represents a protective response, reducing the propensity of palmitate-loaded cells to generate mitochondrial ROS and promote inflammatory signaling. Our findings are surprising in view of many publications reporting that fission promotes mitochondrial ROS production in the setting of nutritional overload [48-50]. However, in these studies ROS generation was usually accompanied by mitochondrial dysfunction and apoptosis, which on its own may contribute to enhanced mitochondrial ROS production. In our model mitochondrial functions are not impaired and no signs of toxicity were present.

Which mechanisms can thus explain decreased ROS production and attenuated inflammatory responses due to mitochondrial fragmentation in palmitate-treated cells? One possibility can be increased uncoupled respiration by fragmented mitochondria [20]. It is known that mild uncoupling is associated with reduced

mitochondrial ROS formation [37] [51]. Although in our system mitochondrial uncoupling was temporarily dissociated from mitochondrial fragmentation, we cannot exclude that slight increases of proton leaks still occur in fragmented mitochondria at later time points following palmitate treatment, contributing to this effect. This hypothesis is supported by our observations that inhibiting fragmentation with Mdivi-1 increased mitochondrial membrane potential and reduced Δψm loss in palmitate-treated macrophages. Another potentially protective effect is increased autophagic removal of damaged mitochondria [29]. However, we did not find evidence for increased mitophagy in our system, at least not within our experimental time frame, suggesting that mitophagy may have only a minor contribution to the observed effects. Interestingly, increasing contacts between mitochondria and ER promotes mitochondrial calcium overload and ROS generation in the context of lipotoxicity [52]. It can be envisaged that mitochondrial fragmentation might disrupt these contacts resulting in less calcium overload and mitochondrial ROS in palmitate-treated macrophages. Future experimental work should reveal whether this is the case.

Mitochondrial fragmentation was postulated to promote insulin resistance in the context of high-fat diet-induced obesity, supported by studies in mice with a knockout of MFN2 [53] or DRP1 [54]. However, a recent study using liver-specific MFN1 knockout mice provided evidence that mitochondrial fragmentation in hepatocytes protects against insulin resistance induced by a high-fat diet [25]. Hepatocytes with fragmented mitochondria oxidized more fatty acids and accumulated less toxic lipids and their intermediates. Remarkably, DRP1 knockout hepatocytes had increased levels of ER-stress [54], generating adaptive responses such as FGF21 expression and increasing systemic energy expenditure. These findings support the notion that mitochondrial fragmentation may be a protective mechanism in the settings of lipotoxicity.

Conclutions

Our data show that alterations in the mitochondrial membrane lipid composition by palmitate provoked DRP1 oligomerization and mitochondrial fission. Attenuating palmitate-induced mitochondrial fragmentation enhanced inflammatory response in macrophages. Therefore, mitochondrial fragmentation may be a protective mechanism to restrain palmitate-induced inflammation.

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Conflict of interest

The authors declare no conflict of interest.

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Figure 1

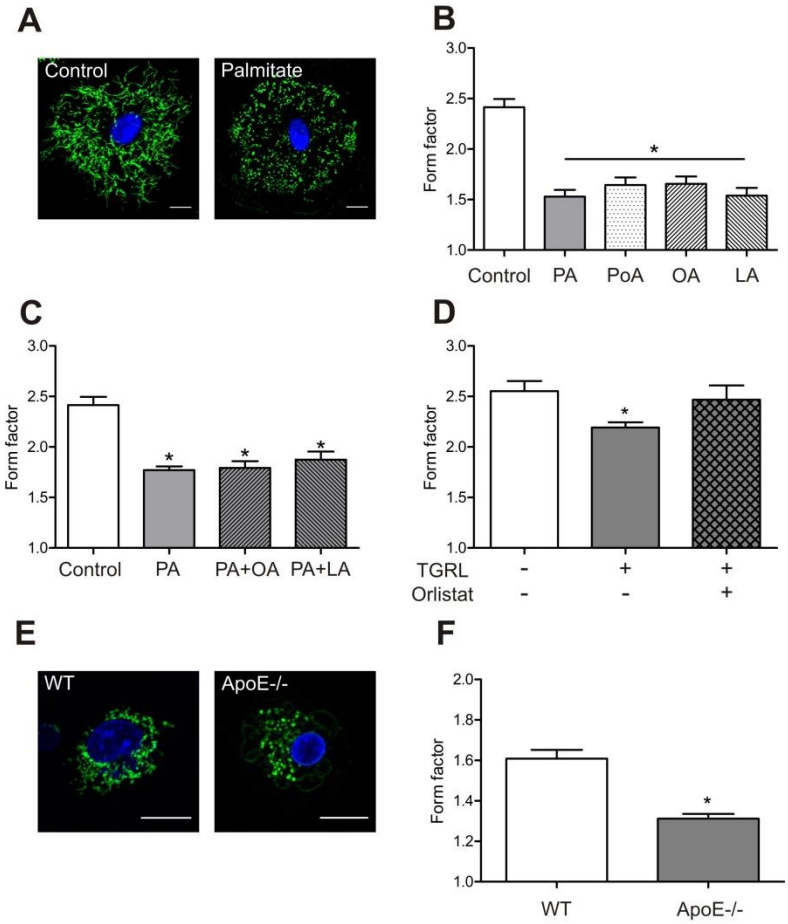


Fig. 1. Fatty acids induce mitochondrial fragmentation.

(A) Representative images of mitochondria stained for mitochondrial complex III (green) and cell nuclei (blue) in primary human macrophages (MΦ) treated with 500 µM palmitate for 6 h. (B and C) Mitochondrial form factor in MΦ treated for 6 h with 500 µM palmitate (PA), palmitoleate (PoA), oleate (OA) and linoleate (LA) alone, or as combinations of 250 µM fatty acids. (D) Mitochondrial form factor in MΦ treated for 6 h with TGRL with or without orlistat. (E) Representative images of mouse peritoneal macrophages stained with MitoTracker Green and Hoechst. (F) Mitochondrial form factor in peritoneal macrophages from wild type and ApoE-/- mice. *, p≤0.05 vs Control. Scale bar = 10 µm.

Figure 2

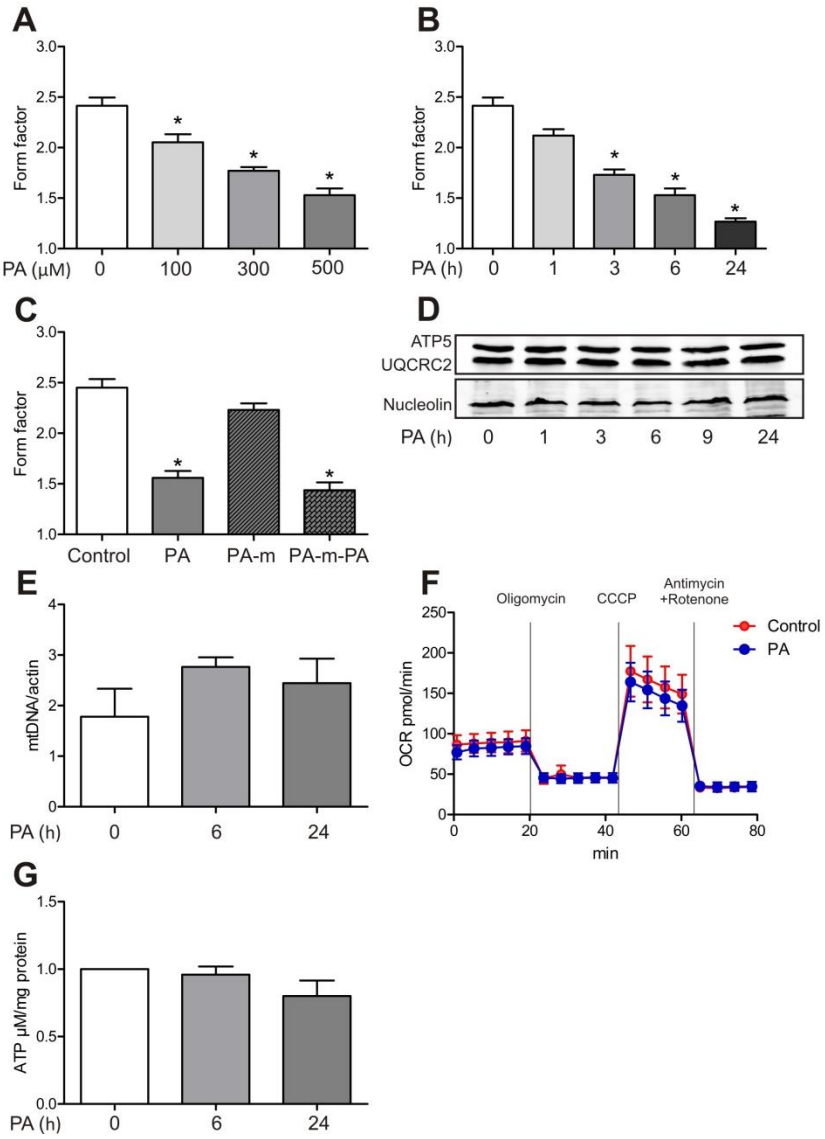


Fig. 2. Palmitate does not cause mitochondrial damage.

(A and B) Mitochondrial form factor in MΦ treated with indicated concentrations of palmitate for 6 h, or with 500 μM palmitate for indicated times. (C) Mitochondrial form factor in MΦ treated for 6 h with 500 μM palmitate followed by 18 h incubation in medium without palmitate (PA-m) and the second 6 h-treatment with palmitate (PA-m-PA). (D and E) Analysis of mitochondrial proteins and mitochondrial DNA (mtDNA) in MΦ treated with 500 μM PA for indicated times. (F and G) OCR and cellular ATP levels (G) in M following 6 h-treatment with 500 μM palmitate. *, p≤0.05 vs Control.

Figure 3

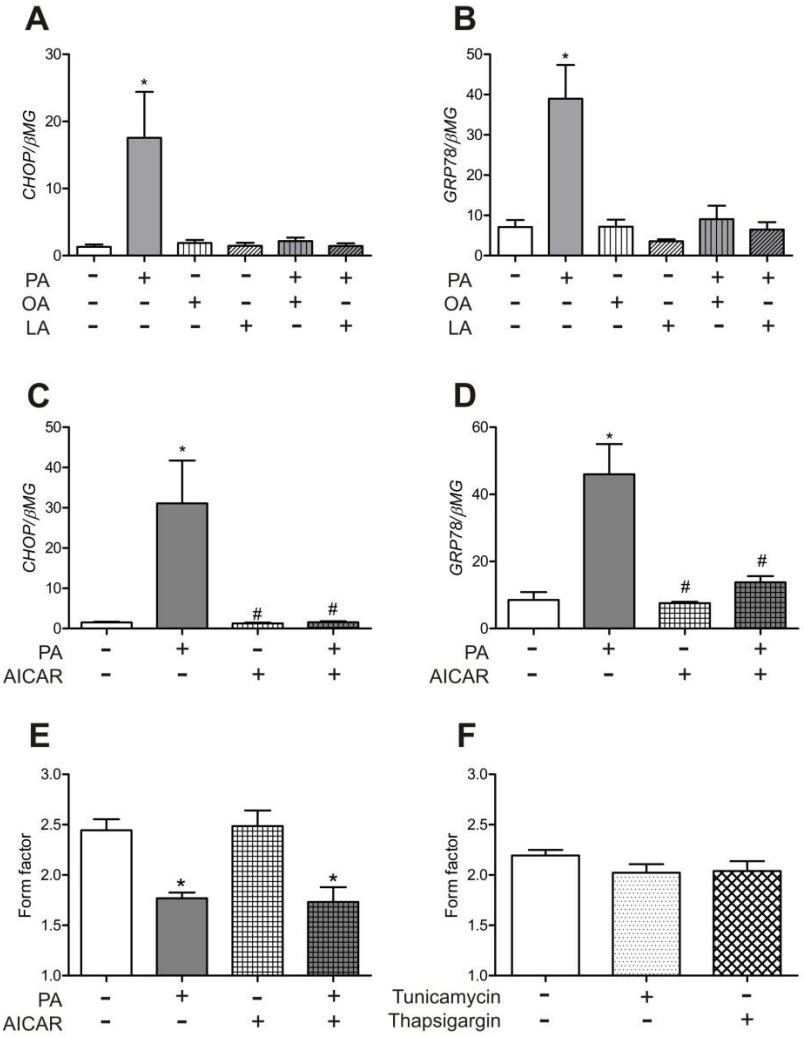


Fig. 3. ER-stress does not regulate palmitate-induced mitochondrial fragmentation.

(A and B) mRNA expression of CHOP and GRP78 in M treated with 500 µM fatty acids for 6 h. (C and D) mRNA expression of CHOP and GRP78 and mitochondrial form factor (E) in MΦ treated with AICAR and palmitate. (F) Mitochondrial form factor in M treated by tunicamycin and thapsigargin. *, p≤0.05 vs Control. #, p≤0.05 vs PA.

Figure 4

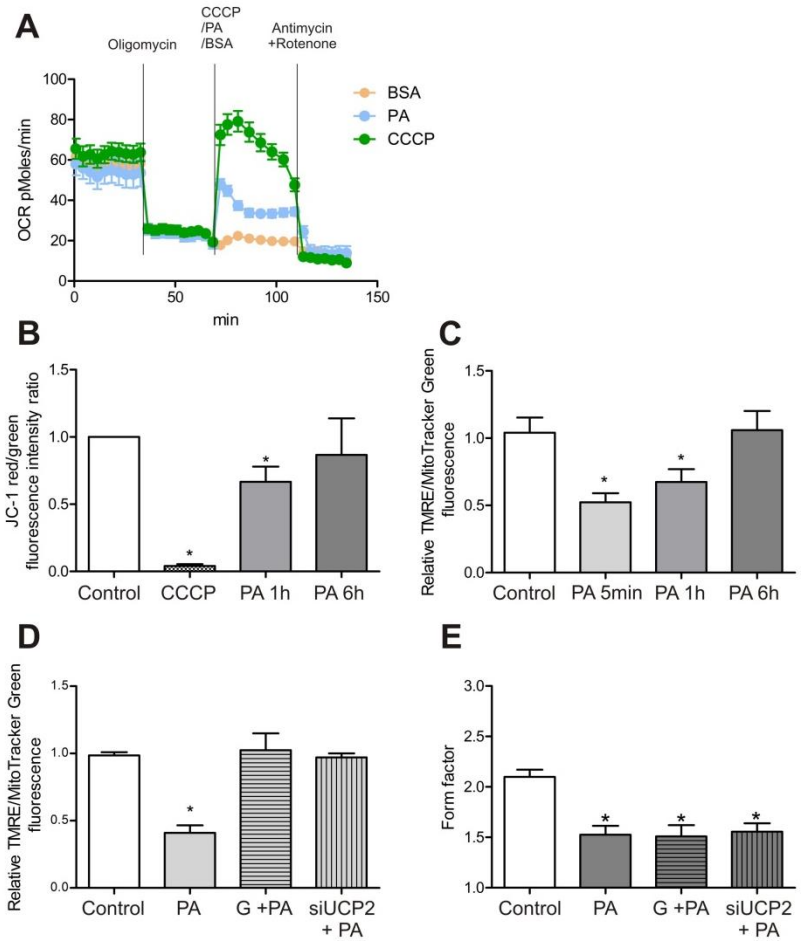


Fig. 4. Loss of mitochondrial membrane potential is not involved in palmitate-induced mitochondrial fragmentation.

(A) OCR in MΦ following injections of indicated reagents. (B and C) Measurements of mitochondrial membrane potential using JC-1 and TMRE in M treated with 500 µM palmitate for indicated times. (D) Mitochondrial membrane potential in M upon UCP2 silencing or genipin treatment prior to treatments with 500 µM palmitate for 5 min. (E) Mitochondrial form factor in M after inhibition of UCP2 activity and following treatment with 500 µM palmitate for 6 h. * p≤0.05 vs Control.

Figure 5

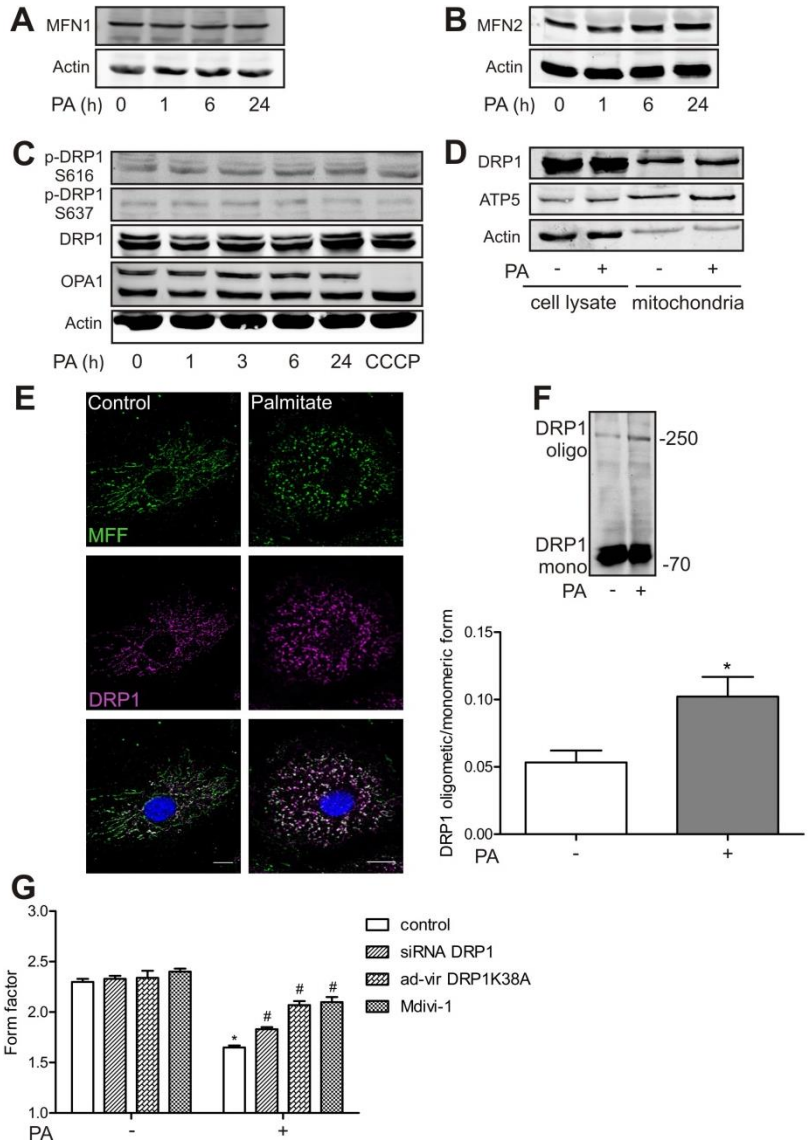


Fig. 5. Palmitate-induced mitochondrial fragmentation is DRP1-mediated.

(A-C) Western analysis of MΦ treated with 500 μM palmitate for indicated times or with 10 μM CCCP for 3 h. (D-F) Subcellular fractionation, immunofluorescence microscopy and DRP1 oligomerization analysis of MΦ treated with 500 μM palmitate for 6 h. (G) Mitochondrial form factor in MΦ transfected with DRP1 siRNA, ad-DRP1K38A or pre-treated with Mdivi-1 and subsequent addition of 500 μM PA for 6 h. *, p≤0.05 vs Control. #, p≤0.05 vs PA. Scale bar = 10 μm.

Figure 6

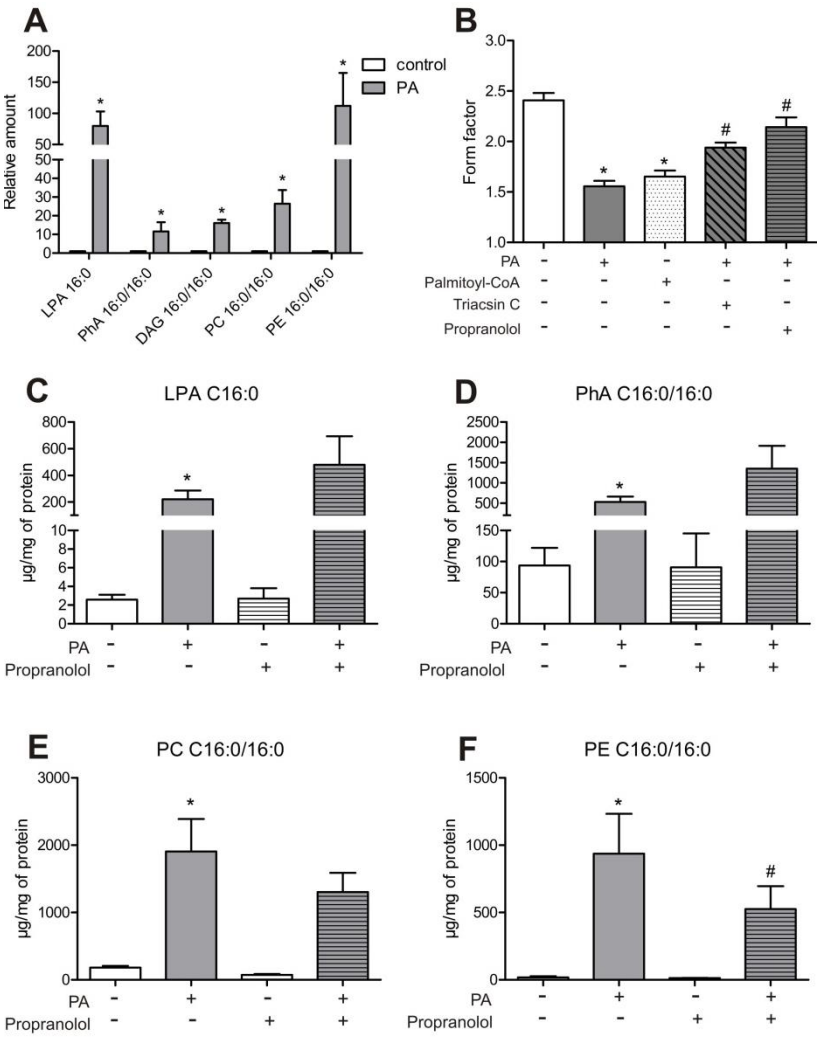
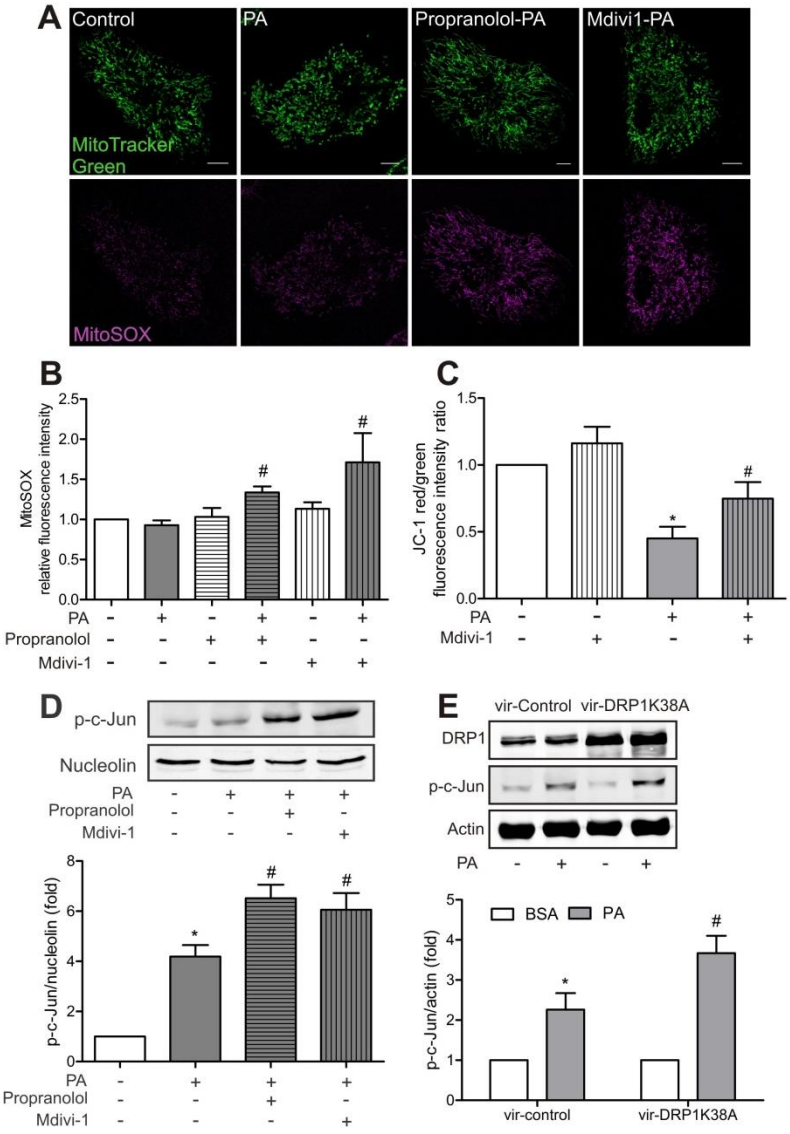


Fig. 6. Palmitate alters mitochondrial lipid composition.

(A) Relative amounts of palmitate-containing lipid species in mitochondrial fractions of MΦ treated with 500 µM palmitate for 6 h. (B) Mitochondrial form factor in MΦ treated with palmitoyl-CoA, triacsin C, propranolol and palmitate. (C-F) Amounts of LPA, PhA, PE, and PC in mitochondrial fractions of MΦ treated with propranolol and palmitate. *, p≤0.05 vs Control. #, p≤0.05 vs PA.

Figure 7


A
Control
PA
Propranolol-PA
Mdivi1-PA
MitoTracker Green
MitoSOX
B
MitoSOX relative fluorescence intensity
PA
Propranolol
Mdivi-1
C
JC-1 red/green fluorescence intensity ratio
PA
Mdivi-1
D
p-c-Jun
Nucleolin
PA
Propranolol
Mdivi-1
p-c-Jun/nucleolin (fold)
E
vir-Control
vir-DRP1K38A
DRP1
p-c-Jun
Actin
PA
BSA
PA
p-c-Jun/actin (fold)
vir-control
vir-DRP1K38A

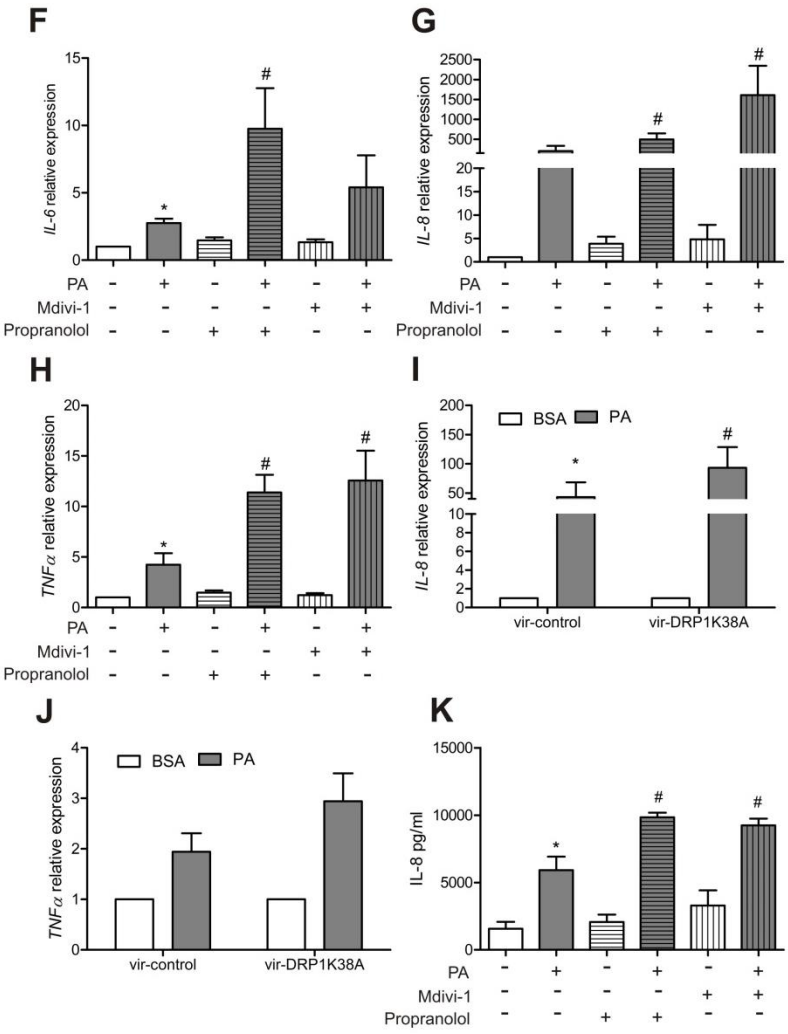
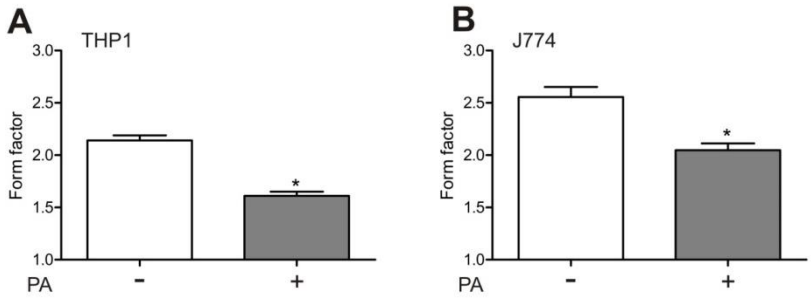


Fig. 7. Mitochondrial fragmentation attenuates inflammatory responses to palmitate.

(A and B) Representative images and FACS analysis of MitoSOX fluorescence in MΦ pre-treated with Mdivi-1 and propranolol and treated with 500 μM palmitate for 3 h. (C) Measurements of mitochondrial membrane potential using JC-1 in MΦ pretreated with Mdivi-1and treated with 500 μM palmitate for 1 h. (D and E) c-Jun phosphorylation in MΦ pre-treated with Mdivi-1 and propranolol or infected with a dominant-negative DRP1K38A adenovirus prior to treatments with palmitate for 6 h. mRNA expression level of IL-6 (F), IL-8 (G) and TNF (H) in MΦ pre-treated with Mdivi-1 and propranolol and treated with palmitate for 6 h. (I and J) mRNA expression level of IL-8 and TNF in MΦ infected with ad-DRP1K38A and treated with palmitate for 6 h. (K) Protein concentration of IL-8 in cell culture supernatants after treatments with Mdivi-1, propranolol and palmitate for 24 h. *, p≤0.05 vs Control. #, p≤0.05 vs PA.

Supplemental Data

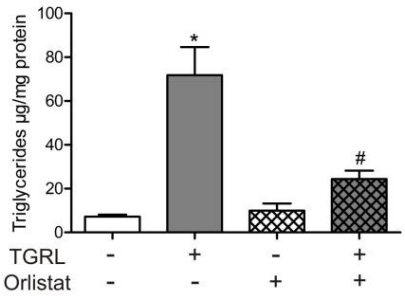
Figure S1



Suppl. Fig. 1

(A and B) Mitochondrial form factor in THP1 and J774 cell lines treated for 6 h with 500 μM palmitate (PA). *, p≤0.05 vs Control.

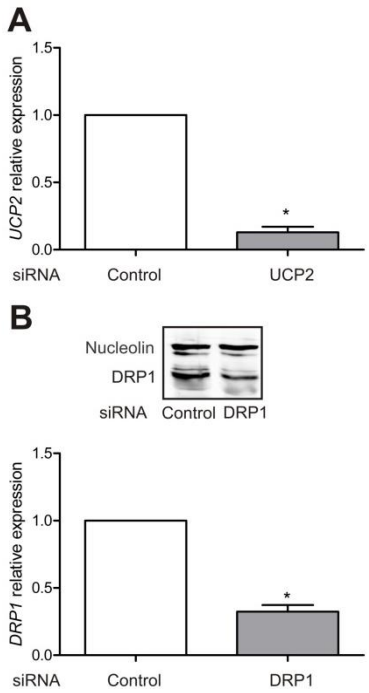
Figure S2



Suppl. Fig. 2

Triglyceride levels in MΦ pre-treated for 1 h with orlistat and treated for 6 h with TGRL. *, p≤0.05 vs Control, #, p≤0.05 vs PA.

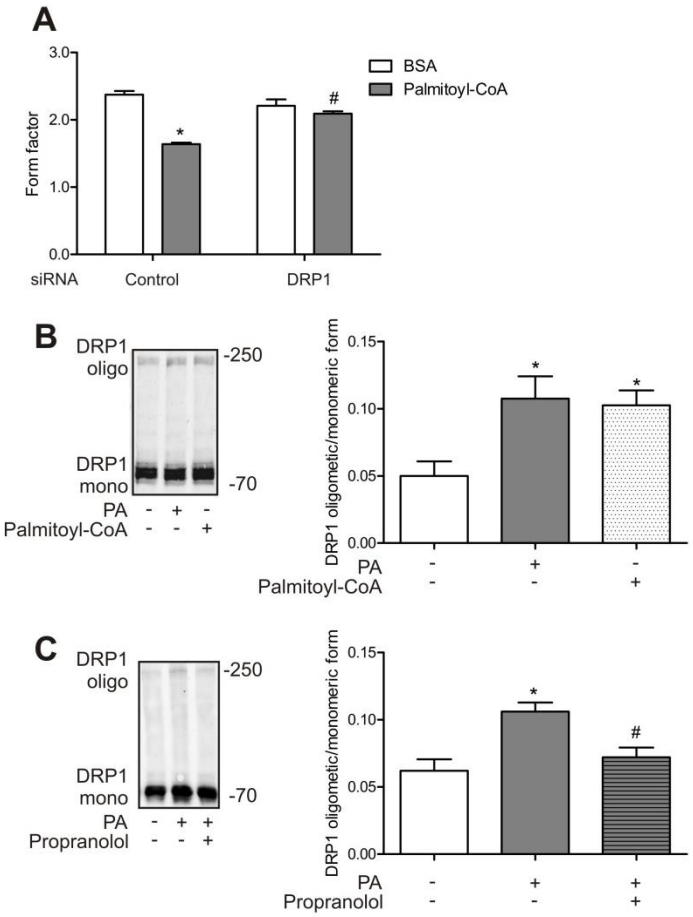
Figure S3



Suppl. Fig. 3

(A) mRNA expression of UCP2 in MΦ transfected with UCP2 siRNA. (B) mRNA expression and Western analysis of DRP1 in MΦ transfected with DRP1 siRNA. *, p≤0.05 vs Control.

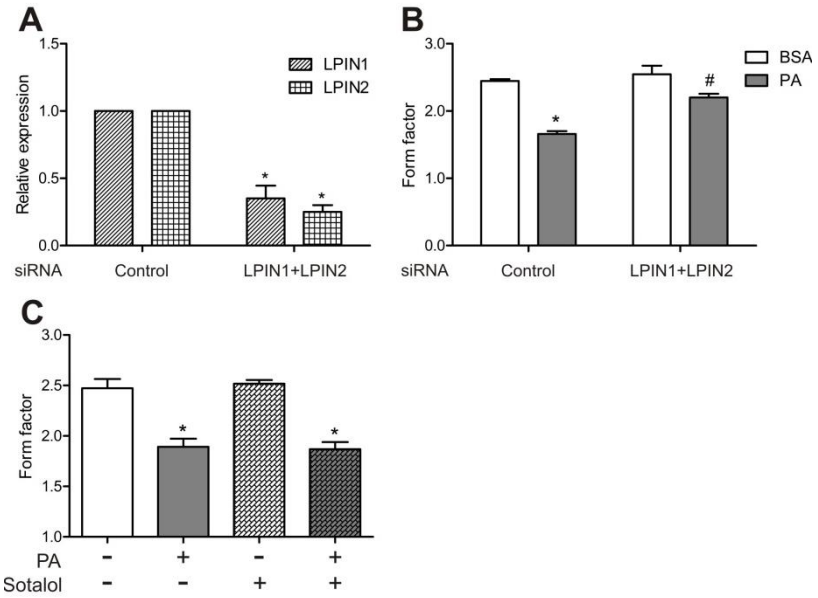
Figure S4



Suppl. Fig. 4

(A) Mitochondrial form factor in MΦ transfected with DRP1 siRNA and treated for 6 h with 500 µM palmitoyl-CoA. (B and C) Western analysis of DRP1 oligomerization in MΦ treated with palmitoyl-CoA, or pretreated with for 1h with propranolol and treated for 6 h with 500 µM PA. *, p≤0.05 vs Control, #, p≤0.05 vs PA.

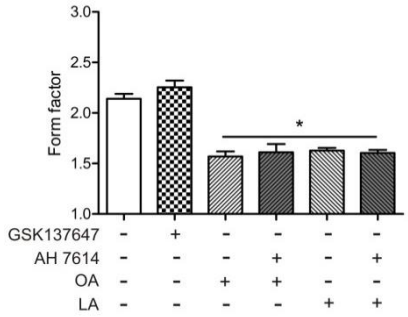
Figure S5



Suppl. Fig. 5

(A) mRNA expression of LPIN1 and 2 in MΦ transfected with LPIN1 and 2 siRNA. (B and C) Mitochondrial form factor in MΦ transfected with LPIN1 and 2 siRNA, or pretreated for 1 h with sotalol and treated for 6 h with 500 µM PA. *, p≤0.05 vs Control, #, p≤0.05 vs PA.

Figure S6



Suppl. Fig. 6

Mitochondrial form factor in MΦ pretreated for 1 h with AH 7614, and treated for 6 h with GSK 137647, 500 µM oleate, or 500 µM linoleate *, p≤0.05 vs Control.

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7. Contribution statement on publications

1. I performed all experiments, data validation and writing of the manuscript.

2. As a co-author I performed selected experiments, including measurement of mitochondrial ROS production.

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10. Curriculum vitae

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11. Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

Mitochondrial dynamics in responce to fatty acids in human macrophages

am Institut der Biochemie 1 / Pathobiochemie unter Betreuung und Anleitung von PD Dr. Dmitry Namgaladze mit Unterstützung durch Prof. Dr. Bernhard Brüne ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Thesis oder Dissertation eingereicht.

Die Grundsätze der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Sicherung guter wissenschaftlicher Praxis in ihrer gültigen Form liegen mir vor und wurden bei derwissenschaftlichen Arbeit eingehalten.

Vorliegende Ergebnisse der Arbeit wurden in folgendem Publikationsorgan veröffentlicht: Zezina E, Snodgrass RG, Schreiber Y, Zukunft S, Schürmann C, Heringdorf DMZ, Geisslinger G, Fleming I, Brandes RP, Brüne B, Namgaladze D. Mitochondrial fragmentation in human macrophages attenuates palmitate-induced inflammatory responses. Biochim Biophys Acta. 2018 Feb 2;1863(4):433-446.

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