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Resolvin E1 and its precursor 18*R*-HEPE restore mitochondrial function in inflammation

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ABSTRACT

Inflammatory disorders such as sepsis are a major cause of morbidity and mortality. Mitochondrial dysfunction is considered a key factor in the pathogenesis of severe inflammation. In the present study, we aimed to investigate the impact of arachidonic acid, omega-3 (*n*-3) fatty acids, and *n*-3-derived lipid mediators 18*R*-HEPE and resolvin (Rv) E1 on mitochondrial function in experimental inflammation. The results revealed that, in contrast to *n*-6 and *n*-3 fatty acids, both 18*R*-HEPE and RvE1 possess anti-inflammatory and anti-apoptotic properties. Both mediators are able to restore inflammation-induced mitochondrial dysfunction, which is characterized by a decrease in mitochondrial respiration and membrane potential, as well as an imbalance of mitochondrial fission and fusion. Furthermore, inhibition of mitochondrial fission by Mdivi-1 and Dynasore reduces levels of the pro-inflammatory cytokines IL-6 and IL-8. These results suggest a novel functional mechanism for the beneficial effects of RvE1 in inflammatory reactions.

1. Introduction

Severe inflammatory disorders such as sepsis and pneumonia are regarded as a leading cause of morbidity and mortality worldwide [1]. Sepsis is characterized by an excessive and uncontrolled inflammatory reaction mainly governed by the interplay of invading pathogens and the subsequent exaggerated response of the host [1]. Dysregulated immune activity with release of pro-inflammatory mediators and oxidative stress-related molecules might cumulate in vascular dysfunction with hypotension and damage of vascular integrity, disturbance of the coagulation system, tissue hypoperfusion and relevant changes in metabolism [2]. These pathophysiological features of severe sepsis often exacerbate clinical symptoms, resulting in the development of multiple organ failure and septic shock, which is associated with poor prognosis [1].

As mitochondria play a critical role in the regulation of inflammation, metabolism and energy supply, mitochondrial dysfunction is now regarded as a major factor in the pathogenesis of sepsis [3]. Several patterns of mitochondrial damage have been described in response to severe inflammation. Pro-inflammatory mediators are capable of reducing or even abolishing mitochondrial respiratory capacity and thus energy production, which activates the intrinsic apoptotic pathways by inhibition of respiratory chain complexes [4, 5]. In addition, excessive generation of reactive oxygen species (ROS) might induce structural damage to mitochondrial proteins and DNA [6, 7]. The extent to which impaired mitochondria are not only victim to severe inflammatory reactions, but may also cause further deterioration, is currently under investigation. During stress and damage, mitochondria are able to release danger-associated molecular patterns (DAMP) such as reactive oxygen species, cytochrome *c*, or mitochondrial DNA (mtDNA), which are suspected to contribute to the vicious circle of sepsis-induced tissue and organ failure [8, 9]. A better understanding of mitochondrial biogenesis, dynamics and mitophagy in the recent years (such as the discovery of mitochondrial fission and fusion) might enhance our

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; 18*R*-HEPE, the EPA-metabolite 18*R*-hydroxyeicosapentaenoic acid; RvE1, resolvin E1; ROS, reactive oxygen species; PBMC, peripheral mononuclear blood cells

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understanding of the mitochondrial stress response and the potential impact on the inflammatory response [9].

Lipids are of special interest in the context of inflammation and sepsis for several reasons. Lipid mediators such as prostaglandins, leukotrienes and resolvins are intimately involved in the initiation and resolution of the inflammatory response [10]. In addition, lipids and lipid emulsions are integral parts of nutritional regimes as they offer high-caloric density to provide adequate metabolic support and supplementation with essential fatty acids for critically ill patients [11, 12]. In sepsis, maintenance of optimal energy supply is essential to prevent bioenergetic mitochondrial failure [12]. In the context of severe inflammation, we recently demonstrated the impact of short- and medium-chain fatty acids on mitochondrial function in severe inflammation [13, 14]. In addition to energy supply, several studies indicate that lipids possess immunomodulatory properties, which could influence the immune response mainly via the generation of pro- and anti-inflammatory lipid mediators [12]. Thus far, supplementation of omega-3 (*n*-3)-derived lipid emulsions containing fish oil (FO) has been investigated in various experimental and clinical studies with controversial results [15–18]. Recently, resolvins were identified as a novel class of promising *n*-3-derived lipids displaying beneficial effects concerning the resolution of inflammation in several disease models [10, 19].

In the present study, we aimed to investigate the impact of the *n*-6 fatty acid arachidonic acid (AA), the *n*-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), RvE1 and its EPA-derived precursor 18*R*-hydroxyeicosapentaenoic acid on mitochondrial function in experimental inflammation. As there is a paucity of data in the human system, especially with respect to 18*R*-HEPE and RvE1, we chose human peripheral mononuclear blood cells (PBMCs) for further analyses, as their main components (lymphocytes and monocytes) play an essential role in the pathogenesis of inflammation.

2. Material and methods

2.1. Preparation and culture of peripheral mononuclear blood cells

The institutional review board (Ethikkommission des Fachbereichs Medizin, Justus-Liebig University Giessen) approved the study and written informed consent was obtained from each healthy volunteer. To isolate human PBMCs, peripheral blood (15 ml) was collected by venepuncture into EDTA-buffered collection tubes (Sarstedt, Nürnberg, Germany) and subsequently subjected to a Ficoll-Hypaque (Sigma-Aldrich, Germany) gradient following the manufacturer's protocol [13].

2.2. Cell culture experiments

To investigate the impact of selected fatty acids and RvE1 on mitochondrial function, PBMCs were cultured for 12 h using RPMI-1640 cell culture medium (Sigma-Aldrich, Munich, Germany) including 10% foetal calf serum (PAA, Linz, Austria) and subsequently incubated with equimolar concentrations (30 μmol/l) of arachidonic acid, docosahexaenoic acid, eicosapentaenoic acid, the EPA-metabolite 18*R*-hydroxyeicosapentaenoic acid (2 μM), or resolvin E1 (50 nM) for 3 h and then subjected to the different experiments. All fatty acids were obtained from Sigma-Aldrich (Munich, Germany). 18*R*-HEPE and RvE1 were from Cayman Chemical (distributed by Biomol, Hamburg, Germany). To mimic inflammatory conditions, cells were incubated with tumour necrosis factor (TNF)-α (10 ng/ml) 1 h prior to the addition of fatty acids or RvE1 as we could achieve a stable and significant inflammatory response after 4 h of TNF-α stimulation based on findings from pre-experiments. This setting was used for all experiments if not indicated different. Mitochondrial fission inhibitors Mdivi-1 or Dynasore (both from Sigma-Aldrich) were administered to cell culture 30 min prior to addition of TNF-α in the indicated experiments.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Concentrations of interleukin-6 (IL-6) and -8 (IL-8) in cell culture supernatants were determined by ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

2.4. High-resolution respirometry

Cellular respiration was measured by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) as described previously [13, 14]. According to the experimental protocol, the respective cell suspensions were transferred into the measuring chambers of the Oxygraph-2k device (37 °C with a stirring speed of 750 rpm). Respiration was recorded in real-time (1-second time intervals) and analysed using DatLab software (Oroboros Instruments, Innsbruck, Austria). To assess mitochondrial respiration, each experiment began by recording basal physiological respiratory activity in intact cells (routine respiration). After reaching steady-state respiratory flux, ATP synthase was inhibited with 2 μg/ml oligomycin (Sigma-Aldrich, Munich, Germany) to determine “leak respiration”. Maximal capacity of the respiratory chain (“Max”) was assessed by 0.5 μM stepwise titration of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, Sigma-Aldrich, Munich, Germany) leading to an uncoupling of oxidative phosphorylation. Finally, non-mitochondrial respiration was measured by sequential addition of the specific complex I and III inhibitor with 5 μM antimycin A (Sigma-Aldrich, Munich, Germany) to the cell suspension (Fig. 1).

2.5. Cell stress and apoptosis antibody array

Relative levels of human cell stress-related and apoptosis-related proteins were determined in pre-treated cell lysates using membrane-based antibody arrays, according to the manufacturer's instructions (Human Cell Stress Antibody Array, Human Apoptosis Array, both purchased from R&D Systems, Wiesbaden, Germany). To evaluate changes in spot intensity, blots were visually evaluated by three independent investigators. Spots marked in the manuscript were whose which all three investigators described as being changed.

2.6. Determination of mitochondrial membrane potential (Δϕ)

Mitochondrial membrane potential (Δϕ) was assessed using the membrane-permeant lipophilic cationic JC-1 dye. We used the commercially available JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemicals, via Biomol Hamburg, Germany) according to the manufacturer's instructions.

Briefly, JC-1 exhibits potential-dependent accumulation in mitochondria. In cells with high Δϕ, JC-1 forms complexes (known as J-aggregates) with intense red fluorescence (590 nm), whereas in cells with low Δϕ, JC-1 remains in the monomeric form and exhibits green fluorescence (530 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

2.7. Measurement of reactive oxygen species (ROS)

ROS concentration was determined using the 2′,7′-dichlorofluorescein diacetate (DCF-DA) method using a commercially available assay (DCFDA Cellular ROS Detection Assay Kit, Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, after diffusion into the cell, DCF-DA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2′,7′–dichlorofluorescein (DCF), which is a highly fluorescent compound being detectable by fluorescence spectroscopy.