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Mitochondrial fragmentation in human macrophages attenuates palmitate-induced inflammatory responses

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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.

1. Introduction

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

Abbreviations: 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; $\Delta\psi_m$, mitochondrial membrane potential

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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, Ca^{2+} 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.

2. Material and methods

2.1. 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 h 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.

2.2. 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.

2.3. 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 3.5–5 × 10^4 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 $CaCl_2$, 2 mM $MgSO_4$, 1.2 mM NaH_2PO_4) 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.

2.4. 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).

2.5. RNA extraction and quantitative real-time PCR

Total RNA from 1 × 10^6 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.

2.6. 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 Na_3VO_4, 0.5% NP-40, 1 mM PMSF, protease inhibitor cocktail). Protein lysates were sonicated, centrifuged at 12000 ×*g* for 10 min at 4 °C, the supernatants were heat-denatured in 5× 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