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ATP-citrate lyase is essential for macrophage inflammatory response

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A B S T R A C T

Growing evidence suggests that energy metabolism and inflammation are closely linked and that cross-talk between these processes is fundamental to the pathogenesis of many human diseases. However, the molecular mechanisms underlying these observations are still poorly understood. Here we describe the key role of ATP-citrate lyase (ACLY) in inflammation. We find that ACLY mRNA and protein levels markedly and quickly increase in activated macrophages. Importantly, ACLY activity inhibition as well as ACLY gene silencing lead to reduced nitric oxide, reactive oxygen species and prostaglandin E2 inflammatory mediators. In conclusion, we present a direct role for ACLY in macrophage inflammatory metabolism.

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1. Introduction

Inflammation is the body's basic response to a variety of external or internal insults, such as infectious agents, physical injury, hypoxia, or disease processes [1]. Macrophages play a major role in the inflammatory process by detecting these insults and releasing various pro-inflammatory molecules including prostaglandins (PGs), reactive oxygen species (ROS), nitric oxide (NO) and cytokines. These factors promote inflammation by causing vasodilation and recruitment of neutrophils, monocytes and by altering the functionality of many tissues and organs. Depending on the inducer, the inflammatory response has a different physiological purpose and pathological consequences. For instance, during microbial infections one of the most potent macrophage activators is the gram-negative bacterial cell wall component lipopolysaccharide (LPS) which leads to the production of a variety of inflammatory mediators [2].

Tumour necrosis factor α (TNFα) and interferon γ (IFNγ) are the endogenous inducers produced in the tissues under stress, damage or otherwise malfunctioning. In this case the inflammation has the physiological purpose to adapt to stress, and restore a homeostatic state. However, a pathological consequence can be the development of inflammatory diseases. A comprehensive list of chronic inflammatory diseases would run to over 100, each of which shows high levels of inflammation. Among them are rheumatoid arthritis, systemic lupus erythematosus and Crohn's disease. Many of these pathological conditions are debilitating and are becoming increasingly common in our aging society. However, the number of safe and effective treatments is limited. To date, the major research effort has concentrated on those mediators responsible for initiation and maintenance of the pathological process. In contrast, little attention has been focused on metabolic signals which can be responsible for induction and/or control of the inflammatory response.

Here we investigate the role of ATP-citrate lyase (ACLY), a cross-link between glucose metabolism and fatty acid synthesis. In the cytoplasm, glucose-derived citrate is transformed, in the presence of ATP, into acetyl-CoA by ACLY [3,4]. Acetyl CoA is an essential substrate for cholesterol, isoprenoids and fatty acid synthesis pathways. Acetyl-CoA is also required for acetylation of nuclear histones in mammalian cells [5]. ACLY is most abundantly expressed in liver and white adipose tissue. Additionally, ACLY expression has been reported to be upregulated in many tumors, nonalcoholic fatty liver disease and other pathological conditions [6]. Surprisingly, we find that ACLY expression levels markedly and quickly increase in normal peripheral blood differentiated macrophages as well as in macrophage cell lines activated by exogenous and endogenous inducers. Furthermore, the specific ACLY activity inhibition or gene silencing is sufficient to reduce production of inflammatory mediators. Overall these results indicate a central role for ACLY in inflammation. In light of the evidence presented here, the ability of ACLY to integrate energy metabolism and inflammatory signaling makes it a particularly attractive target in human inflammatory diseases.

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2. Materials and methods

2.1. Cell culture

Mononuclear cells were isolated from heparinized blood of healthy adult volunteers and differentiated into macrophages as described previously [7]. Human monocytic/macrophage cells from hystiocytoma, U937 cells (HTL 94002, Interlab Cell Line Collection, Genoa, Italy), were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2 in a water-saturated atmosphere. U937 cells were differentiated with 10 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma–Aldrich, St Louis, MO, USA).

2.2. Activating stimuli

U937/PMA (differentiated U937) cells were treated for 16 h with 200 ng/ml *Salmonella* typhimurium LPS (Sigma–Aldrich), for 1 h with 5 ng/ml TNFα (Sigma–Aldrich), 10 ng/ml IFNγ (ImmunoTools GmbH, Friesoythe, Germany) or combined IFNγ and TNFα. Human macrophages differentiated from peripheral blood mononuclear cells (see Supplementary information) were treated for 1 h with 200 ng/ml LPS, 5 ng/ml TNFα, 10 ng/ml IFNγ or combined IFNγ and TNFα. Where indicated U937/PMA cells were treated with 20 μM TPCK (Sigma–Aldrich), 10 μM NIFU (Sigma–Aldrich), 500 μM HCA (Sigma–Aldrich), 5 μM SB-204990 ((+)-(3R*,5S*)-3-carboxy-11-(2,4-dichlorophenyl)-3,5-dihydroxyundecanoic acid, a gift from GlaxoSmithKline) or 250 nM RAD (Sigma–Aldrich) 1 h before stimulation with LPS, TNFα, IFNγ, or combined IFNγ and TNFα.

2.3. RNA interference

RNA interference experiments were performed as described previously [8] by using a specific pre-designed small interfering RNA (siRNA) targeting human ACLY (s917, Life Technologies, Paisley, UK). After 24 h, the medium was replaced with fresh medium and the siRNA-transfected U937/PMA cells were treated with LPS, TNFα, IFNγ, or combined IFNγ and TNFα. ROS and NO were measured 24 h after the addition of inducers. siRNA (C6A-0126, Life Technologies) with no significant similarity to human, mouse, or rat gene sequences was used as negative control [9].

2.4. Real-time PCR, SDS–PAGE and Western blotting

Total RNA was extracted and reverse transcripted as reported [10]. Real-time PCR was performed as previously described [11] by using human ACLY (Hs00982738_m1) and human β-actin (4326315E) taqMan® assays (Life Technologies). For immunoblot analysis, U937 cells were rinsed with ice-cold PBS and lysed using RIPA buffer. Thirty micrograms of total proteins were heated at 100 °C for 5 min, separated on 4–8% SDS polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were then blocked for 1 h in a PBS solution containing 2% bovine serum albumin and 0.1% Tween 20, and then treated at room temperature with anti-ACLY (Aviva Systems Biology, San Diego, CA, USA) or anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. The immunoreaction was detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

2.5. NO, ROS and PGE_2 detection

Nitrite formation was detected by using 1H-naphthotriazole from 2,3-diaminonaphthalene (DAN, Life Technologies) [12]. For ROS analysis, U937/PMA activated cells were incubated with 10 μM DCFH2-DA (Life Technologies) for 30 min. The fluorescence was revealed by GloMax plate reader (Promega, Madison, WI, USA) [13]. PGE_2 was detected by the PGE_2 Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's instructions.

3. Results

3.1. Expression of ACLY in macrophages from peripheral blood

Inflammatory response is an energy-intensive process and metabolic changes occur in cells that participate in inflammation, such as activated macrophages [14]. In view of the central role of ACLY in energy metabolism, we tested whether inflammatory stimuli affected ACLY gene expression. First of all, macrophages from peripheral blood were treated with LPS, TNFα, IFNγ and by a combination of TNFα and IFNγ. Fig. 1A shows that both exogenous and endogenous inducers produced an increase in ACLY mRNA levels. Interestingly, this ACLY overexpression was observed at 1 h after all treatments, in agreement with the increase in protein levels. Among the different inducers, the combination of TNFα and IFNγ was more efficient in upregulating ACLY gene expression (Fig. 1A). These findings clearly show that multiple stimuli trigger ACLY overexpression in immune cells.

3.2. ACLY gene upregulation in LPS-activated macrophages

To further investigate the modulation of ACLY gene expression in inflammation, we used human differentiated U937 (U937/PMA) cells. We induced inflammation by LPS exposure and analyzed ACLY mRNA and protein levels. A markedly increase of about 2,5-fold ACLY activation was evident at 16 h after stimulation compared to untreated cells (Fig. 1B and S1A).

The main pathway reported for LPS-TLR4-induced signaling acts through nuclear factor κB (NF-kB) [15]. However, the signal transducer and activator of transcription (STAT) signaling can also be activated during LPS treatments [16]. To clarify the molecular mechanisms responsible for ACLY gene upregulation during LPS-activation we performed in silico analysis of the human ACLY gene promoter and we found two NF-kB and three STAT responsive elements. We tested the effect of the LPS-induced pathways on ACLY gene activation by using tosylphenylalanylchloromethane (TPCK) and nifuroxazide (NIFU), specific inhibitors of NF-kB [17] and STAT signaling [18], respectively. When U937/PMA cells were treated with LPS in the presence of TPCK or nifuroxazide, a reduction of ACLY mRNA and protein levels was observed as compared to cells treated with LPS alone (Fig. 1C and D). These results indicate that ACLY gene upregulation in microbial pathogen-induced macrophages is under control of both NF-kB and STAT transcription factors.

3.3. TNFα and IFNγ upregulate ACLY gene expression

To understand the effect of endogenous inducers on ACLY gene expression, we treated U937/PMA cells with TNFα and IFNγ alone or in combination. Surprisingly, at 1 h after stimulation, ACLY mRNA increased of about 50% when TNFα and IFNγ alone were used and even more in TNFα + IFNγ-activated U937/PMA as compared with untreated cells (Fig. 1E and S1B–D). A greater amount of ACLY protein was also detected after macrophage induction with respect to control cells (Fig. 1E).

It is known that TNFα acts by binding to its receptors TNFRs and leading to the activation of NF-kB [19]. As we found NF-kB responsive elements in ACLY gene promoter, we tested the involvement

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