Biochemical and Biophysical Research Communications 404 (2011) 1060–1064

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Adiponectin inhibits lymphotoxin-β receptor-mediated NF-κB signaling in human umbilical vein endothelial cells

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ARTICLE INFO

Article history:
Received 16 December 2010
Available online 30 December 2010

Keywords:
Adiponectin
AdipoR1
LTBR
Yeast two-hybrid screen
NF-κB

ABSTRACT

Adiponectin exerts anti-diabetic and anti-atherogenesis properties through its 2 receptors (AdipoR1 and AdipoR2). However, the signaling pathways responsible for the anti-inflammatory effects of adiponectin are largely unknown. In this study, we identified the lymphotoxin (LT)-β receptor (LTBR) as an interacting partner of human AdipoR1 by using a yeast two-hybrid screening. The interaction between LTBR and AdipoR1 was confirmed by co-immunoprecipitation and co-localization analysis. Furthermore, adiponectin incubation inhibited lymphotoxin-induced NF-κB activation and the expression of adhesion molecules in human umbilical vein endothelial cells. These results indicated that AdipoR1 interacted with LTBR and mediated the inhibition of LTBR-activated NF-κB pathway.

1. Introduction

Adiponectin/Acrp30 is an abundant adipocyte-derived factor with antidiabetic, and insulin-sensitizing metabolic effects [1]. Increasing evidences suggest that adiponectin also protects against cardiovascular diseases especially atherogenesis. It has been reported that the adiponectin levels are negatively correlated with the risk of cardiovascular disease in obese individuals and in patients with diabetes and hyperglycemia [2–4]. Adiponectin ameliorates the progression of vascular disease in mice. Neointimal formation is attenuated in mechanically injured arteries of adiponectin knock-out (Adipoq−/−) mice [5]. Adiponectin also reduces atherosclerosis in apolipoprotein E-deficient mice [6,7]. These data indicate that adiponectin is a vascular-protecting adipokine.

Adiponectin exerts its vascular-protecting properties by improving endothelial function and having anti-inflammatory effects in the vascular wall. Nuclear factor-kappa B (NF-κB) plays an important role in vascular inflammation [8]. In resting situations, the inhibitory protein I-kB binds to NF-κB and retains NF-κB in the cytosol, while IκB is phosphorylated and degraded under stimulation and NF-κB is then translocated into the nucleus, where it stimulates the transcription of inflammatory genes [8]. It has been reported that adiponectin inhibits endothelial NF-κB signaling, and further reduces adhesion molecule expression [9]. However, the molecular mechanism underlying adiponectin-inhibited NF-κB activation remains to be identified.

The physiological effects of adiponectin are mediated by its two receptors (AdipoR1 and AdipoR2) [10]. AdipoR1 and AdipoR2 have been shown to be involved in adiponectin-activated AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor (PPAR)-α, and the p38 mitogen-activated protein kinase (MAPK) pathways [10], and further mediate the physiological effects of adiponectin on glucose and lipid metabolism. Adiponectin receptors are also expressed in endothelial cells [11], and overexpression of these receptors potentiates the anti-inflammatory action of globular adiponectin in vascular endothelial cells [12], indicating a role of adiponectin receptors in mediating the vascular-protecting effects of adiponectin.

To identify proteins that participate in the adiponectin-activated signaling pathways, we carried out a yeast two-hybrid screen using AdipoR1 as the bait and identified lymphotoxin-β receptor (LTBR) as a potential AdipoR1-binding protein. LTBR belongs to the tumor necrosis factor (TNF) receptor family and plays a role in the inflammatory effects in endothelial cells [13,14]. In our study, we confirmed the interaction between AdipoR1 with LTBR. Meanwhile, adiponectin incubation inhibited lymphotoxin-induced NF-κB activation and the expression of adhesion molecules in human umbilical vein endothelial cells. These results indicated that adiponectin inhibited LTBR-mediated NF-κB pathway through AdipoR1.

Abbreviations: AdipoR, adiponectin receptor; LTBR, lymphotoxin-β receptor; NF-κB, nuclear factor-kappa B; HUVEC, human umbilical vein endothelial cell; GFP, green fluorescent protein.

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doi:10.1016/j.bbrc.2010.12.110

2. Materials and methods

2.1. Cell culture

COS-7 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C. Human umbilical vein endothelial cells (HUVECs) were purchased from Cascade Biologics and cultured in medium 200 supplemented with low serum growth supplement (Cascade Biologics).

2.2. Plasmid construction

The constructions of pDBLeu-AdipoR1, pMyc-AdipoR1, and pGFP-AdipoR1 were described previously [15]. The cDNA fragment of full human LTBR was amplified from human liver cDNA library by PCR with primers LTBR-For (5′-G*GAATTC*AATGACTGAGCAGATG-3′) and LTBR-Rev (5'-G*AAGATCT*CTAGCGTGTGCCAATG-3') and ligated into a eukaryotic vector of pFLAG-CMV2 (Sigma) to create FLAG-tagged fusion protein expression vector pFLAG-LTBR. LTBR DNA was amplified with primers LTBR-DsRed2-For (5′-CCC*AAGCTT*CGATGACTGAGCAGATG-3′) and LTBR- DsRed2-Rev (5′-CG*GGATCC*CTAGCGTGTGCCAATG-3′), and fused to the DsRed2 DNA sequence by cloning into the *Hin*dIII and *Bam*HI sites of vector pDsRed2-C1 (Clontech) to generate pDsRed2-LTBR.

2.3. Yeast two-hybrid system

Yeast two-hybrid screening was performed according to the manufacturer's instructions (Invitrogen). Briefly, a bait strain was created by transforming pDBLeu-AdipoR1 into *Saccharomyces cerevisiae* strain MaV204. The bait strain was then co-transformed with a human liver cDNA library constructed in the Gal4 activating domain (AD) vector pPC86 (Invitrogen). The co-transformants were plated onto SD minimal yeast media lacking histidine, leucine, and tryptophan (SD -His/-Leu/-Trp) plates, containing 20 mM 3-aminotriazole. The yeast colonies grown on the triple deficient media were assayed for β-galactosidase activity. Positive prey plasmids were isolated and sequenced. To confirm the interaction in yeast, purified prey plasmids were re-transformed with the pDBLeu-AdipoR1 or with the empty bait vector pDBLeu back into the yeasts and the colonies grown on the triple deficient media were tested for β-galactosidase activity.

2.4. Co-immunoprecipitation and Western blots

COS-7 cells were co-transfected with pFLAG-LTBR together with pMyc, pMyc-AdipoR1 together with pFLAG, or pFLAG-LTBR together with pMyc-AdipoR1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were washed twice with ice-cold phosphate buffered saline and lysed in 400 μl ice-cold lysis buffer (100 mM Tris–HCl, pH 7.5, 300 mM NaCl, 0.5% NP-40, 1 mM DTT) containing the protease inhibitor cocktail tablets (Sigma). The cell lysates were collected, incubated with 1 μg of anti-FLAG antibody (Cell Signaling Technology) for 3 h, and then precipitated with 20 μl of protein A/G-agarose (Santa Cruz) at 4 °C for 1 h. The protein A/G-agarose pellets were washed three times with the lysis buffer and resuspended in 20 μl SDS loading buffer, heated at 95 °C for 5 min, and separated by SDS–PAGE. Immunocomplexes were analyzed by Western blot using either anti-FLAG antibody or anti-myc antibody (Cell Signaling Technology), respectively, followed by anti-mouse IgG conjugated with HRP (Santa Cruz). Immunodetection was performed with an enhanced chemiluminescence (ECL) kit (Amersham).

2.5. Imaging with confocal microscopy

HUVECs seeded in 35 mm dishes were transfected with 1 μg pGFP-AdipoR1 and 1 μg pDsRed2-LTBR. After 24 h, the transfected HUVECs were fixed with 4% paraformaldehyde at 4 °C and visualized with a Leica confocal microscopy (TCS-SP, Leica). Images were obtained with an excitation wavelength of 488 nm and an emission wavelength of 500–520 nm used for GFP, and an excitation wavelength of 543 nm and an emission wavelength of 550–570 nm used for DsRed2.

2.6. NF-κB activation assay

NF-κB activation was detected as reported previously [16]. Briefly, HUVECs were transfected with pNFκB-Luc, a cis-reporter plasmid containing the luciferase reporter gene linked to five repeats of NF-κB binding sites (Stratagene). After 24 h, HUVECs were treated with or without 10 μg/mL of adiponectin (R&D systems) for 24 h, and then incubated with or without 100 ng/mL of lymphotoxin-α1β2 for another 8 h. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

2.7. RNA isolation, reverse transcription and real-time quantitative PCR (qPCR)

Total RNA was isolated using Trizol reagent (Invitrogen) and 2 μg total RNA was used for cDNA synthesis. Real-time quantitative PCR analysis was performed using SYBR Premix Ex Taq (Takara) in an ABI7900 system (Applied biosystem). Primers 5′-TGCCAAAGCTGCTCTAGCCTTG-3′ and 5′-TGTTGTTGCCAGTGTTCAGCCAG-3′ were used for E-selectin amplification. Primers 5′-TCCCCTGGACCCCGGATTGC-3′ and 5′-AGGGAATGAGTAGAGCTCCACCTG-3′ were used for VCAM-1 amplification. Primers 5′-TGCTTTAGCTTGGAAATTCCGGAGC-3′ and 5′-GTCGACTGGGGCGCGTGATC-3′ were used for ICAM-1 amplification. Primers 5′-CGAGCACAGAGCCTCGCCTTT-3′ and 5′-TCATCATCCATGGTGAGCTGGCG-3′ were used for β-actin gene amplification. The expression levels of E-selectin, VCAM-1 and ICAM-1 were normalized to β-actin.

3. Results

3.1. LTBR is a binding partner of AdipoR1 in yeast

To identify intracellular proteins interacting with the AdipoR1, the yeast two hybrid system was applied to screen a human liver cDNA library by using AdipoR1 as the bait. Out of 1×10^6 total transformants screened, 12 clones were found to grow on nutritional deficient plates and activate the β-galactosidase assay and one was identified as LTBR. The isolated clone comprises the full amino acids of LTBR. The plasmid pPC86-LTBR was transformed back into the competent MaV203 cells along with the original bait plasmid pDBLeu-AdipoR1 or alternatively, with the bait vector pDBLeu. Transformants turned blue in X-gal analysis only when AdipoR1 and LTBR were simultaneously expressed (Fig. 1A), demonstrating a true interaction between AdipoR1 and LTBR in yeast.

3.2. Interaction of LTBR and AdipoR1 in mammalian cells

To determine whether LTBR interacts with AdipoR1 in mammalian cells, the expression constructs encoding FLAG-tagged LTBR and Myc-tagged AdipoR1 were co-transfected into COS-7 cells. The cell lysates were subjected to immunoprecipitation assays with anti-FLAG antibody followed by Western blot analysis with anti-FLAG and anti-Myc antibody, respectively. Data showed that anti-FLAG antibody was able to precipitate Myc-AdipoR1 protein