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Fatty acid transport protein 1 enhances the macrophage inflammatory response by coupling with ceramide and c-Jun N-terminal kinase signaling

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ABSTRACT

Macrophages are important cells that need to be controlled at the site of inflammation. Several factors are involved in chronic inflammation and its timely resolution. Free fatty acids drive the inflammatory response in macrophages and contribute to the vicious cycle of the inflammatory response. However, the identity of the uptake pathways of fatty acids is not fully clear in macrophages and how the inflammatory responses are regulated by the uptake of fatty acids remain poorly understood. We investigated the relationship between fatty acid transport protein (FATP) and the inflammatory response signaling pathway in macrophages as the first report. The FATP family has composed six isoforms, FATP1-6. We found that FATP1 is the most highly expressed isoform in macrophages. Forced expression of FATP1 enhanced production of inflammatory cytokines, such as TNFα and IL-6 concomitant with the increased uptake of fatty acids, increased level of ceramide, and increased phosphorylation of c-Jun N-terminal kinase (JNK). The enhancement by FATP1 was abolished by treatment with a JNK inhibitor, NF-κB inhibitor, or ceramide synthesis inhibitor. siRNA-mediated knockdown of FATP1 strongly inhibited the production of TNFα and IL-6. Similarly, an inhibitor of FATP1 inhibited the production of TNFα and IL-6. Finally, an inhibitor of FATP1 attenuated the production of inflammatory cytokines in bronchoalveolar lavage fluid in an LPS-induced acute lung injury in vivo mouse model. In summary, we propose that FATP1 is an important regulator of inflammatory response signaling in macrophages. Our findings suggest that ceramide-JNK signaling is important to terminate or sustain inflammation.

1. Introduction

Fatty acids (FA) are essential components of the cellular energy production and are precursor molecules for all lipid classes, including those that form biological membranes [1]. Free FA act as powerful signaling molecules to regulate numerous cellular processes associated with lipid metabolism and the development of metabolic syndromes, such as diabetes [2]. Furthermore, free FA act as endogenous ligands for specific receptors, including toll-like receptor (TLR) 2 and TLR4 [3–5]. TLRs are expressed in innate immune cells, such as macrophages, and induce the production of inflammatory cytokines [6]. LPS, an exogenous TLR4 ligand, affects triglyceride retention, ceramide biosynthesis, lipolysis, and FA oxidation as well induces inflammatory responses [7,8], indicating that TLR signaling is involved in FA metabolism. FA oxidation regulates macrophage polarization and affects the production of inflammatory cytokines [9–11]. Thus, FA contribute to the vicious cycle of the inflammatory response in macrophages [12]. However, the mechanism of inflammatory response regulation mediated by FA remains to be clarified.

Fatty acid transport protein (FATP) is found in the plasma membrane and intracellular organelles, has fatty acyl-CoA ligase activity, and is an important molecule in FA uptake [13–15]. The FATP family contains six isoforms: FATP1-6 [13–15]. A recent study has shown that FATPs are expressed in macrophages [16]. Although FA regulate the inflammatory response, the relationship between FA and FATPs is not known. Our aim was to understand the importance of FATP on the inflammatory cascade in macrophages. In this study, we investigated the involvement of FATPs in the inflammatory response mediated by FA using bone marrow-derived macrophages (BMDM), mouse macrophage cell line RAW264.7 cells, and a LPS-induced acute lung injury (ALI) in

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vivo mouse model and found that among FATPs, FATP1 regulates the production of inflammatory cytokines through ceramide and the c-Jun N-terminal kinase (JNK) signaling pathway.

2. Material and methods

2.1. Regents

LPS from *E. coli* [0111:B4] was purchased from Sigma (St. Louis, MO). Recombinant INFγ and IL-4 were purchased from PeproTech (Rocky Hill, NJ). Sp600125, APDC, and U0126 were purchased from Cell Signaling Technology (Danvers, MA). C2 ceramide was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). FB1 was purchased from Cayman Chemical (Ann Arbor, MI). FATP1 inhibitors (DS22420314 and DS29530755) were gifts from DAIICHI SANKYO COMPANY, LIMITED (Tokyo, Japan).

2.2. Animals

Male C57BL/6 mice (8–10 weeks old) were obtained from CLEA Japan, Inc. (Tokyo, Japan). All procedures used in this study complied with the institutional policies of the Osaka Prefecture University Animal Care and Use Committee.

2.3. BMDM

BMDM were prepared as described previously [17]. Briefly, BM cells from mice tibias and femurs were obtained by flushing with DMEM. BM cells were cultured in DMEM supplemented with 20% FBS, L-glutamine, and 30% L929 supernatant containing M-CSF. After 5 days, BMDM were resuspended in DMEM supplemented with 5% FBS.

2.4. Cell cultures

RAW264.7 cells were gifts of S. Maeda and Y. Yoshioka (Setsunan University, Osaka, Japan). RAW264.7 cells were grown in DMEM 4.5 g/L (25 mM) glucose supplemented with 10% FBS, 1 × L-glutamine, and antibiotics/antimycotics at 37 °C in a 5% CO_2 humidified incubator.

2.5. Establishment of stably transfected cells

For overexpression, cDNA of the murine FATP1, FATP4, and FATP6 genes was cloned into the pcDNA3.1 vector (Genscript Corporation, Piscataway, NJ). RAW264.7 cells were transfected with either the empty vector, FATP1 vector, FATP4 vector or FATP6 vector using an electroporation system (Gene Pulser Xcell; BIO-RAD, Hercules, CA). Cells were grown to subconfluence, trypsinized, plated at a low density, and selected in the presence of 1000 μg/mL G418 (Roche, Madison, WI) for 2 weeks. Colonies were isolated by digestion with trypsin/EDTA for 3 min at 37 °C within stainless steel cloning rings. Two to three independent clones were established for each expression vector. Stably transfected RAW264.7 cells were maintained in 400 μg/mL G418.

2.6. Small interfering RNA transfection

RAW264.7 cells were transfected with pooled siRNA against FATP1 (GE Healthcare, Buckinghamshire, UK) or nontargeted control siRNA (GE Healthcare), using Viromer BLUE (Lipocalyx, Halle, Germany). The siRNA concentration was 25 nM. RAW264.7 cells were then cultured for 48 h to achieve efficient knockdown.

Table 1
Sequences of primers used in quantitative PCR.

Name		5′-3′
FATP1	F	AGTGCGTCATCTACGGGTTG
	R	GGCAGATTTCACCTATGTACTGC
FATP2	F	TACTTCGGGAACCACAGGTC
	R	TCCGCAAAGCTAAAGTAGCC
FATP3	F	GGGCCCTGAAACTAATGTAGC
	R	AATCCCTGGCACTGTAGAACC
FATP4	F	CTTCATCAAGACGGTCAGGAG
	R	GGAAGGTCCAGTGAGTGTCTG
FATP5	F	AGTGGAAATCTCCTGCCATATTC
	R	GGTAGGACGTCATAGACCACATC
FATP6	F	TTCACTCCAAGAAAAGCTGAGTC
	R	AACACTTGCAACTGGCTAATCAC
TNFa	F	ATGAGCACAGAAAGCATGATCCGC
	R	CCAAAGTAGACCTGCCCGGACTC
IL-6	F	AAGGGCCAGGGATCTGTAAG
	R	TCTCTTGTTGCTCCCCAAAG
Nos2	F	CCAAGCCCTCACCTACTTCC
	R	CTCTGAGGGCTGACACAAGG
IL-10	F	GCTCTTACTGACTGGCATGAG
	R	CGCAGCTCTAGGAGCATGTG
HPRT	F	GTTGGATACAGGCCAGACTTTGTTG
	R	GAGGGTAGGCTGGCCTATAGGCT

Forward (F) and reverse (R) sequences are listed (5′-3′) for *FATP1*, *FATP2*, *FATP3*, *FATP4*, *FATP5*, *FATP6*, *TNFa*, *IL-6*, *Nos2*, and *HPRT*.

2.7. Proliferation assay

Cell proliferation was evaluated by the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI) [18]. Briefly, cells were incubated in 96-well culture plates for 48 h. After incubation, MTS solution was added to the culture plates. The absorbance at a wavelength of 490 nm was measured at 2 h after the addition of MTS solution, and the reference absorbance was 690 nm.

2.8. Phagocytic activity assay

RAW264.7 cells were tested for their ability to ingest FITC-labeled latex beads using the Phagocytosis Assay Kit (Cayman Chemical) according to the manufacturer's instructions. The fluorescence was determined using a microplate reader for detecting excitation and emission at 485 nm and 535 nm, respectively.

2.9. RNA isolation and qPCR

Total RNA was isolated as previously described [19] with minor modifications. RNA was used to synthesize complementary cDNA using SuperScript Reverse Transcriptase (Roche). The primers used for amplification are described in Table 1. mRNA expression was quantified using quantitative real-time PCR (qPCR) analysis based on the intercalation of SYBR Green (Toyobo, Osaka, Japan). Amplification of HPRT mRNA was used as an endogenous control to account for the differences in the amount and quality of RNA added to each reaction.

2.10. ELISA for cytokine

The cytokine concentrations were determined in the culture supernatants or BALF using ELISA (eBioscience, San Diego, CA) and the multiplex assay (BIO-RAD) according to the manufacturer's instructions.