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Lactoferrin promotes autophagy via AMP-activated protein kinase activation through low-density lipoprotein receptor-related protein 1

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ABSTRACT

Lactoferrin (LF) is a multifunctional, iron-binding glycoprotein in mammalian secretions, such as breast milk, and has several beneficial effects for human health. However, how these effects are exerted at the cellular level is still largely unknown. In this study, we investigated the effects of LF on autophagy activity in NIH/3T3 mouse fibroblasts. LF from bovine milk was found to increase LC3-I to LC3-II conversion and LC3-positive cytosolic punctate structures because of increased autophagy flux. Knockdown of the putative LF receptor low-density receptor-related protein 1 (LRP1) completely abolished LC3 conversion in cells by LF treatment. Moreover, exposure to LF increased the phosphorylation levels of AMPK in cells, and treatment of dorsomorphin, a pharmacological inhibitor of AMPK signaling, attenuated LC3 conversion by LF. Therefore, we concluded that the beneficial effects of LF might be due to an increase of autophagy activity via AMPK signaling through the LRP1 receptor. These findings provide a novel insight into the physiological role of LF for the maintenance of cellular and tissue homeostasis.

1. Introduction

Lactoferrin (LF) is an iron-binding glycoprotein that belongs to the transferrin family, is produced in epithelial cells of exocrine glands, and exists not only in breast milk at high concentration but also in saliva, bile, and lacrimal fluid. Neutrophils also produce and secrete LF in response to inflammation [1,2]. LF is considered to have anti-bacterial properties because LF binds iron and ferric ions are essential for growth in many bacteria [3]. LF also contributes to a number of immunological responses [4] and possesses anti-cancer activity [5]. A recent study showed that LF reduces body mass and fat content in obese mice [6]. Furthermore, a clinical study revealed that treatment of enteric-coated LF reduced visceral fat accumulation in obese Japanese women and men [7]. Thus, there is growing interest in the application of LF for human health. Even though LF has several well-known beneficial effects on human health, it is unclear how these effects are exerted on the cellular level.

Macroautophagy (hereafter referred to as autophagy) is the process of delivering cytoplasmic components, such as proteins and organelles, by an autophagosome, a double-membrane vesicle, to lysosomes for degradation [8]. This degradation system is evolutionarily conserved from yeast to human. The primary function of autophagy is to enhance cell survival during nutrient deprivation and to maintain cellular and tissue homeostasis by controlling the quality of proteins and organelles [9]. Abnormal autophagy has been implicated in many human diseases, including metabolic and neurodegenerative diseases, and cancer [10,11]. Accordingly, natural products and chemical compounds that modulate autophagy have attracted attention for human health and the treatment of disease.

In this study, we assessed the effect of LF from bovine milk on autophagy in the NIH/3T3 mouse fibroblast cell line. Treatment of LF increased the conversion of microtubule-associated protein 1A/1B-light chain 3-I (LC3-I) to LC3-II, and the number of LC3-positve cytosolic puncta. These increases were caused by enhanced autophagy flux. Furthermore, we found that the AMP-activated protein kinase (AMPK) signaling pathway through low-density lipoprotein (LDL) receptor-related protein 1 (LRP1), a multifunctional cell surface receptor, has a crucial role in LF-promoted autophagy.

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2. Materials and methods

2.1. Reagents and antibodies

Lactoferrin from bovine milk (129–04121) and Dulbecco's Modified Eagle's Medium (D-MEM; 044–29765) were obtained from Wako Pure Chemical Industries. Fetal bovine serum (FBS; FB-1061) was obtained from Biosera. Chloroquine diphosphate (CQ; C6628) and dorsomorphin (DM; P5499) were purchased from Sigma-Aldrich. Lipofectamine RNAiMAX Transfection Reagent (13778075) was from Thermo Fisher Scientific. The primary antibodies used in this study were rabbit polyclonal antibody against LC3 (MBL, PM036), rabbit polyclonal antibody against LRP1 (Abcam, ab92544), rabbit polyclonal antibody against phospho-AMPK (Thr172) (Cell Signaling Technology, 2535), rabbit polyclonal antibody against AMPK (Cell Signaling Technology, 2532), and mouse monoclonal antibody against β-actin (Sigma-Aldrich, A1978). The secondary antibodies conjugated with horseradish peroxidase (HRP) for rabbit (7074) and mouse (7076) IgG were also from Cell Signaling Technology. Alexa Fluor 488 conjugated goat anti-rabbit IgG was from Thermo Fisher Scientific (A11008).

2.2. Cell culture and treatment

NIH/3T3 mouse fibroblasts were maintained in D-MEM containing 10% FBS in a humidified incubator with 5% CO_2 at 37 °C. Twenty-four hours before LF treatment, cells were seeded onto a 6-well culture plate at a density of 3×10^5 cells/well. After plating, cells were exposed to LF (10, 50, 100, and 250 μg/ml) or phosphate-buffered saline (PBS) for 24 h. For the autophagy flux assay, cells were treated or untreated with LF (100 μg/ml) in the presence or absence of 50 μM CQ for 16 h. For the inhibition of AMPK signaling, cells were treated or untreated with LF (100 μg/ml) in the presence or absence of 10 μM DM for 24 h.

2.3. Western blot analysis

Cultured cells were washed with ice-cold PBS and lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and Protease Inhibitor Cocktail (Sigma-Aldrich, P8340). Lysates were centrifuged at 17,700 × *g* for 10 min at 4 °C, separated by electrophoresis on SDS-polyacrylamide gels, and transferred to an Immobilon-P membrane (Merck Millipore, IPVH00010). Following transfer, membranes were blocked with 3% bovine serum albumin in PBS or 3% non-fat dried milk in PBS containing 0.1% Tween-20 (PBS-T), and subsequently immunoblotted with appropriate primary antibodies diluted in blocking buffer. After washing by PBS-T, the membranes were incubated with HRP-conjugated secondary antibody (1:10,000 in PBS-T) for an additional hour. Detection was performed with the Luminata Forte Western HRP substrate (Merck Millipore, WBLUF0100) using ChemiDoc MP (Bio-Rad Laboratories). The band signals were measured using ImageJ software ver. 1.46r (http://rsb.info.nih.gov/ij/).

2.4. Immunofluorescence microscopy

Cells cultured on chamber slides were washed with PBS, fixed with 3.7% formaldehyde in PBS for 20 min at 24 °C, and permeabilized with 0.1% Tween 20 in PBS for 5 min. Non-specific binding of antibody was eliminated by incubating cells in blocking solution containing 3% BSA for 1 h at 24 °C. Afterwards, cells were incubated with primary antibodies against LC3 (1:200 in blocking solution). Primary antibodies were detected by incubation with Alexa Fluor 488 conjugated secondary antibody for 1 h at 24 °C, and slides were mounted with Vectashield mounting medium (Vector Laboratories, H-1500). Slides were observed using a fluorescence microscope (Olympus). The number of LC3-positive puncta per cell was manually counted from at least 30 cells from 3 independent experiments for each experimental condition.

2.5. siRNA transfection

For siRNA-mediated knockdown of endogenous *Lrp1*, cells were transfected with 10 nM *Lrp1*-targeting or *enhanced green fluorescent protein* (*EGFP*)-targeting siRNA using Lipofectamine RNAiMAX for 48 h. Afterwards, cells were treated with LF (100 μg/ml) for 24 h as described above. The siRNA targeting *EGFP* was used as a control. The target sequences are as follows: *Lrp1*; 5′-CGA GGG GUA UCG CUA UCG A-3′, *EGFP*; 5′- GCC ACA ACG UCU AUA UCA U -3′. The interference efficiency was evaluated by western blot analysis.

2.6. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). The significance of differences between groups was determined by Student's *t*-test for comparison between two groups, or by an analysis of variance with Tukey's multiple comparison test for comparison of more than two groups using GraphPad Prism version 6.0 (GraphPad software, Inc.). The criterion for significance was a *P* value less than 0.05 in all cases.

3. Results

To determine the effects of LF on autophagy, we initially assessed the conversion status of LC3, a hallmark of autophagy, in NIH/3T3 mouse fibroblasts. Endogenous LC3 is post-translationally processed into LC3-I, a soluble cytosolic form of the protein. If autophagy is induced, LC3-I is conjugated to phosphatidylethanolamine to form lipid-bound LC3-II, which associates with autophagosome membranes. Thus, the amount of LC3-II is correlated with the number of autophagosomes [12,13]. Treatment with LF (0–250 μg/ml) resulted in a dose-dependent increase of LC3-II levels (Fig. 1A and B). We also found that the lowest concentration of LF (10 μg/ml) used in this study was sufficient to induce a significant increase in LC3 conversion.

Next, we investigated the effects of LF on autophagosome formation. NIH/3T3 cells treated with LF (0 or 100 μg/ml) for 24 h were immunostained with anti-LC3 antibody. As shown in Fig. 1C, LC3-positive cytosolic puncta were observed in LF-treated NIH/3T3 cells, but rarely observed in pretreatment cells. The number of LC3-positive puncta significantly increased in LF-treated cells when compared with that of untreated cells (Fig. 1D).

The accumulation of LC3-II protein indicates either increased autophagy activity or blockage of downstream steps in the autophagy process, such as autophagosome-lysosome fusion or lysosomal degradation. Therefore, assessment of “autophagy flux” in the presence of CQ, which inhibits lysosomal degradation, was performed. As shown in Fig. 2A and B, treatment with LF or CQ alone resulted in the expected increase in LC3-II levels. However, LF treatment in the presence of CQ significantly increased LC3-II levels when compared with that of LF alone. These results strongly indicate that the increased levels of LC3-II induced by LF are the result of the promotion of autophagy, and not the result of the impairment of autophagosome-lysosome fusion or lysosomal degradation.

Further experiments were conducted to examine the mechanism of action of LF in the promotion of autophagy. First, the major LF receptor LRP1 [14], a ubiquitously expressed, large endocytic receptor belonging to the LDL receptor family in mammalian cells,

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