The effect of itaconic acid on mitochondrial substrate-level phosphorylation

PhD Thesis

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INTRODUCTION

Itaconic acid is an unsaturated dicarboxylic acid. It is produced in industrial scale from *cis*-aconitate by the extramitochondrial *cis*-aconitate decarboxylase, an enzyme encoded by the *cad1* gene in *Aspergillus terreus* and is used as a monomer for the production of a plethora of products including resins, plastics, paints, and synthetic fibers. Itaconic acid has been identified in a small number of metabolomic studies of mammalian tissue specimens, such as activated macrophages, *Mycobacterium tuberculosis*-infected lung tissue, urine and serum samples, and glioblastomas. More recently, it has been shown that lipopolisaccharide (LPS)-induced human and mouse macrophages produce itaconic acid from *cis*-aconitate through an enzyme exhibiting similar activity as in *Aspergillus terreus*, coded by the gene *cis*-aconitate decarboxylase 1 (*Acod1*) (previous name: immunoresponsive gene 1, *Irg1*). The latter finding confirmed earlier reports suggesting the presence of itaconate in macrophage-like tumor cell lines and primary murine macrophages in the low millimolar range. Acod1-mediated itaconate production contributes to the antimicrobial activity of macrophages by inhibiting isocitrate lyase, a key enzyme of the glyoxylate shunt. The glyoxylate shunt is not present in animals, but is essential for the survival of bacteria growing on fatty acids or acetate as limiting carbon source.

Although the applications of itaconic acid and its derivatives extend to dental, ophthalmic, and drug delivery fields, and in

complexation with benzylammonium it is used to prepare water soluble coating for food packaging to reduce bacteria contamination, it does not enter the food chain to an appreciable degree. Still, it was shown to be extensively metabolized when administered *per os* to cats, dogs and murine animals.

The group of Henry A. Lardy reported more than 50 years ago that exogenously added itaconate to isolated mitochondria is oxidized as most members of the citric acid cycle. The same group elucidated the pathway of itaconate metabolism towards pyruvate and acetyl CoA; however, at the time, the identity of succinate-CoA ligase (referred to as “succinate-activating enzyme”, or “P enzyme”) and its role in substrate-level phosphorylation (SLP) was not yet revealed.

OBJECTIVES

We assumed that itaconic acid exerts bioenergetic effects on adenine (or guanine) nucleotide production in the mitochondrial matrix *via* succinate-CoA ligase.

We set as an aim:

- to investigate specific bioenergetic effects of increased itaconate production mediated by LPS-induced stimulation of *cis*-aconitate decarboxylase (Acod1) in macrophages;

- to investigate the dose-dependent effect of exogenously added itaconate to isolated liver mitochondria, under defined metabolic conditions to reveal the mechanism(s) of itaconate on SLP.

METHODS

Animals: Mice were of C57Bl/6 background; of either sex and between 2 and 3 months of age.

Isolation of mitochondria: Following cervical dislocation, the liver was rapidly removed, minced, washed and homogenized in ice-cold isolation buffer. For mitochondria isolation we used differentiated centrifugation. Protein concentration was determined by protein assay.

Determination of membrane potential (ΔΨm) in isolated liver mitochondria: ΔΨm of isolated mitochondria was estimated fluorimetrically with safranine O. Fluorescence was recorded in a Hitachi F-7000 spectrofluorimeter at a 5 Hz acquisition rate or at a 1 Hz rate using the O2k-Fluorescence LED2-Module of the Oxygraph-2k. Experiments were performed at 37^{o}C.

Mitochondrial respiration: Oxygen consumption was estimated polarographically using an OROBOROS Oxygraph-2k. Experiments were performed at 37^{o}C. Oxygen concentration and oxygen flux were recorded using DatLab software (Oroboros Instruments).

Cell cultures:

BMDMs (Bone marrow-derived macrophages) originated from mice. Cells were first cultured in Minimum Essential Medium α complemented with fetal bovine serum, L-glutamine, penicillin/streptomycin in the presence of mouse macrophage

colony-stimulating factor (M-CSF). After 2 days, non-adherent cells were plated on petri dishes and cultured in the same medium but M-CSF was supplied as a conditioned medium from CMG14-12 cells. Medium/cytokine was changed in every two days.

TIPMs (Thioglycollate-induced peritoneal macrophages) were obtained by lavage of the peritoneal cavity of C57BL/6 mice which were injected 3 days previously with sodium thioglycollate. The cells were plated and cultured similarly as for the BMDMs.

RAW-264.7 cells were cultured in RPMI 1640 medium containing L-glutamine, supplemented with fetal bovine serum and penicillin/streptomycin. The medium was changed every 2 days. Cells were plated on petri dishes for Western blot analysis, or on cover glass for image analysis. Eight hours after plating, fresh media with or without ultrapurified LPS was added and the cells were cultured for additional 12 hours before cell lysis or imaging.

COS-7 cells were grown in DMEM with glutamine, FCS, and streptomycin-penicillin. On reaching confluence, cultures were harvested by trypsinization and were transfected by electroporation according to the manufacturer's instructions.

Mitochondrial membrane potential (ΔΨm) measurement in cultured BMDM and RAW-264.7 cells: For ΔΨm, cells were loaded with TMRM and submitted to a temperature controlled (34^{o}C) incubation. After identifying the transfected cells, time lapse

epifluorescence microscopy was carried out – for TMRM as well as for fluorescein-tagged siRNA or scrambled siRNA.

Image analysis: Image analysis was performed in Image Analyst MKII (Novato, CA). Due to significant migration of cells during the measurements, first time series of images were "maximum intensity" projected into a single frame (pixel by pixel) and regions of interests (ROIs) were subsequently selected by an automated algorithmic tool of the software. The ROIs were subsequently assigned to individual cells and TMRM intensities corresponding to individual cells were plotted over time.

Measurement of in situ mitochondrial oxidation and glycolytic activity: Real-time measurements of oxygen consumption rate (OCR − reflecting mitochondrial oxidation) and extracellular acidification rate (ECAR − considered as a parameter of glycolytic activity) were performed on a microfluorimetric XF96 Analyzer (Seahorse Bioscience, North Billerica, MA, USA). Cells were seeded 1-2 days before measurement in Seahorse XF96 cell culture and were treated with ultrapurified LPS. Before measurement growth media was changed to XF assay media. O2 tension and pH values were detected and OCR, ECAR values were calculated by the XF96 Analyzer software. Data were normalized to total protein content, measured with BCA protein assay kit.

Western blot analysis: Cells were harvested by trypsinization, washed, solubilised in RIPA buffer containing a cocktail of protease inhibitors and frozen at −80^{o}C. Frozen pellets were thawed on ice, their protein concentration was determined using the bicinchoninic acid assay and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed using rabbit polyclonal anti-ACOD1, mouse monoclonal anti-FLAG and mouse monoclonal anti-β actin primary antibodies. Immunoreactivity was detected using the appropriate peroxidase-linked secondary antibody.

Fluorescein-tagged siRNA and cell transfections: RAW-264.7 cells were transfected with siRNA specific to murine *Acod1* or scrambled siRNA (the corresponding non-targeting control) before a subsequent treatment with LPS, or vehicle. Prior to transfections, cells were plated in their regular media in the absence of antibiotics.

***Acod1*-FLAG plasmid transfections:** pCMV6-FLAG-*Acod1* overexpressing plasmid was transfected into RAW-264.7 or COS7 cells cultures cells using Lipofectamine 2000 and further incubated for 24-48 hours.

Immunocytochemistry: RAW-264.7 cell cultures were transfected with the pCMV6-FLAG-*Acod1* overexpressing plasmid in OPTIMEM (reduced serum medium without antibiotics). Prior to fixation, cells were treated with Mitotracker Orange. Subsequent

immunocytochemistry of the cultures was performed by fixing the cells with paraformaldehyde, followed by permeabilization by TX-100. Cultures were treated with 10% donkey serum overnight at $4^{o}C$ followed by bathing in 1% donkey serum and anti-FLAG antibody for 1 hour at room temperature. Cells were subsequently decorated by using the appropriate Alexa 492-linked secondary antibody and visualized using the imaging setup.

Determination of SDH activity: The activity of SDH (succinate dehydrogenase) in isolated liver mitochondria was determined by spectrophotometric assay

RESULTS

Here, we investigated specific bioenergetic effects of increased itaconate production mediated by lipopolysaccharide (LPS)-induced stimulation of *Acod1* in murine bone marrow-derived macrophages (BMDM) and RAW-264.7 cells.

In rotenone-treated macrophage cells, stimulation by LPS led to impairment in substrate-level phosphorylation (SLP) of *in situ* mitochondria, deduced by a reversal in the directionality of the adenine nucleotide translocase operation.

In RAW-264.7 cells, the LPS-induced impairment in SLP was reversed by short-interfering RNA (siRNA) − but not scrambled siRNA − treatment directed against *Acod1*.

LPS dose-dependently inhibited oxygen consumption rates (61-91%) and elevated glycolysis rates (>21%) in BMDM but not RAW-264.7 cells, studied under various metabolic conditions.

In isolated mouse liver mitochondria treated with rotenone, itaconate dose-dependently (0.5-2 mM) reversed the operation of adenine nucleotide translocase, implying impairment in SLP, an effect that was partially mimicked by malonate. However, malonate yielded greater ADP-induced depolarizations (3-19%) than itaconate.

CONCLUSIONS

We postulate that itaconate abolishes substrate-level phosphorylation due to:

1. a “CoA trap” in the form of itaconyl-CoA that negatively affects the upstream supply of succinyl-CoA from the α-ketoglutarate dehydrogenase complex;

2. depletion of ATP (or GTP), which are required for the thioesterification by succinate-CoA ligase;

3. inhibition of complex II leading to a buildup of succinate which shifts succinate-CoA ligase equilibrium toward ATP (or GTP) utilization.

Our results support the notion that *Acod1*-expressing cells of macrophage lineage lose the capacity of mitochondrial SLP for producing itaconate during mounting of an immune defense.

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