Role of Adenylyl Cyclase-Associated Protein 1 (CAP1) in Mediating Resistin Actions in Mouse Liver Cells

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Abstract

Resistin is a pro-inflammatory adipokine produced by the white adipose tissue (WAT) adipocytes and macrophages. Obesity results in chronic inflammation of the WAT, marked by an increase in resistin and other inflammatory cytokines, and by infiltrating leukocytes. Elevated resistin levels are believed to play a major role in the development of insulin resistance in the peripheral tissues. Adenylyl cyclase-associated protein 1 (CAP1) was recently identified as a receptor for resistin. In the present study we aimed to investigate whether CAP1 mediates resistin actions which may affect insulin sensitivity in the liver. As a model we used BNL CL.2 mouse liver cell line. Concentration- and time-dependent experiments demonstrated that resistin upregulated TNFα, SOCS3, IL-1α, and IL-6 mRNA expression maximally when used in concentration of 12.5 ng/ml for 6 hours. In order to determine the CAP1 involvement in mediating resistin actions in the liver, we transfected BNL CL.2 cells with CAP1 siRNA and performed a real-time PCR array measuring the expression of 84 key genes involved in insulin signaling, adipokine signaling, and inflammation. Results demonstrated that resistin upregulated mRNA expression of IL-6; this effect was ameliorated when CAP1 was downregulated. Knock-down of CAP1 facilitated mRNA expression of genes involved in insulin signaling and adipokine signaling pathways, while it resulted in downregulation of infiltrating leukocyte markers expression. Taken together these results indicate that CAP1 is a mediator of resistin actions in the liver.

Introduction

Resistin is a cytokine produced mainly by the white adipose tissue (WAT) that plays a role in modulating insulin sensitivity of peripheral tissues. [1, 2, 3] Multiple clinical and *in vivo* studies involving genetic or diet-induced obesity models found that serum resistin levels correlate with WAT mass reaching highest levels in states of obesity. [4, 5, 6] Conversely, weight loss is accompanied by a decrease in serum resistin levels. [7]

Adenylyl cyclase-associated protein 1 (CAP1) was recently identified as a receptor for resistin. [8] To date, there are no studies committed to examining the role of CAP1 in mediating resistin actions.

Aim

To examine whether CAP1 mediates resistin actions and affects insulin sensitivity in the liver.

Materials & Methods

Reagents

Mouse recombinant resistin was purchased from Sigma-Aldrich. The lyophilized form was reconstituted with water to a concentration of 100 μg/ml and further to a series of dilutions of 50, 25, and 12.5 μg/ml, which were used for cell treatment.

Cell line

Mouse embryonic liver BNL CL.2 cells were purchased from ATCC. Cells were grown in DMEM Medium supplemented with 10% FBS and 1x Antibiotic/Antimycotic Solution.

Quantitative RT-PCR analyses

RNA was extracted by using TRIzol/chloroform/iso-propanol method. For RT conversion, samples were normalized to 1 mg/ml RNA and RT reaction was performed by using qScript cDNA Synthesis Kit (Quanta Bio) and SimpliAmp Thermal Cycler (Applied Biosystems). Quantitative PCR analyses were performed by using PowerUp SYBR Green Master Mix and QuantStudio 3 Real-Time PCR System (Applied Biosystems). The specific primer sequences used for amplification are listed in Table 1. Each experiment was performed at least three times in duplicates, and for PCR analysis each samples was run twice.

Gene	Forward Primer [5'-3']	Reverse Primer [5'-3']	Tm [°C]	Reference
TNFα	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG	60	PMID: 20144135
SOX3	GCGGGCAGCTTTCGTTATCC	TCACCGACTGGGTGCTGAC	60	PMID: 15984405
IL-1α	CTCTGAGACTTCCATCCAGATAC	TGGAATCCAGGGGAAACACTG	60	ietf-designed
IL-15	CATCCATCTCGTGCTACTTGTGTT	CATCTATCCAGTTGGCCTCTGTTT	60	PMID: 28975741
CAP1	CGATCCATTATGGCTGACATG	GCGGCCGCTTATCCAGCAATT	60	PMC3774368
18s	AACCTGGTTGATCCTGCCAGT	GGCACCAGACTTGCCCTC	60	PMC3940700

Table 1. List of primers used for qRT-PCR analyses.

Western blot analyses

Protein was extracted by using Pierce™ RIPA Lysis Buffer and protein concentrations were quantified by using Pierce™ BCA Protein Assay Kit and BioTek® plate reader and Gen5™ data analysis software. Normalized protein extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blotted with anti-CAP1 [EPR8339(B)] (Abcam, Cat. # ab155079) and anti-GAPDH (14C10) Rabbit mAb (Cell Signaling Technology, Cat. # 2118) and Pierce™ Goat Anti-Rabbit Horseradish Peroxidase Conjugated Secondary Antibody (Thermo Fisher Scientific). Protein bands were visualized by using SuperSignal West Pico Chemiluminescent Substrate and MyECL™ Imager (Thermo Fisher Scientific).

siRNA transfection

For transfection, 0.25·10^5/10^6 cells were seeded in 6-well plates 24 hours prior to each experiment to achieve 60-80% confluency. UltraMEM™ Reduced Serum Medium (protein-free basal medium with selenium and without L-glutamine) (Lonza) in the absence of antibiotic was used during transfection to achieve optimal efficacy. Transfection was performed by using Lipofectamine® RNAiMAX and Silencer® Select predesigned CAP1 or negative control siRNAs (Thermo Fisher Scientific).

PCR array

Total RNA from MCF-7 cells was extracted by using TRIzol®/chloroform/isopropanol method. RNA extracts were normalized to 0.5 μg/ml and reverse transcribed by using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) and SimpliAmp™ Thermal Cycler. Mouse Insulin Resistance RT2 Profiler PCR array (Cat. # PAMM-156Z, QIAGEN, Gaithersburg, MD, USA) using RT² SYBR Green ROX qPCR Mastermix (Cat. # 330520, QIAGEN Sciences, MD, USA) and QuantStudio™ 3 Real-Time PCR System was performed to profile the expression of 84 genes involved in the mechanisms behind non-insulin dependent *diabetes mellitus*. Data were analyzed by using PCR Array Data Analysis online software (QIAGEN Sciences, Gaithersburg, MD, USA) and ΔΔCt method to evaluate the relative quantification, and a set of controls were used to assess the reverse transcription performance, genomic DNA contamination, and PCR performance.

Statistical analyses

All experiments were performed multiple times. Statistical analysis was performed using GraphPad Prism 7 software (GraphPad, La Jolla, CA, USA). Significant differences were analyzed using Student's *t* test and two-tailed distribution. Results were considered to be statistically significant if $p < 0.05$.

Results

Time- and concentration-response experiments for resistin treatment of BNL CL.2 cells

Initially, to utilize the most appropriate concentration of resistin for further treatments, we stimulated BNL CL.2 cells with 12.5, 25, and 50 ng/ml of resistin and by using qRT-PCR analysis we measured mRNA levels of known target genes of resistin (TNFα, SOCS3, IL-1α, and IL-15). Based on the results from this experiments (Figure 1), we performed all further experiments using concentration of resistin of 12.5 ng/ml for 6 hours.

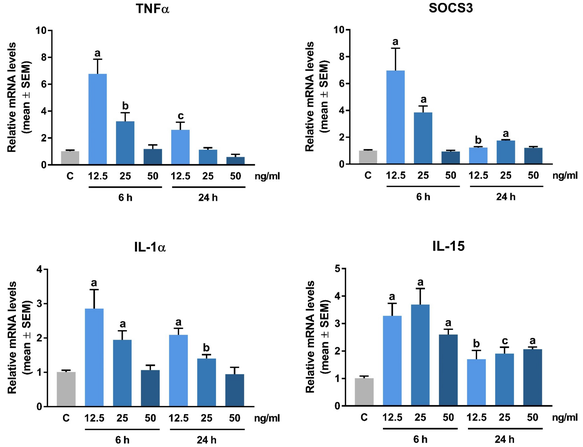

Figure 1. Time- and concentration-response experiment for resistin treatment of BNL CL.2 cells. TNFα: a, $p < 0.0001$; b, $p < 0.0061$; c, $p < 0.0317$; SOCS3: a, $p < 0.05$; b, $p < 0.0182$; IL-1α: a, $p < 0.05$; b, $p < 0.0079$; IL-15: a, $p < 0.0056$; b, $p < 0.0035$; c, $p < 0.0064$.

Role of CAP1 in mediating resistin action on insulin signaling pathway

In order to investigate whether CAP1 plays a role in mediating resistin actions on insulin signaling in the liver, we knocked-down CAP1 gene expression in BNL CL.2 cells by using siRNA approach (Figure 2). A successful 70% inhibition of CAP1 mRNA and protein levels was achieved.

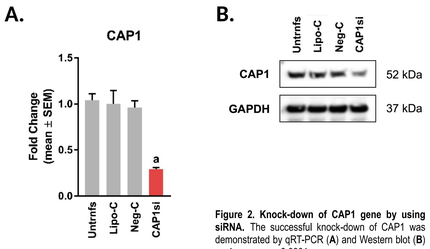

Figure 2. Knock-down of CAP1 gene by using siRNA. The successful knock-down of CAP1 was demonstrated by qRT-PCR (**A**) and Western blot (**B**) analyses. a, $p < 0.0001$.

We further treated BNL CL.2 cells with resistin, both untransfected and CAP1 siRNA-transfected and performed a qPCR array to profile the expression of 84 genes involved in the mechanisms behind the non-insulin dependent diabetes (Table 2 and Figure 2). Results from the analysis demonstrated that treatment affected the expression of 77 genes two- or more than two-folds (Figure 3). Our data indicated that when CAP1 was knocked-down, there was upregulation of genes involved in the insulin and adipokine signaling pathways as well as downregulation of infiltrating leukocyte markers (Table 3 and Figure 3).

Description
Acetyl-CoAcarboxyme A carboxylase alpha
Acetyl-CoAcarboxyme A carboxylase beta
Acyl-CoA synthetase long-chain family member 1
Acyl-CoA synthetase long-chain family member 4
Adiponectin, C1Q and collagen domain containing
Adiponectin receptor 1
Adiponectin receptor 2
Thymoma viral proto-oncogene 3
Arachidonate 5-lipoxygenase
Apolipoprotein E
Caspase 1
Chemokine (C-C motif) ligand 12
Chemokine (C-C motif) receptor 4
Chemokine (C-C motif) receptor 5
Chemokine (C-C motif) receptor 6
CD36 antigen
CD3 antigen, epsilon polypeptide
CCAAT/enhancer binding protein (C/EBP), alpha
Conserved helix-loop-helix ubiquitous kinase
Cellular nucleic acid binding protein
Cytokine receptor-like factor 2
Citrate synthase
Chemokine (C-X-C motif) receptor 3
Chemokine (C-X-C motif) receptor 4
EGF-like module containing, mucin-like, hormone receptor-like sequence 1
Fatty acid binding protein 4, adipocyte
Fatty acid synthase
Glycogen synthase 1, muscle
Hexokinase 2
Interferon gamma
Insulin-like growth factor 1
Insulin-like growth factor 1 receptor
Inhibitor of kappaB kinase beta
Interleukin 1B receptor 1
Interleukin 1 beta
Interleukin 1 receptor, type I
Interleukin 23 receptor
Interleukin 6
Insulin receptor
Insulin receptor substrate 1
Insulin receptor substrate 2
Janus kinase 2
Leptin
Leptin receptor
Lipase, hormone sensitive
Lipoprotein lipase
Leukotriene A4 hydrolase
Mitogen-activated protein kinase kinase 1
Mitogen-activated protein kinase 3
Mitogen-activated protein kinase 9
Mechanistic target of rapamycin (serine/threonine kinase)
Nicotinamide phosphoribosyltransferase
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, a
NLR family, pyrin domain containing 3
Oxidized low density lipoprotein (lectin-like) receptor 1
Phosphoenolpyruvate carboxykinase 1, cytosolic
Phosphodiesterase 3B, cGMP-inhibited
Pyruvate dehydrogenase kinase, isoenzyme 2
Pancreatic and duodenal homeobox 1
Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide
Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)
Peroxisome proliferator-activated receptor alpha
Peroxisome proliferator activated receptor gamma
Peroxisome proliferator-activated receptor, gamma, coactivator 1 alpha
Protein tyrosine phosphatase, non-receptor type 1
PYD and CARD domain containing
Retinol binding protein 4, plasma
V-rel reticuloendotheliosis viral oncogene homolog A (avian)
Resistin
Ribosomal protein S6 kinase, polypeptide 1
Stearoyl-Coenzyme A desaturase 1
Serine (or cysteine) peptidase inhibitor, clade E, member 1
Solute carrier family 27 (fatty acid transporter), member 1
Solute carrier family 2 (facilitated glucose transporter), member 4
Suppressor of cytokine signaling 3
Sterol regulatory element binding transcription factor 1
Sterol regulatory element binding factor 2
Signal transducer and activator of transcription 3
Toll-like receptor 4
Tumor necrosis factor
Tumor necrosis factor receptor superfamily, member 1a
Tumor necrosis factor receptor superfamily, member 1b
Uncoupling protein 1 (mitochondrial, proton carrier)
Very low density lipoprotein receptor
Actin, beta
Beta-2 microglobulin
Glyceraldehyde-3-phosphate dehydrogenase
Glucuronidase, beta
Heat shock protein 90 alpha (cytosolic), class B member 1

Gene Symbol	Fold Regulation	Gene Symbol	Fold Regulation
Resistin vs. Control		Il18r1	-2.46
Il6	2.18	Il1b	-12.66
Pdk2	2.02	Il1r1	2.00
Tnfrsf1b	-2.32	Il23r	-12.50
Ucp1	-2.91	Insr	2.36
CAP1si vs. Control		Irs1	2.32
Adipor1	-2.05	Irs2	2.60
Igf1	2.30	Jak2	2.17
Il6	2.40	Lep	-4.89
Lep	2.32	Lipe	2.22
Lpl	2.02	Lpl	-15.81
Pck1	2.67	Lta4h	2.16
Srebf2	2.41	Map2k1	2.42
Tnfrsf1b	-2.92	Mapk9	2.35
CAP1si + R vs. Control		Mtor	2.08
Acsl4	2.60	Nampt	2.28
Adipoq	-25.37	Nfkbia	2.25
Adipor1	2.25	Nlrp3	-10.70
Adipor2	2.53	Olr1	-4.10
Akt3	2.29	Pck1	-13.22
Ccl12	-13.23	Pde3b	2.17
Ccr4	-28.31	Pdk2	2.05
Ccr5	-15.81	Pdx1	3.23
Ccr6	-17.02	Pik3ca	2.13
Cd36	-7.49	Pik3r1	2.15
Cd3e	-4.58	Pparg	2.27
Cebpa	2.04	Ptpn1	2.65
Chuk	2.05	Rela	2.47
Cnbp	2.23	Rps6kb1	2.32
Cs	2.24	Slc27a1	2.17
Cxcr3	-12.92	Slc2a4	-3.74
Cxcr4	-26.11	Socs3	2.60
Adgre1	-24.84	Srebf1	2.50
Fasn	2.37	Srebf2	2.73
Gys1	2.35	Stat3	2.24
Hk2	2.16	Tlr4	2.07
Ifng	-9.75	Tnf	-11.74
Igf1	-20.40	Tnfrsf1a	2.40
Igf1r	2.41	Ucp1	-6.02
Ikbkb	2.24	Vldlr	2.05

Table 2. List of genes used for PCR array.

Table 3. List of genes regulated more than two-folds by treatment. Red color designates genes upregulated more than two-folds, blue color designates genes downregulated more than two-folds, and **black** color designate a gene upregulated two-folds.

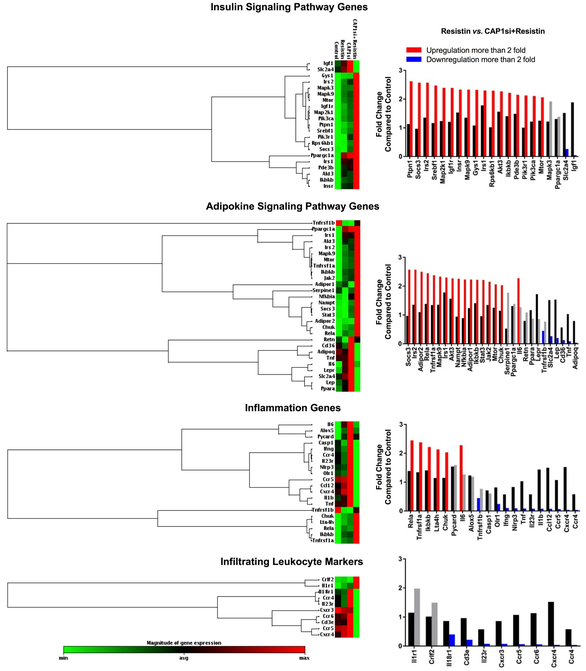

Figure 3. Effect of CAP1 downregulation on resistin-mediated gene expression. Bar graphs show gene expression as fold-change compared to control. First column represents resistin-treated BNL CL.2 cells, second column—CAP1 siRNA-transfected cells treated with resistin.

Conclusions

To the best of our knowledge, this is the first study to demonstrate the role of CAP1 in mediating resistin signaling pathway in mouse liver cells. Further studies aiming to clarify the mechanisms of interaction between resistin and CAP1 are ongoing.

Literature

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