Original Paper

MACROD1/LRP16 Enhances LPS-Stimulated Inflammatory Responses by Up-Regulating a Rac1-Dependent Pathway in Adipocytes

Li Zang^a Quan Hong^b Guoqing Yang^a Weijun Gu^a Anping Wang^a
Jingtao Dou^a Yiming Mu^a Di Wu^b Zhaohui Lyu^a

^aDepartment of Endocrinology, Chinese PLA General Hospital, Beijing, ^bDepartment of Kidney, Chinese PLA General Hospital, Beijing, China

Key Words

Adipocytes • MAPK1 • ERK • LRP16 • Rac1

Abstract

Background/Aims: Chronic inflammation contributes to the development of type 2 diabetes mellitus by targeting the insulin receptor substrate protein-1 (IRS-1) signaling pathway. Previous studies showed that Leukemia related protein 16 (LRP16) reduced insulin stimulated glucose uptake in adipocytes by impairing the IRS-1 signaling pathway. We explored the mechanism by which LRP16 promotes the inflammatory response. **Methods:** We screened LRP16 induced proteins in the lipopolysaccharide (LPS)-stimulated inflammatory response using liquid chromatography-mass spectrometry (LC-MS) and analyzed the potential biological functions of these proteins using online bioinformatics tools. mRNA expression and protein expression of target genes were measured by real time PCR and Western blot, respectively. **Results:** A total of 390 differentially expressed proteins were identified. The mitogen-activated protein kinase (MAPK) signaling pathway was the primary activated pathway in LRP16-expressing cells. Overexpression of LRP16 activated ERK1/2 and Rac1, which are two key players related to the MAPK signaling pathway. Furthermore, knock down of endogenous LRP16 by RNA interference (RNAi) reduced Rac1 expression, ERK activation, and inflammatory cytokine expression in human adipocytes stimulated by LPS. The stimulatory effect of LRP16 was diminished by suppressing Rac1 expression and treating the cells with the ERK specific inhibitor, PD98059. **Conclusion:** These findings revealed the functions of LRP16 in promoting the inflammatory response through activating the Rac1-MAPK1/ERK pathway in human adipocytes.

© 2018 The Author(s)
Published by S. Karger AG, Basel

L. Zang and Q. Hong contributed equally to this work

Zhaohui Lyu

Department of Endocrinology, Chinese PLA General Hospital
28 Fu Xing Road, Beijing 100853 (China)
Tel. +86-10-55499101, Fax +86-10-68168917, E-Mail metabolism301@126.com

KARGER

Introduction

Leukemia related protein 16 (LRP16), also known as MACRO domain containing 1 (MACROD1), is a co-activator of estrogen receptor alpha (ER α). LRP16 was first cloned from acute myeloid leukemia cells in our laboratory [1]. Ma *et al.*, showed that LRP16 expression gradually increased during 3T3-L1 preadipocyte differentiation, suggesting that LRP16 plays a crucial role in adipocyte maturation [2]. LRP16 expression has been found both in the cytosol and nucleus, and nuclear LRP16 plays different biological actions [3]. LRP16 is considered a crucial regulator of the transcription factor nuclear factor κ B (NF- κ B) because it integrates into its transcriptional complex [4]. LRP16 was identified as a negative regulator of insulin through impairing the insulin receptor substrate protein-1 (IRS-1) signaling pathway [5]. However, the mechanism by which LRP16 impairs the IRS-1 signaling pathway is still poorly understood.

One of the earliest etiologic factors of type 2 diabetes mellitus is the development of peripheral insulin resistance, which is caused by abnormalities in the IRS-1 signaling pathway [6]. Previous *in vitro* and *in vivo* evidence suggests that chronic inflammation contributes to the pathogenesis of diabetes mellitus by inhibiting IRS-1 signaling [7] and by modulating inflammatory cytokines such as tumor necrosis factor alpha (TNF α), interleukin-1 (IL-1) and IL-6[8]. As a pivotal molecule, NF- κ B has been shown to induce insulin resistance in multiple organs (fat, muscle and liver) [9, 10]. Both NF- κ B and mitogen activated protein kinase (MAPK) signaling pathways contribute to adipocyte inflammation [11]. Therefore, developing anti-inflammatory targets is a reasonable strategy to treat type 2 diabetes mellitus. Protein screening and validation can provide important information about LRP16-mediated inflammation in adipocytes. Considering that insulin resistance induced by chronic mild inflammation widely occurs in human visceral fat tissue, we used human preadipocyte-visceral (HPA-v) cells in this study.

Materials and Methods

Reagents

The LRP16 plasmid was described in our previous reports [12]. LRP16 and Rac1 specific siRNA were chemically synthesized by GenChem Co (Shanghai, China). LPS was purchased from Sigma Aldrich (St. Louis, Missouri, USA). The ERK specific inhibitor, PD98059, was obtained from New England Biolabs. DMEM and fetal bovine serum (FBS) were obtained from Gibco (Paisley, USA). Lipofectamine 2000 was purchased from Invitrogen. SuperFect transfection reagent was obtained from QIAGEN. Dexamethasone was obtained from Steraloids. Insulin, indomethacin, isobutylmethylxanthine, and peroxisome proliferator-activated receptor (PPAR) agonist were purchased from Sigma Aldrich. Rabbit anti-Rac1 antibody was purchased from Sigma Aldrich. Rabbit antibodies against TNF α , c-Jun, c-fos, NF- κ B, p65, IL-1 β , IL-2, IL-4, IL-6, and phosphorylated (p)-ERK1/2 were purchased from Abcam (Cambridge, Massachusetts, USA). Goat anti-rabbit IgG horse radish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA).

Cell culture, cell transfection and cell differentiation

HPA-v cells were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. Plasmid encoding LRP16 or the empty vector pcDNA3.1 (control) was transfected into the human preadipocytes using SuperFect reagent. Cells stably expressing LRP16 or empty control vector were screened by incubating cells in culture medium supplemented with 0.4 μ g/mL G418. LRP16, Rac1 specific siRNA or siRNA negative control was transfected into the cells using Lipofectamine 2000 according to the manufacturer's recommendations. Differentiation of wide-type human preadipocytes into mature adipocytes was previously reported [13]. Briefly, cells were maintained in culture medium containing dexamethasone (0.5 μ M), insulin (5 μ g/mL), indomethacin (100 μ M), isobutylmethylxanthine (0.25 mM), and PPAR agonist (100 μ M) for two weeks to induce differentiation.

Protein preparation

Mature adipocytes transfected with plasmid encoding LRP16 and control cells were lysed in protein lysis buffer containing 1% NP-40, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 50 mM pH 7.6 Tris-HCl, 0.5% deoxycholic acid, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride. After a 30 min incubation on ice, samples were centrifuged at 12,000 r/min for 30 min at 4°C. The supernatant was collected and protein concentration was determined by the bicinchoninic acid (BCA) method (Bio-Rad, Hercules, CA, USA). A total of 100 µg protein was incubated with 10 mM DTT at 56°C for 1 h, followed by alkalization by 50 mM iodoacetamide (IAM) for 45 min. After diluting with 25 mM NH₄HCO₃, protein samples were digested with 1% trypsin at 37°C and digestion was terminated with 0.1% formic acid (FA). After centrifuging at 13,000 r/min for 10 min, the supernatant was collected and stored at -80°C until MS analysis.

LC-MS analysis

The multidimensional protein identification device is composed of an LTQ-ion trap (Thermo Fisher Scientific, Waltham, USA) mass spectrometer with an electrospray ionization (nano-ESI) source. High-throughput liquid chromatography-mass spectrometry (LC-MS) analysis was performed as previously described [14-16]. In brief, 100 µg of peptides were loaded onto a biphasic capillary column by high-pressure nitrogen. The mobile phase consisted of solution A (5% AN, 0.1% FA), solution B (80% AN, 0.1% FA), and solution C (800 mM ammonium acetate, 5% AN, 0.1% FA). For MS analysis, the elution from the column was loaded onto a C18 analytical capillary chromatography column and a fraction from the column was ionized by electrospray, with a spray voltage of 2.0 kV and at 200°C. Data-dependent acquisition mode was applied for MS data collection. XCalibur (Thermo Fisher Scientific, Waltham, USA) was used to control the elution gradient of high performance liquid chromatography (HPLC) and MS scanning.

Data analysis and protein functional groupings

LC-MS data were processed using Progenesis LC-MS software (Nonlinear Dynamics Corp, Newcastle upon Tyne, UK) using the default settings. The NCBI human database (March 2011) was applied for protein identification. The RefSeq of proteins was transformed into UniProtKB at: <http://www.uniprot.org> [17]. Differentially expressed proteins were analyzed according to the Gene Ontology (GO) classification. Pathway networks and protein functional grouping were generated using the online platform according to the pathway- biocarta and pathway-kegg analyses: <http://bioinfo.capitalbio.com/mas>.

Real-time PCR assay

Mature adipocytes over-expressing LRP16, LRP16 and Rac1 knock down cells, and their wide-type control cells were plated into 60-mm diameter dishes. The cells were stimulated with LPS (120 ng/ml) for 40 min, treated with the ERK inhibitor, PD98059 (100 ng/ml), and collected. The TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used for total RNA isolation. Isolated RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (1 to 2 µg) (Invitrogen). Real-time quantitative PCR was carried out using a Taq-Man PCR Master Mix and an iCycler system (Bio-Rad). The relative expression of gene (fold-change vs. control) was calculated by the 2^{-ΔΔCt} method [18]. Data were quantified from three independent experiments.

Western blot assay

A total of 50 µg protein was separated by 7.5% SDS-PAGE. After electrophoresis, proteins were transferred to a PVDF membrane, blocked with 5% skim milk, and incubated with primary antibodies for 2 h at room temperature. Subsequently, membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactivity was visualized with a super enhanced chemiluminescent (ECL) detection reagent (Shanghai Yeasen Biological Technology, China). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal loading control. The relative expression of target protein was normalized to GAPDH.

Statistical analysis

Data analysis was performed using SPSS 13.0 (SPSS Inc, Chicago, IL, USA) software. The results were expressed as means $\pm$ standard deviation (SD). Statistical analysis of two groups was performed using individual Student's *t* tests, and statistical analysis of more than two groups was performed using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test or two-way ANOVA followed by Bonferroni post. A *P* value < 0.05 was considered statistically significant.

Results

LC-MS analysis

HPA-v cells stably transfected with plasmid encoding LRP16 showed a 2-fold increase in LRP16 expression compared to control (Fig. 1A). 1, 075 total proteins and 390 differentially expressed proteins were identified in the cells stably overexpressing LRP16 relative to the control cells. The top 30 differentially expressed proteins are listed in Table 1. According to the LC-MS analysis criteria, the proteins with 20 > ratio > 1.5 were considered up-regulated, while proteins with 0.05 < ratio < 0.66 were considered down-regulated. Of all the differently expressed proteins, one protein was absent in the Uniprot database (accession No is AF202922_1).

Functional grouping of differentially expressed proteins

Among these proteins, 353 are related to cellular component, 303 to molecular functions, 337 to biological processes, and 93 to enzyme (Fig. 2A). The subgroup analysis is as follows: (1) proteins related to cell component include cell part, organelle part, membrane and macromolecular complex; (2) proteins related to molecular function include binding, catalytic activity, structural molecule activity and transporter activity; and (3) in the biological process category, most proteins (304) are relevant to cellular processes and other biological processes including biological regulation, single-organism process, response to stimulus, cellular component organization, or biogenesis (Fig. 2B–D).

Pathway analysis

Significant pathways were identified by analyzing differentially expressed proteins (Tables 2–4). The major pathways identified by the pathway-bioclarta analysis were transcription factor cAMP-response element binding protein (CREB) and its extracellular signals, electron transport reaction in mitochondria, eukaryotic protein translation, and how progesterone initiates oocyte membrane. The major pathways identified by the pathway-KEGG analysis were ribosome, Alzheimer's disease, Parkinson's disease, systemic lupus erythematosus, oxidative phosphorylation and MAPK signaling pathway. By analyzing the relationship between these pathways and differentiated genes, MAPK1 (ERK) and Rac1 were repeatedly found to be involved in the same pathways.

Fig. 1. Western blot analysis of the LRP16 expression in cells. (A) Compared with cells transfected with empty vector control, stable overexpression of plasmid encoding LRP16 increased LRP16 expression in cells. (B) RNA interference decreased LRP16 expression in cells.

Table 1. Top 30 differentially expressed proteins

Accession	Uniprot AC	Protein name	Ratio (T/L)
NP_733936.1	G5E949	G5E949	12.52
NP_612366.1	Q8NE86	MCU	10.39
NP_060654.1	Q9NVJ2	ADP-ribosylation factor-like protein 8B	10.33
NP_060843.2	P57105	human synaptotagmin 2 binding protein	9.85
NP_055586.1	O75150	E3 ubiquitin-protein ligase	9.45
NP_001867.2	B2RAQ8	carnitine O-palmitoyltransferase 1	8.07
NP_525127.1	Q96FQ6	S100 calcium binding protein A16	7.96
AF_202922.1			7.67
NP_005175.2	B4DJ51	calmodulin 149 aa protein	6.78
NP_002195.1	P26006	integrin alpha-3	6.78
NP_004695.1	O43818	U3 small nucleolar RNA-interacting protein 2	6.07
NP_065843.3	Q6PIU2	neutral cholesterol ester hydrolase 1	5.94
NP_057479.2	Q9P035	3-hydroxyacyl-CoA dehydratase 3	5.92
NP_001907.2	P08574	cytochrome c1	5.88
NP_064576.1	P82650	28S ribosomal protein S22	5.76
NP_958439.1	P00533	EGFR	5.70
NP_006631.2	Q9UHD8	guanine nucleotide binding protein gamma 2	5.61
NP_004159.2	P31040	succinate dehydrogenase flavoprotein subunit	5.56
NP_115886.1	B4DP29	zinc finger protein 559	5.54
NP_002957.1	P60903	protein S100-A10	5.43
NP_002297.2	P17931	galectin-3	5.41
NP_005727.1	P61163	alpha-centractin 376 aa protein	5.40
NP_079428.2	Q8TB37	iron-sulfur protein NUBPL	5.39
NP_003507.1	Q6FI13	histone H2A type 2-A	5.34
NP_055051.1	O15228	dihydroxyacetone phosphate acyltransferase	5.25
NP_002898.2	P46063	RECQ1	5.02
NP_079106.3	Q8NF37	PCAT1	4.91
NP_005909.2	P40926	malate dehydrogenase	4.90
NP_001388.1	P42892	endothelin-converting enzyme 1	4.87
NP_036375.1	Q9Y2D2	S35A3	4.72

Fig. 2. Identification of differentially expressed proteins using GO analysis. We identified (A) 353 proteins in cellular components, 303 in molecular function, 373 in biological processes, and 93 in enzyme. The number of proteins in cellular components (B), molecular functions (C), biological processes (D), and enzyme (E) are presented.

MAPK and Rac1 protein analysis

We analyzed protein interaction using the Molecular Annotation System (MAS) (pathway-biocardia networks and pathway-KEGG networks of all proteins). MAPK was found to interact with many other proteins. The MAPK pathway-biocardia networks showed that MAPK can interact with Rac1. The MAPK pathway-KEGG networks also suggested that MAPK can interact with Rac1. Activation of the MAPK signaling pathway has been reported in diabetic rats [19]. In addition, the MAPK signaling pathway is involved in inflammation and cell apoptosis caused by chronic intermittent hypoxia in rat pancreatic tissues [20]. MAPK signaling also contributes to the inflammatory response in adipocytes [11]. Based on these findings, we hypothesized that the Rac1/MAPK1 signaling pathway plays a role in the LRP16-mediated inflammatory response in adipocytes. MAPK signaling pathway related proteins are shown in Table 5.

Table 2. Top 15 pathway-biocarta analyses of all differentially expressed proteins

Pathway	Count	p-Value	q-Value	Gene
Transcription factor CREB and its extracellular signals	4	3.85681E-06	1.61504E-05	RAC1;GNAS;MAPK1;PRKAR2A
Electron Transport Reaction in Mitochondria	3	5.31956E-06	2.03663E-05	SLC25A4;SDHA;NDUFA1
Eukaryotic protein translation	3	2.20214E-05	7.66461E-05	EIF6;EIF4A1;EIF2S2
How Progesterone Initiates Oocyte Membrane	3	9.31568E-05	0.000265004	PIN1;MAPK1;PRKAR2A
Synaptic Proteins at the Synaptic Junction	3	0.000115887	0.000313715	CASK;SPTBN1;SPTAN1
Regulation of BAD phosphorylation	3	0.000171537	0.000442038	BAX;MAPK1;PRKAR2A
Trefoil Factors Initiate Mucosal Healing	3	0.000242103	0.000592544	EGFR;CTNNB1;MAPK1
Phosphoinositides and their downstream targets.	3	0.000242103	0.000592544	RAB5A;RAC1;AP2M1
Angiotensin II mediated activation of JNK Pathway	3	0.000329123	0.000766999	EGFR;RAC1;MAPK1
Agrin in Postsynaptic Differentiation	3	0.000434067	0.000969417	EGFR;RAC1;MAPK1
The SARS-coronavirus Life Cycle	2	0.000861189	0.001784064	NCL;FBL
The role of FYVE-finger proteins in vesicle transport	2	0.000861189	0.001784064	EGFR;RAB5A
Rho-Selective Guanine Exchange Factor AKAP13	2	0.000861189	0.001784064	AKAP13;PRKAR2A
krebPathway	2	0.001143334	0.002244788	SDHA;IDH2
Regulation And Function Of ChREBP in Liver	2	0.001143334	0.002244788	YWHAB;PRKAR2A
MAPK Signaling Pathway	2	0.089353362	0.066890226	RAC1;MAPK1

Table 3. Top 35 pathway-KEGG analyses of all differentially expressed proteins

Pathway	Count	p-Value	q-Value	Gene
Ribosome	15	2.22E-16	5.95E-14	RPL35A;RPL4;RPS19;RPL13A, etc.
Alzheimer's disease	15	8.98E-13	2.67E-11	NDUFB10;CYC1;COX411;UQCRCB, etc.
Parkinson's disease	13	6.53E-12	1.35E-10	NDUFB10;CYC1;SLC25A4;COX411, etc.
Systemic lupus erythematosus	13	1.36E-11	2.42E-10	H2AFY;H2AFZ;SNRPD1;HIST1H2BE, etc.
Oxidative phosphorylation	12	1.42E-10	2.23E-09	NDUFB10;CYC1;COX411;UQCRCB, etc.
Focal adhesion	13	1.12E-09	1.57E-08	FLNB;EGFR;SRC;ITGA3, etc.
Tight junction	11	1.68E-09	2.20E-08	CASK;KRAS;SRC;CSNK2A2, etc.
Long-term potentiation	8	3.29E-08	2.52E-07	KRAS;RAP1B;PPP1CA;PLCB3, etc.
GnRH signaling pathway	9	3.69E-08	2.67E-07	EGFR;KRAS;SRC;GNAS, etc.
Adherens junction	8	4.98E-08	3.25E-07	EGFR;SRC;CSNK2A2;CTNNB1, etc.
Colorectal cancer	8	8.09E-08	4.93E-07	DVL2;EGFR;KRAS;CTNNB1, etc.
Aminoacyl-tRNA biosynthesis	6	2.62E-07	1.47E-06	NARS;WARS;GARS;KARS, etc.
Melanogenesis	8	4.27E-07	2.20E-06	DVL2;KRAS;CTNNB1;GNAS, etc.
Citrate cycle (TCA cycle)	5	2.27E-06	1.03E-05	PDHA1;DLD;SDHA;MDH2, etc.
Gap junction	7	2.64E-06	1.18E-05	EGFR;KRAS;SRC;GNAS, etc.
MAPK signaling pathway	8	4.69E-04	1.03 E-03	RAC1;MAPK1;MAPKSP1

Table 4. MAPK signaling pathway related proteins

Accession	Uniprot AC	protein name	Ratio (T/L)
NP_958439.1	P00533	EGFR	5.696289176
NP_061485.1	P63000	RAC1	3.643827645
NP_002736.3	Q499G7	MAPK1	3.317763577
NP_004976.2	P01116	K-Ras	3.05334588
NP_068805.1	Q9UHA4	Lam-TOR3	2.958535664
NP_001448.2	O75369	filamin-B	2.670906598
NP_001104026.1	Q6NXF2	filamin-A	2.259681564
NP_001010942.1	P61224	ras-related protein Rap-1b	2.009803077

LRP16 is required for the expression of Rac1, phosphorylated ERK1/2 and its downstream genes

Overexpression of LRP16 increased MAPK1 and Rac1 expression by more than 3.31 and 3.64 fold, respectively, based on LC-MS analysis (Fig. 3A). We also demonstrated that LPS stimulated the expression of Rac1, TNF α , c-Jun, activator protein 1 (AP-1) (c-fos), NF- κ B (p65), IL-1 β , IL-2, IL-4, IL-6 and p-ERK1/2 (Fig. 3B).

Table 5. Primer sequences for real time PCR

Genes	Primers
Rac1	5'-TGGCTAAGGAGATTGGTGCT-3' and 5'-CAAAGGTTCCAAGGGACAGG-3'
TNF α	5'-CAGCCTCTTCTCCTTCCTGA-3' and 5'-GGAAGACCCCTCCCAGATAGA-3'
c-Jun	5'-CCTGCGTCTTAGGCTTCTCC-3' and 5'-GCCTCCGCACTCTTACTTG-3'
c-fos	5'-CTCAGACAGTGCCCGAGATG-3' and 5'-GTTCTCATGCGCT TCCTCT-3'
p65	5'-CGATCTGTTTCCCTCATCT-3' and 5'-ATTGGGTGCGTCTTAGTGGT-3'
IL-1 β	5'-TACAGTGGCAATGAGGATGA-3' and 5'-TGTAGTGGTGGTCCGAGATT-3'
IL-2	5'-GAATGGAATTAATAATTACAAGAATCCC-3' and 5'-TGTTTCAGATCCCTTTAGTTCCAG-3'
IL-4	5'-CACAAGTGGATATCACCTT-3' and 5'-GCTCGAACACTTTGAATATT-3'
IL-6	5'-AATCATCACTGGTCTTTTGGAG-3' and 5'-GCATTTGTGGTTGGGTCA-3'
β -actin	5'-TTCCTGGGCATGGAGTCTCT-3' and 5'-TGATCTTCATTGTGCTGGGTG-3'

Fig. 3. LRP16 upregulates protein expression in the Rac1-MAPK1/ ERK1/2 signaling pathway. (A) MAPK1 and Rac1 expression in human adipocytes overexpressing LRP16 and control cells as detected by LC-MS/MS. (B) Western blot analysis of Rac1, TNF α , c-Jun, c-fos, p65, IL-1 β , IL-2, IL-4, IL-6, and p-ERK1/2 protein expression. Protein expression was significantly increased following LPS stimulation, **P<0.01. (C) Western blot analysis of Rac1, TNF α , c-Jun, c-fos, p65, IL-1 β , IL-2, IL-4, IL-6, and p-ERK1/2 protein expression. Protein expression was significantly increased in response to LRP16 overexpression, **P<0.01. (D) Western blot analysis of Rac1, TNF α , c-Jun, c-fos, p65, IL-1 β , IL-2, IL-4, IL-6, and p-ERK1/2 protein expression. Protein expression was significantly decreased in response to LRP16 knock down, **P<0.01. Data were quantified from three independent experiments and representative images are shown.

To verify the influence of LRP16 on MAPK1 and Rac1 protein expression, we analyzed protein expression of Rac1, phosphorylated ERK1/2 and downstream genes (e.g. TNF α , c-Jun, c-fos, p65, IL-1 β , IL-2, IL-4, IL-6) in LRP16 over-expressing or silenced cells in response to LPS. LRP16 overexpression significantly increased p-ERK1/2 expression and its downstream genes following LPS stimulation (Fig. 3C). Transfection of siRNA targeting LRP16 efficiently reduced LRP16 expression in cells (Fig. 1B). We also demonstrated that siRNA mediated knock down of LRP16 attenuated p-ERK1/2 expression and reduced expression of downstream genes (Fig. 3D). Consistent with the increase in p-ERK1/2, overexpression of LRP16 increased Rac1 expression, which was further supported by the loss-of-function experiment using siRNA-LRP16 (Fig. 3C-D).

Consistent with the western blot results, LRP16 also induced mRNA expression of ERK downstream genes, c-Jun, c-fos, p65, and inflammatory cytokines (e.g. TNF α , IL-1 β , IL-2, IL-4, IL-6), and siRNA mediated knock down of LRP16 attenuated the expression of these genes (Fig. 4).

Rac1 is required for LRP16-induced ERK1/2 phosphorylation

To determine whether LRP16-induced ERK1/2 phosphorylation is mediated by Rac1, we examined ERK1/2 phosphorylation in siRNA-Rac1 expressing human adipocytes. As expected, overexpression of LRP16 stimulated p-ERK1/2 and its downstream genes following LPS stimulation. However, this effect was markedly diminished in the Rac1-knock down cells indicating that Rac1 is required for LRP16 induced ERK1/2 phosphorylation (Fig. 5).

Fig. 4. LRP16 upregulates mRNA expression in the Rac1-MAPK1/ ERK1/2 signaling pathway. (A) Real-time PCR analysis of Rac1, TNF α , c-Jun, c-fos, p65, IL-1 β , IL-2, IL-4, IL-6, and p-ERK1/2 mRNA expression. Gene expression was significantly increased in response to LPS stimulation, **P<0.01. (B) Real-time PCR analysis of Rac1, TNF α , c-Jun, c-fos, p65, IL-1 β , IL-2, IL-4, and IL-6 mRNA expression. Gene expression was significantly increased in response to LRP16 overexpression, **P<0.01. (C) Real-time PCR analysis of Rac1, TNF α , c-Jun, c-fos, p65, IL-1 β , IL-2, IL-4, IL-6, and p-ERK1/2 mRNA expression. Gene expression was significantly decreased in response to LRP16 knock down, **P<0.01. Data were quantified from three independent experiments.

Fig. 5. Knock down of Rac1 decreases gene and protein expression in the MAPK1/ERK1/2 signaling pathway. (A) Real-time PCR analysis of Rac1, TNFα, c-Jun, c-fos, p65, IL-1β, IL-2, IL-4, and IL-6 mRNA expression. Gene expression was significantly decreased in response to Rac1 knock down. (B) Western blot analysis of Rac1, TNFα, c-Jun, c-fos, p65, IL-1β, IL-2, IL-4, IL-6, and p-ERK1/2 protein expression. Protein expression was significantly decreased in response to Rac1 knock down. Data were analyzed by two-way ANOVA: ∞ Significantly increased/decreased ($P < 0.001$) by both factors (LRP16 and siRNA-Rac1). Data were analyzed by one-way ANOVA followed by a Tukey's post hoc test: ** $P < 0.01$ compared to LRP16⁻, siRNA-Rac1⁻ group; ## $P < 0.01$ compared to LRP16⁺, siRNA-Rac1⁻ group. Data were quantified from three independent experiments and representative images are shown.

ERK is required for LRP16 regulation of the inflammatory response in human adipocytes

Given that ERK1/2 phosphorylation was induced by LRP16, we next asked whether pharmacological inhibition of ERK1/2 alters regulation of its downstream genes and inflammatory factors in LRP16 expressing adipocytes. LRP16 expressing adipocytes exposed to PD98059, a pharmacological inhibitor of ERK, reduced mRNA and protein expression of c-fos, p65, c-Jun, and inflammatory factors TNFα, IL-1β, IL-2, IL-4 and IL-6 compared to wide-type control cells. However, PD98059 had no significant effect on Rac1 mRNA and protein expression (Fig. 6).

Fig. 6. Inhibition of ERK1/2 decreases expression of transcription factors and inflammatory cytokines. (A) Real-time PCR analysis of TNFα, c-Jun, c-fos, p65, IL-1β, IL-2, IL-4, and IL-6 mRNA expression. Gene expression was significantly decreased in response to ERK1/2 inhibition. (B) Western blot analysis of TNFα, c-Jun, c-fos, p65, IL-1β, IL-2, IL-4, IL-6, and p-ERK1/2 protein expression. Protein expression was significantly decreased in response to ERK1/2 inhibition. Data were analyzed by two-way ANOVA: ∞ Significantly increased/decreased ($P < 0.001$) by both factors (LRP16 and PD98059). Data were analyzed by one-way ANOVA followed by a Tukey's post hoc test: ** $P < 0.01$ compared to LRP16⁻, PD98059⁻; ## $P < 0.01$ compared to LRP16⁺, PD98059⁻. Data were quantified from three independent experiments and representative images are shown.

Discussion

We identified 390 differentially expressed proteins in LRP16 over-expressing human adipocytes. Among these proteins, we identified MAPK1 and Rac1, which are related to the MAPK signaling pathway. Using gain-of-function and loss-of-function assays we revealed a novel function of LRP16: LRP16 stimulates the inflammatory response through a Rac1 and ERK1/2 dependent pathway.

Previous studies suggested that insulin resistance is associated with chronic inflammation, and several mediators, including pro-inflammatory cytokines and adipocytokines, released from adipocytes have been identified as factors involved in the development of type 2 diabetes mellitus [7]. The pro-inflammatory cytokines include TNFα, IL-1β, IL-6, and various

adipocytokines [21, 22]. As a crucial signaling pathway to regulate inflammatory response, NF- κ B as well as several other transcription factors and kinases are involved in this process [7]. IRS-1 signaling plays an essential role in chronic inflammation associated with type 2 diabetes mellitus. This pathway is activated by tyrosine phosphorylation of IRS-1. LRP16 was identified as a negative regulator of insulin through impairing the IRS-1 signaling pathway [23], but the mechanism by which LRP16 impairs IRS-1 signaling is largely unknown. LRP16 was reported to be a crucial regulator of NF- κ B, which causes insulin resistance through altering tyrosine phosphorylation of IRS-1 [4]. Given the putative role of NF- κ B in regulating the chronic inflammatory response, we speculate that LRP16 disrupts the IRS-1 pathway in the chronic inflammatory response.

LC-MS screening identified 390 differentially expressed proteins in the mature adipocytes stably transfected with plasmid encoding LRP16 and control cells. MAPK1 and Rac1 protein expression increased with overexpression of LRP16. Furthermore, our analyses indicated that MAPK1 and Rac1 can interact with each other. Pathway analyses indicated that MAPK and Rac1 share a similar profile in terms of involved pathways. ERK, a constitutive component of the MAPK1 family, has been shown to regulate the inflammatory response in adipocytes [24, 25]. The LC-MS profiling results led us to investigate the possible roles of ERK and Rac1 in the LRP16 mediated inflammatory response in human adipocytes.

As a member of the MAPK1 family, phosphorylated ERK1/2 translocates from the cytoplasm to the nucleus and participates in the inflammatory response by activating NF- κ B, AP-1, c-fos, and c-Jun [26, 27]. Ectopic expression of LRP16 induced phosphorylation of ERK1/2, and expression of Rac1, transcription factors (c-fos, p65, and c-Jun), and inflammatory cytokines (TNF α , IL-1 β , IL-2, IL-4, IL-6). Importantly, knock down of LRP16 reduced expression of these ERK mediated downstream genes and cytokines, indicating that LRP16 is required for the inflammatory response in human adipocytes. We further demonstrated that the stimulatory effect of LRP16 on the inflammatory response was markedly diminished by knock down of Rac1 and inhibiting the function of ERK1/2 with the chemical inhibitor, PD98059. Inhibiting ERK1/2 reduced expression of transcription factors and inflammatory cytokines. Overall, we uncovered a novel signaling pathway in adipocytes: LRP16 regulates the inflammatory response in LPS-stimulated human adipocytes *via* a Rac1 and ERK1/2-dependent pathway. However, it should also be noted that we did not measure specific changes in ERK1 or ERK2 mRNA and protein expression in response to LPS and/or LRP16 in this current study. Therefore, we could not determine the involvement of the specific ERK subtype based on the present data.

Previously we showed that LRP16 induced insulin resistance in adipocytes and skeletal muscle cells by impairing the IRS-1 signaling pathway and peroxisome proliferator-activated receptor- γ (PPAR γ) activity, respectively [23, 28]. In combination with the results from the current study, we have a better understanding of the role LRP16 plays in the inflammation regulatory network and clarified the exact roles of the NF- κ B and Rac1-MAPK1/ERK signaling pathways in the inflammatory response.

Conclusion

In summary, we show that LRP16 promotes the inflammatory response through modulating Rac1 and activating ERK1/2. Our conclusion is based on pathway analysis and screening results from LC-MS profiling using LRP16 expressing human adipocytes. These findings uncover a novel mechanism of LRP16-mediated inflammation in type 2 diabetes mellitus. However, our *in vitro* findings have not yet been validated physiologically. Further *in vivo* studies and clinical data are required to explore the clinical significance of this pathway.

Disclosure Statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

References

- 1 Yu L, Han WD, Lou FD, Wang QS, Zhao Y, Caligiuri MA: Cloning of leukemia associated gene LRP16 in acute myeloid leukemia. *Junyi Jinxiu Xueyuan Xuebao* 2000;21:81-84.
- 2 Ma XL, Mu YM, Jin MM, Wang BA: Expression of LRP16 protein during 3T3-L1 preadipocyte differentiation. *J Chinese PLA Postgrad Med Sch* 2011;32:486-488.
- 3 Yuanguang M, Zhiqiang W, Xiaoyun Y, Yali Z, Meixia C, Yiling S, Jie Y, Xiaobing F, Weidong H: Keratin 18 attenuates estrogen receptor α -mediated signaling by sequestering LRP16 in cytoplasm. *BMC Cell Biology* 10, 1(2009-12-26) 2009;10:96-96.
- 4 Wu Z, Li Y, Li X, Ti D, Zhao Y, Si Y, Mei Q, Zhao P, Fu X, Han W: LRP16 integrates into NF-kappaB transcriptional complex and is required for its functional activation. *PLoS One* 2011;6:e18157.
- 5 Zang L, Xue B, Lu Z, Li X, Yang G, Guo Q, Ba J, Zou X, Dou J, Lu J, Pan C, Mu Y: Identification of LRP16 as a negative regulator of insulin action and adipogenesis in 3T3-L1 adipocytes. *Horm Metab Res* 2013;45:349-358.
- 6 Sesti G: Pathophysiology of insulin resistance. *Best Practice & Research Clinical Endocrinology & Metabolism* 2006;20:665-679.
- 7 Tilg H, Moschen AR: Inflammatory mechanisms in the regulation of insulin resistance. *Molecular Medicine* 2008;14:222-231.
- 8 Karstoft K, Pedersen BK: Exercise and type 2 diabetes: focus on metabolism and inflammation. *Immunol Cell Biol* 2016;94:146-150.
- 9 Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF- α - and obesity-induced insulin resistance. *Science* 1996;271:665-668.
- 10 Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor- α in human obesity and insulin resistance. *J Clin Invest* 1995;95:2409-2415.
- 11 Wang Z, Lee Y, Eun JS, Bae EJ: Inhibition of adipocyte inflammation and macrophage chemotaxis by butein. *Eur J Pharmacol* 2014;738:40-48.
- 12 Yang J, Zhao YL, Wu ZQ, Si YL, Meng YG, Fu XB, Mu YM, Han WD: The single-macro domain protein LRP16 is an essential cofactor of androgen receptor. *Endocr Relat Cancer* 2009;16:139-153.
- 13 Gagnon A, Foster C, Landry A, Sorisky A: The role of interleukin 1 β in the anti-adipogenic action of macrophages on human preadipocytes. *J Endocrinol* 2013;217:197-206.
- 14 Washburn MP, Wolters D, Yates JR, 3rd: Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol* 2001;19:242-247.
- 15 Delahunty C, Rd YJ: Protein identification using 2D-LC-MS/MS. *Methods* 2005;35:248-255.
- 16 Romijn EP, Yates JR, 3rd: Analysis of organelles by on-line two-dimensional liquid chromatography-tandem mass spectrometry. *Methods Mol Biol* 2008;432:1-16.
- 17 Apweiler R, Bairoch A, Wu CH, Barker WC, Boeckmann B, Ferro S, Gasteiger E, Huang H, Lopez R, Magrane M, Martin MJ, Natale DA, O'Donovan C, Redaschi N, Yeh LS: UniProt: the Universal Protein knowledgebase. *Nucleic Acids Res* 2004;32:D115-119.
- 18 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2 $^{-\Delta\Delta C_T}$ Method. *Methods* 2001;25:402-408.
- 19 Chen Q, Mo R, Wu N, Zou X, Shi C, Gong J, Li J, Fang K, Wang D, Yang D, Wang K, Chen J: Berberine Ameliorates Diabetes-Associated Cognitive Decline through Modulation of Aberrant Inflammation Response and Insulin Signaling Pathway in DM Rats. *Front Pharmacol* 2017;8:334.
- 20 Wang Y, Hai B, Niu X, Ai L, Cao Y, Li R, Li Y: Chronic intermittent hypoxia disturbs insulin secretion and causes pancreatic injury via the MAPK signaling pathway. *Biochem Cell Biol* 2017;95:415-420.
- 21 Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H, Zick Y: A molecular basis for insulin

- resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J Biol Chem* 1997;272:29911-29918.
- 22 Jager J, Gremeaux T, Cormont M, Le Marchand-Brustel Y, Tanti JF: Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. *Endocrinology* 2007;148:241-251.
- 23 Yuan JS, Reed A, Chen F, Stewart CN, Jr.: Statistical analysis of real-time PCR data. *BMC Bioinformatics* 2006;7:85.
- 24 Sell H, Eckel J, Dietze-Schroeder D: Pathways leading to muscle insulin resistance--the muscle--fat connection. *Arch Physiol Biochem* 2006;112:105-113.
- 25 Bost F, Aouadi M, Caron L, Binetruy B: The role of MAPKs in adipocyte differentiation and obesity. *Biochimie* 2005;87:51-56.
- 26 Sabio G, Davis RJ: TNF and MAP kinase signalling pathways. *Semin Immunol* 2014;26:237-245.
- 27 Arthur JS, Ley SC: Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol* 2013;13:679-692.
- 28 Zang L, Mu YM, Lu ZH, Xue B, Ma XL, Yang GQ, Ba JM, Dou JT, Lu JM: [LRP16 gene causes insulin resistance in C2-C12 cells by inhibiting the IRS-1 signaling and the transcriptional activity of peroxisome proliferator activated receptor gamma]. *Zhonghua Yi Xue Za Zhi* 2011;91:1408-1412.