Biochimica et Biophysica Acta 1843 (2014) 2775–2783

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

The effect of anti-inflammatory properties of ferritin light chain on lipopolysaccharide-induced inflammatory response in murine macrophages

Yumei Fan [a,b,c,1], Jie Zhang [a,b,c,1], Linlin Cai [a,b,c,1], Shengnan Wang [a,b,c], Caizhi Liu [a,b,c], Yongze Zhang [a,b,c], Linhao You [a,b,c], Yujian Fu [a,b,c], Zhenhua Shi [a,b,c], Zhimin Yin [d], Lan Luo [e], Yanzhong Chang [a,b,c,*], Xianglin Duan [a,b,c,*]

[a] Laboratory of Molecular Iron Metabolism, College of Life Science, Hebei Normal University, Shijiazhuang 050024, PR China
[b] Key Laboratory of Animal Physiology, Biochemistry and Molecular Biology of Hebei Province, College of Life Science, Hebei Normal University, Shijiazhuang 050024, PR China
[c] Key Laboratory of Molecular and Cellular Biology of Ministry of Education, College of Life Science, Hebei Normal University, Shijiazhuang 050024, PR China
[d] Jiangsu Province Key Laboratory for Molecular and Medical Biotechnology, College of Life Science, Nanjing Normal University, Nanjing 210046, PR China
[e] State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210093, PR China

ARTICLE INFO

Article history:
Received 17 February 2014
Received in revised form 18 June 2014
Accepted 19 June 2014
Available online 28 June 2014

Keywords:
Ferritin light chain
Inflammatory response
LIP
ROS
MAPKs
NF-κB

ABSTRACT

Ferritin light chain (FTL) reduces the free iron concentration by forming ferritin complexes with ferritin heavy chain (FTH). Thus, FTL competes with the Fenton reaction by acting as an antioxidant. In the present study, we determined that FTL influences the lipopolysaccharide (LPS)-induced inflammatory response. FTL protein expression was regulated by LPS stimulation in RAW264.7 cells. To investigate the role of FTL in LPS-activated murine macrophages, we established stable FTL-expressing cells and used shRNA to silence FTL expression in RAW264.7 cells. Overexpression of FTL significantly decreased the LPS-induced production of tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), nitric oxide (NO) and prostaglandin E2 (PGE2). Additionally, overexpression of FTL decreased the LPS-induced increase of the intracellular labile iron pool (LIP) and reactive oxygen species (ROS). Moreover, FTL overexpression suppressed the LPS-induced activation of MAPKs and nuclear factor-κB (NF-κB). In contrast, knockdown of FTL by shRNA showed the reverse effects. Therefore, our results indicate that FTL plays an anti-inflammatory role in response to LPS in murine macrophages and may have therapeutic potential for treating inflammatory diseases.

1. Introduction

Ferritin, an iron storage molecule, is mainly localized in the cytoplasm, trace amounts are also detected in the serum and other biological fluids [1]. The cytoplasmic form of mammalian ferritin consists of two subunits: ferritin heavy chain (FTH) and ferritin light chain (FTL). FTH has high ferroxidase activity and converts Fe (II) to Fe (III), and FTL transfers iron from the ferroxidase center to the iron core to improve the overall iron sequestering process [2]. High amounts of ferritin have been observed in inflammatory macrophages [3]. Lipopolysaccharide (LPS) addition also induces ferritin expression in the lung [4]. These data suggest that the increase in intracellular ferritin in macrophages is related to the secretion of factors by phagocytes. Recently, the functions of FTH in immunity and the cell apoptosis process have gained more attention [5,6]. However, little is known about the functions and mechanism of FTL in the inflammatory response, and new functions for the iron storage protein are yet to be discovered.

LPS, the major outer membrane component in Gram-negative bacteria, can activate monocytes/macrophages and induce a variety of immune responses to severe infections, such as septic shock and systemic inflammatory response syndrome (SIRS) [7,8]. The development of the inflammatory process involves the production of pro-inflammatory cytokines and inflammatory mediators, such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), nitric oxide (NO) and prostaglandin E2 (PGE2), which are the main factors that mediate the LPS-induced toxic effects [9–11]. The inhibition of the excess production of pro-inflammatory factors is required for

Abbreviations: FTL, ferritin light chain; LPS, lipopolysaccharide or endotoxin; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; ERK, extracellular-signal-related kinase; NF-κB, nuclear factor-κB; TfR1, transferrin receptor 1; LIP, labile iron pool; ROS, reactive oxidative species; DCFH-DA, 2′,7′-dichlorfluorescein-diacetate; SIH, salicylaldehyde isonicotinoyl hydrazone

* Corresponding authors at: No. 1 2nd Ring Rd East, Laboratory of Molecular Iron Metabolism, College of Life Science, Hebei Normal University, Shijiazhuang, Hebei Province, 050024, PR China. Tel./fax: +86 311 80787586.
E-mail addresses: chang7676@163.com (Y. Chang), xlduan0311@163.com (X. Duan).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbamcr.2014.06.015
0167-4889/

decreasing inflammatory reactions, which are the main cause of inflammatory diseases. Previous reports showed that MAPKs and NF-κB signaling pathways are closely related to the LPS-induced synthesis of TNF-α and IL-1β in macrophages. These signaling pathways are also involved in inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in macrophages, which catalyze the production of NO and PGE2, respectively [12–15]. LPS-induced reactive oxygen species (ROS) upregulation is a potent inducer of MAPKs and NF-κB signaling pathways [16]. Free iron, which is found in the cellular labile iron pool (LIP), acts as a catalytic agent for Fenton reactions and participates in the generation of free radicals that possess unpaired electrons such as $HO^{\cdot}$. Free radicals are generally known as ROS and result in oxidative damage [17].

We hypothesized that FTL may exert an anti-inflammatory role in the LPS-induced inflammatory response that involves inhibiting LIP upregulation and ROS production. In the current study, we developed a RAW264.7 murine macrophage-based cell line that either stably overexpressed the FTL protein or was depleted of FTL through shRNA technology. These cells were used to clarify the role of FTL in the LPS-induced inflammatory response in activated macrophages. Our results demonstrated the role for FTL in LPS-induced activation of signaling pathways and the development of the inflammatory process. Our findings suggest that FTL exerts an anti-inflammatory role in LPS-induced inflammatory response, and modulation of FTL may be a potential approach for anti-inflammatory therapy.

2. Materials and methods

2.1. DNA constructs

The pcDNA3-Flag-FTL (pFTL) construct was generated by inserting the mouse *Ftl* cDNA (NCBI accession number: NM_010240) into pcDNA3 (Invitrogen, Carlsbad, CA, USA). The Flag epitope tag was introduced at the 3′ end of the coding sequence of the gene. The FTL shRNA expression vector, pRNA-U6.1/neo-shFTL (shFTL), was constructed using pRNA-U6.1/neo (GenScript, Piscataway, NJ, USA). To generate an intermediate plasmid for cloning shRNA targeted to mouse FTL, oligonucleotides sense: 5′-gatccgaacctgaatcaggccctcttcaagagagagggcctga ttcaggttctttttttggaaa-3′ and antisense: 5′-agcttttccaaaaaagaacctgaatc aggccctctctcttgaagagggcctgattcaggttcg-3′ were annealed *in vitro*, and the resultant double-stranded DNA fragments were subcloned into the BamHI-HindIII sites of pRNA-U6.1/neo. All of the constructs were confirmed by DNA sequencing.

2.2. Cell culture and stable transfection

RAW264.7 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, People's Republic of China). RAW264.7 cell clones stably expressing pFTL or shFTL were constructed by transfecting the cells with pFTL or shFTL using lipofectamine™ 2000. Cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) with 10% fetal bovine serum, G418 (600 μg/ml), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO_2. The transfected cells were cultured in the presence of G418, which was gradually increased to 800 μg/ml. After a period of 4–5 weeks, the G418-resistant colonies were picked. Corresponding empty vector clones were used as controls. These clones were then expanded and screened for the following experiments.

2.3. Antibodies and reagents

Antibodies for FTL, ferritin, TfR1 and NF-κB (p65 subunit) were purchased from Abcam (Hong Kong, China). Antibodies for p38, p44/42 (ERK1/2), IκBα, p-p44/42 (p-ERK1/2) (Thr202/Tyr204), p-p38 (Thr180/Tyr 182) and p-IκBα (Ser32/36) were obtained from Cell Signaling Technology (Danvers, MA, USA). The polyclonal antibody for iNOS was obtained from Cayman (Ann Arbor, MI, USA). The polyclonal antibody for COX-2 was obtained from Abcam (Burlingame, CA, USA). All secondary antibodies used for western blotting were purchased from Cell Signaling Technology. The Flag-tag antibody, LPS, calcein-AM and DCFH-DA were purchased from Sigma. G418 was from Amresco.

2.4. Reverse transcription-PCR analysis

Total RNA was extracted with TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. A reverse transcription reaction of 2 μg of each total RNA was performed at 42 °C for 1 h. PCR was performed using the Mastercycler Gradient RT-PCR System (Eppendorf) with the following primers: TNF-α: sense 5′-agcacagaaagcatga-3′, antisense 5′-cagagcaatgactcca-3′; IL-1β: sense 5′-aagctctccacctc-3′, antisense 5′-ctgatgtaccagttg-3′; iNOS: sense 5′-cccttccgaagtttctggcagc-3′, antisense 5′-ggctgtcagagcctcgtggctt-3′; COX-2: sense 5′-tctccaacctc tcctatcac-3′, antisense 5′-gcacgtagtcttcgatcact-3′; β-actin: sense 5′-agccatgtacgtagccatcc-3′, antisense 5′-tttgatgtcacgcacgattt-3′. PCR products were resolved on 1.2% agarose gels and stained with ethidium bromide. β-actin was used as a loading control where indicated.

2.5. Western blot

Cells were rinsed twice with ice-cold PBS and solubilized in lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM b-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min. Cell lysates were centrifuged at 15,000 ×*g* at 4 °C for 15 min [9]. The supernatant was collected, and the protein concentration was measured with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Aliquots of the extract containing 50 μg of protein were denatured in SDS and subjected to SDS–PAGE followed by transfer onto polyvinylidene difluoride (PVDF) membranes for 90 min at RT. The membranes were blocked with blocking buffer containing 5% nonfat milk for 1.5 h at room temperature and then incubated with primary antibody overnight at 4 °C. The antibody–antigen complexes were visualized using Super Signal West Pico (Pierce). The total density of the protein bands was detected with the LAS4000 System (FujiFilm).

2.6. ELISA assay

RAW264.7 cells were seeded in 24-well plates at 2.5×10^5 cells/well 24 h before the experiments. After LPS treatment, media were collected and centrifuged at 10,000 rpm for 5 min. The contents of TNF-α, IL-1β and PGE2 were determined by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using the mouse ELISA kits (Roche) for TNF-α and IL-1β and PGE_2 (Cayman) according to the manufacturers' instructions.

2.7. Nitrite analysis

RAW264.7 cells were seeded in 24-well plates at 2.5×10^5 cells/well 24 h before the experiment. Cells were treated with LPS (100 ng/ml) for 12 h. NO synthesis was then determined by assaying the culture supernatants for nitrite using the Griess reagent (1% sulfanilic acid, 0.1% N-1-naphthyl-ethylenediamine dihydrochloride, and 5% phosphoric acid) as described in [9]. Absorbance was measured at 550 nm.

2.8. Immunofluorescence assay

For the detection of the intracellular localization of the NF-κB p65 subunit, cells were grown on glass coverslips at a seeding density of 2×10^4 cells/ml. After LPS (100 ng/ml) treatment for 30 min, the