

Involvement of receptor-interacting protein 140 in palmitate-stimulated macrophage infiltration of pancreatic beta cells

RUNMEI ZOU^{1,2}, JUNLI XUE¹, QI HUANG¹, ZHE DAI¹ and YANCHENG XU¹

¹Department of Endocrinology, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071;

²Children's Medical Center, The Second Xiangya Hospital of Central South University, Changsha, Hunan 410011, P.R. China

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Abstract. Receptor-interacting protein 140 (RIP140) in macrophages stimulates the nuclear factor- κ B subunit RelA to activate tumor necrosis factor (TNF)- α and interleukin (IL)-6 transcription. However, under lipotoxic conditions, the involvement of RIP140 in the infiltration of beta cells by macrophages remains unknown. In the present study, murine RAW264.7 macrophages were transfected with a RIP140 overexpression plasmid or siRNA prior to macrophage activation with 500 μ M palmitate. Palmitate-free conditioned media was then collected and added to murine insulinoma MIN6 cells. Significant decreases were observed in cell viability ($P<0.01$), glucose-stimulated insulin secretion ($P<0.01$) and levels of peroxisome proliferator-activated receptor- γ coactivator-1 α ($P<0.05$), phosphoenolpyruvate carboxykinase and proliferating cell nuclear antigen mRNA ($P<0.01$) in MIN6 cells. In addition, conditioned media from palmitate-treated and RIP140-upregulated macrophages significantly increased the levels of uncoupling protein-2 ($P<0.01$), inducible nitric oxide synthase 1 ($P<0.01$) and pancreatic and duodenal homeobox 1 ($P<0.05$) mRNA and levels of activated Jun N-terminal kinase (JNK) ($P<0.01$) and extracellular signal-regulated kinase (ERK) 1/2 ($P<0.01$). In turn, the conditioned media was found to be significantly enriched in TNF- α and IL-6 (both $P<0.05$). These results were the opposite of those obtained from MIN6 cells treated with conditioned media from palmitate-treated and RIP140-knockdown macrophages. MIN6 cells were transfected with RIP140 overexpression plasmid or siRNA prior to treatment with 500 μ M palmitate and supernatant was collected for use in macrophage chemotaxis assays. In the palmitate-activated and RIP140-overexpressing MIN6

cells, TNF- α and IL-6 secretion increased significantly (both $P<0.05$) and macrophage chemotaxis towards MIN6 cells was enhanced. By contrast, downregulating RIP140 in MIN6 cells had the opposite effect. These data suggest that RIP140 in macrophages mediates the transcription of inflammatory cytokines when concentration of palmitate is high. Macrophage RIP140 may also impair beta cell function by activating the JNK and ERK1/2 signaling pathways and promoting specific gene transcription. Furthermore, expression of RIP140 in pancreatic beta cells may stimulate macrophage chemotaxis, thus triggering local low-grade inflammation.

Introduction

Dysfunction of pancreatic beta cells in type II diabetes is associated with high circulatory levels of free fatty acids and glucose. Previous results have demonstrated that levels of free fatty acids are higher in diabetic patients than in healthy individuals (1). High levels of saturated fatty acids, including palmitate, lauric acid and linoleic acids, activate signaling from toll like receptors (TLRs) and their downstream Jun N-terminal kinase (JNK) and nuclear factor- κ B (NF- κ B) pathways, thus promoting the release of interleukin (IL)-6, tumor necrosis factor (TNF)- α and other inflammatory cytokines from macrophages (2,3). An increase in fat mass associated with an inflammatory response may be involved in the development of insulin resistance, as indicated by a previous study in obese mice, where it was observed that an increased mass of adipose tissue led to macrophage infiltration of adipose tissue (4). Higher levels of circulating free fatty acids and inflammatory cytokines were also observed, which may then act on skeletal muscle cells, adipocytes and other cells to influence insulin signaling (5,6). In muscle cells, the conditioned media from palmitate-stimulated macrophages has been demonstrated to activate protein kinase C- θ and - ϵ to promote inflammatory cytokine expression, thus contributing to insulin resistance (7). Conditioned media from palmitate-treated macrophages may also provoke insulin resistance in muscle cells at the levels of Akt signaling, glucose transporter type 4 translocation and glucose uptake (8). Furthermore, it has been observed in a co-culture of hepatocytes and macrophages that lipopolysaccharide-induced TNF- α factor (LITAF) was involved in macrophage infiltration of hepatocytes, with knockdown of

Correspondence to: Professor Yancheng Xu, Department of Endocrinology, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuhan, Hubei 430071, P.R. China
E-mail: xjl100901@whu.edu.cn

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LITAF leading to improvement of insulin resistance in human hepatocytes (9). Similarly, prolonged exposure of beta cells to inflammatory cytokines may induce excessive production of reactive oxygen species and endoplasmic reticulum stress, leading to cell apoptosis and a decrease in insulin secretion (10,11). Collectively, these data suggest that insulin signaling and beta cell function may be altered by inflammatory processes. However, the underlying mechanism for the effects of macrophage-derived conditioned media on beta cell function remains unknown.

Receptor interacting protein 140 (RIP140) was first identified as a co-repressor of the estrogen receptor (12), with high expression of RIP140 observed in reproductive (13) and metabolic tissues, including adipose, liver and muscular tissue (14). RIP140 is now an established co-repressor of various nuclear receptors, including the thyroid receptor, peroxisome proliferator activated receptor (PPAR) (15), liver X receptor (LXR)- α (16), glucocorticoid receptor (17) and retinoic acid receptor (18), whereby it regulates the transcription of genes involved in glucose and lipid metabolism (16). In some instances, RIP140 also acts as a co-activator of nuclear receptors, by stimulating histone acetylase and cyclic adenosine monophosphate-responsive element binding protein (CBP), as the co-activator of NF- κ B, thus promoting pro-inflammatory cytokine transcription (19). Intracellular cholesterol and a high fatty diet may increase the level of RIP140 in macrophages, whereas microRNA miR-33 reduces the inflammatory activity of macrophages by inhibiting RIP140 co-activation of NF- κ B (20). It has been demonstrated that macrophage treatment with a low dose of lipopolysaccharide (LPS) stimulated phosphorylation of RIP140 by spleen tyrosine kinase, thus stimulating RIP140 interaction with the NF- κ B subunit RelA. In turn, this triggered the degradation of RIP140, resulting in decreased transcription of cytokine genes and a reduced inflammatory response (21). Additionally, mice with RIP140 knockdown in macrophages exhibit a decreased population of circulating monocytes, along with a reduction in inflammatory M1 (pro-inflammatory phenotype) and expansion in M2 (anti-inflammatory phenotype) macrophages, which subsequently improved insulin resistance induced by a high-fat diet (22).

Co-activation of NF- κ B by RIP140 mediates the release of inflammatory cytokines. However, under lipotoxic conditions, the influence of RIP140 on the infiltration of pancreatic beta cells by macrophages and the subsequent effects on beta cell function remain unknown. In the present study, conditioned media from palmitate-stimulated macrophages was used to stimulate beta cells and the chemotaxis of macrophages towards beta cells was evaluated. It was determined that RIP140 promoted inflammatory cytokine expression in macrophages under high palmitate conditions and impaired beta cell function. In addition, under lipotoxic conditions, RIP140 enhanced the chemotaxis of macrophages towards pancreatic beta cells through the release of inflammatory cytokines, thus triggering local low-grade inflammation and impairing beta cell function.

Materials and methods

Materials and reagents. A total of 50 mM palmitate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was

dissolved in 1 ml 99% ethanol and mixed with 9 ml 10% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA), from which a 5 mM palmitate-10% BSA stock solution was prepared, as previously described (23). Antibodies against phosphorylated (p)-JNK (cat. no. 9255S), JNK (cat. no. 3708S), p-ERK1/2 (cat. no. 9106S) and ERK1/2 (cat. no. 4696S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against RIP140 (cat. no. ab42125) and β -actin (cat. no. sc-130300) were purchased from Abcam (Cambridge, MA, USA) and Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), respectively. Goat anti-rabbit immunoglobulin (Ig)-G (heavy and light chain) secondary antibody conjugated to horseradish peroxidase (HRP; cat. no. BS13278) was obtained from Bioworld Technology, Inc. (St. Louis Park, MN, USA). ELISA kits for mouse TNF- α (cat. no. EK0527) and IL-6 (cat. no. EK0411) were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China).

Cell culture and treatment. Murine RAW264.7 macrophages (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare, Logan, UT, USA) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere (5% CO₂) for 24 h. The mouse pancreatic beta cell line MIN6 (American Type Culture Collection) was cultured overnight in Dulbecco's modified Eagle medium (DMEM; Hyclone; GE Healthcare) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc. Waltham, MA, USA), 0.001% beta-mercaptoethanol and 1% penicillin-streptomycin at 37°C in a humidified atmosphere (5% CO₂, 95% air). Macrophages were transiently transfected with pEGFP-N1-RIP140 plasmid (or pEGFP-N1 plasmid as a control) or RIP140 siRNA (or scramble siRNA as a control) at 37°C for 24 h, then treated with 500 μ M palmitate at 37°C for 24 h. Supernatant was then collected in order to detect the concentrations of TNF- α and IL-6 using the relevant ELISA kits, according to the manufacturer's instructions. Macrophages were washed several times with phosphate-buffered saline (PBS) and fresh RPMI 1640 media with 10% FBS was added. Following 24 h incubation at 37°C, palmitate-free conditioned media was collected, centrifuged (10,000 \times g, 5 min, room temperature) and added to MIN6 cells for a 24 h incubation, as previously described (7). MIN6 cells were then collected for cell viability, gene expression and western blot analyses. For chemotaxis assays, MIN6 cells were transiently transfected and treated with palmitate as above and culture supernatant was collected.

Plasmid construction and cell transfection. A mouse RIP140 overexpression plasmid was constructed, as previously described (24), for transfection into the RAW264.7 macrophages and MIN6 cells. Briefly, total DNA from RAW264.7 macrophages was extracted using a DNA extraction kit (cat. no. DP304; Beijing Transgen Biotech Co., Ltd., Beijing, China) and a fragment of RIP140 (Reference Sequence: NM 173440.2) was amplified by polymerase chain reaction (PCR) using the following primers: Forward, 5'-GACTCGAGG ATCTTACTGACGAGAGGAGCT-3', and reverse, 5'-ACG GTACCCAGGTACACATTTCTTCTGACTC-3'. A TransTaq High Fidelity DNA polymerase was obtained from Beijing

Transgen Biotech Co., Ltd. (Beijing, China) and the cycling conditions were as follows: 4 cycles for 30 sec each at 94°C, 1 cycle for 30 sec at 58°C, 1 cycle for 4 min at 72°C, 30 cycles for 30 sec each at 94°C, 1 cycle for 30 sec at 64°C and 4 min at 72°C. *KpnI* (GGTACC) and *XhoI* (CTCGAG) restriction sites were included to facilitate ligation of the cDNA product into a pEGFP-N1 plasmid (Biovector Science Lab, Inc., Beijing, China) using *KpnI*, *XhoI* and T4 DNA ligase (all from Fermentas Inc., Burlington, Canada), according to the manufacturer's protocol. The sequence of pEGFP-N1-RIP140 was verified by DNA sequencing. The sequences of RIP140 siRNA were as follows: Forward, 5'-CGGCGUUGACAUCAAAGAAAdTdT-3' and reverse, 3'-dTdTGCCGCAACUGUAGUUUCUU-5'. Transient transfection was conducted with Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Lipofectamine 2000 and 250 µl optimum-minimal essential medium (Opti-MEM; Gibco; Thermo Fisher Scientific, Inc.) were mixed, incubated at room temperature for 5 min, then 5 µg plasmid or 200 pmol/l siRNA was added to a separate 250 µl Opti-MEM. The two solutions were mixed and incubated for 20 min at room temperature, then added to serum-free medium-cultured RAW264.7 cells. The mixture was replaced with fresh medium after 6 h incubation at 37°C.

Total RNA extraction and reverse transcription (RT)-PCR. Total RNA was extracted with a Spin Column RNA Extraction kit (Sangon Biotech Co., Ltd., Shanghai, China) and transcribed to cDNA using Moloney Murine Leukemia Virus reverse transcriptase (Promega Corporation, Madison, WI, USA). PCR for RIP140 was performed using a SYBR-Green PCR Master Mix kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. The cycling conditions were as follows: An initial denaturation step at 95°C for 5 min, followed by 40 PCR cycles for 5 sec each at 95°C, 1 cycle for 30 sec at 58°C and 1 cycle for 30 sec at 72°C. Semiquantitative RT-PCR was performed using Taq DNA polymerase (Takara Bio, Inc.) for comparison of the cDNAs developed, under the following conditions: An initial denaturation at 95°C for 5 min, followed by 30 PCR cycles for 5 sec each at 95°C, 1 cycle for 30 sec at 56°C and 1 cycle for 30 sec at 72°C. Semiquantification was performed using mouse β-actin as an internal standard. Levels of target bands in the agar gel were analyzed by AlphaEaseFC 4.0 software (Alpha Innotech Corporation; ProteinSimple, San Jose, CA, USA). The primers of all genes quantified are listed in Table I.

Western blotting. MIN6 cells were washed three times with PBS and lysed on ice in radioimmunoprecipitation assay buffer containing 50 mM Tris hydrochloride (pH 7.4), 150 mM sodium chloride (NaCl), 1% NP-40 lysis buffer, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride and 1 mM phosphatase inhibitor (Applygen Technologies Inc, Beijing, China) for 30 min, then centrifuged at 10,000 × g at 4°C for 10 min. The supernatant was collected and protein concentration was determined using a BCA Protein Assay kit (Applygen Technologies, Inc., Beijing, China), according to the manufacturer's protocol. SDS-PAGE, trans-blotting and subsequent immunodetection were performed, as previously described (25). Briefly, blots were incubated with primary antibodies (anti-RIP40, 1:1,000;

anti-p-JNK, 1:250; and anti-JNK, anti-p-ERK and anti-ERK, 1:500) at 4°C overnight, then with HRP-conjugated secondary IgG (1:5,000) at 37 °C for 1 h. β-actin was used as an internal control. Levels of target protein within bands were determined using AlphaEaseFC 4.0 software.

Cell viability. MIN6 cells (5×10³ cells/ per well) were cultured in 96 well plates in complete DMEM at 37°C. After 24 h of culture, cells were treated with 100 µl conditioned media from the following RAW264.7 cells: i) Treated with BSA, ii) treated with 500 µM palmitate, iii) transfected with pEGFP-N1 control plasmid, iv) transfected with pEGFP-N1-RIP140 plasmid, v) transfected with scramble siRNA and vi) transfected with RIP140 siRNA. Groups 3-6 were treated with 500 µM palmitate for 24 h post-transfection. Cell viability was then measured by a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Specifically, 5 mg/ml MTT solution (Sigma-Aldrich; Merck KGaA) was added to each well prior to 4 h incubation at 37°C. The resulting formazan precipitate was dissolved by adding 100 µl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. Color intensity was then detected at 570 nm by a microplate reader (Thermo Labsystems, Beverly, MA, USA). Three independent experiments were performed.

Glucose-stimulated insulin secretion assay. MIN6 cells were exposed to the conditioned media of RAW264.7 cells for 24 h. MIN6 cells were then washed twice with PBS, incubated in Krebs-Ringer bicarbonate 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (KRBH buffer: 115 mM NaCl, 24 mM sodium bicarbonate, 5 mM potassium chloride, 1 mM magnesium chloride, 25 mM HEPES, 0.5% BSA, pH 7.4) with 2.8 mM glucose for 40 min. Supernatant was then collected for measurement of basal insulin secretion (BIS). Fresh KRBH buffer containing 25 mM glucose was added to MIN6 cells and cells were incubated at 37°C for 60 min. Supernatant was then collected to measure glucose-stimulated insulin secretion (GSIS). Insulin concentrations were measured with an ELISA kit (cat. no. CSB-E05071m; Cusabio Biotech Co., Ltd., Wuhan China), according to the manufacturer's protocol. The experiment was performed three times.

Chemotaxis assays. Chemotaxis assays were performed on Transwell plates (24-well format, 8 µm; Corning Incorporated, Corning, NY, USA). A total of 500 µl conditioned media from MIN6 cells was added to the lower chamber. RAW264.7 cells were washed with PBS three times and suspended in RPMI 1640 medium containing 0.5% FBS, then seeded into the upper chamber (10⁴ cells in total). Chambers were incubated for 6 h at 37°C. Cells that remained in the upper chamber were removed while cells in the lower chamber were fixed with 4% paraformaldehyde for 20 min at room temperature, followed by incubation with 1% crystal violet (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. Stained cells were manually counted under a light microscope in 6 randomly selected fields. The experiment was performed three times.

Chemotaxis of murine peritoneal macrophages was also measured. Peritoneal macrophages were isolated from 3 male c57BL/6 mice obtained from the Animal Center of Wuhan

University, Wuhan, China (mean age, 5.0 ± 0.5 weeks; mean weight, 19.0 ± 0.5 g). The research protocol was approved by Medical Ethical Committee of Zhongnan Hospital (Wuhan, China). The mice were sacrificed by cervical dislocation and soaked in 75% ethyl alcohol for 5 min, injected intraperitoneally with 5 ml serum-free RPMI 1640 medium, then placed in a supine position for 5 min. The abdominal cavity was opened and 5 ml peritoneal fluid was aspirated and centrifuged at $1,000 \times g$ at 4°C for 5 min. Isolated cells were maintained in RPMI1640 medium supplemented with 10% FBS at 37°C for 24 h. After adhering to the culture dishes, macrophages were harvested and added to the upper Transwell chamber and conditioned media from MIN6 cells was added to the lower chamber. Chambers were incubated for 6 h at 37°C and chemotaxis assays were performed as before.

Statistical analysis. Data are presented as the mean \pm standard deviation. When two groups were being compared, differences between mean values were determined by Independent Sample t tests. One-way analysis of variance followed by a Turkey test was used to determine differences between the mean values of >2 groups. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc. Chicago, IL, USA) and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

TNF- α and IL-6 levels are elevated by RIP140 in palmitate-treated macrophages. Expression of RIP140, TNF- α and IL-6 at the mRNA and protein level in palmitate-treated RAW264.7 macrophages was determined by semiquantitative RT-PCR and ELISA, respectively. It was observed that the level of RIP140 mRNA expression in RAW264.7 cells was significantly increased by $500 \mu\text{M}$ palmitate, compared with control cells treated with BSA alone ($P < 0.05$; Fig. 1A). Expression of the inflammatory cytokines TNF- α and IL-6 was also significantly increased at the mRNA and protein levels by $250 \mu\text{M}$ and $500 \mu\text{M}$ palmitate, relative to control cells. Relative to $250 \mu\text{M}$ palmitate, expression of TNF- α and IL-6 was also significantly increased at the mRNA and protein levels by $500 \mu\text{M}$ palmitate ($P < 0.05$; Fig. 1B-E). The level of RIP140 expression was subsequently regulated by transfection of RAW264.7 macrophages with RIP140 plasmid or siRNA. As demonstrated by western blot analysis, the level of RIP140 protein expression was significantly increased in cells transfected with pEGFP-N1-RIP140 plasmid, relative to wild type control cells ($P < 0.05$; Fig. 2A and B). In addition, transfection with RIP140 siRNA significantly decreased RIP140 mRNA, relative to control cells transfected with scramble siRNA ($P < 0.05$; Fig. 2C and D). The influence of these transfections on inflammatory cytokine expression in palmitate-treated macrophages was subsequently evaluated. In RAW264.7 macrophages treated with $500 \mu\text{M}$ palmitate, it was observed that the expression of TNF- α and IL-6 were significantly increased at the mRNA and protein levels by the RIP140 overexpression plasmid, relative to control cells transfected with pEGFP-N1 plasmid alone ($P < 0.01$; Fig. 3). In addition, TNF- α and IL-6 expression was significantly decreased by RIP140 siRNA, relative to scramble siRNA control cells ($P < 0.01$; Fig. 3). These data suggest that RIP140

Table I. Primer sequences.

Genes (accession no.)	Primer sequences (5' to 3')
RIP140 (NM 73440.2)	
Forward	GGCAGCAAACCTGAATTC GGC
Reverse	CTCACCGGGCACGGCACATC
β -actin (NM 0007393.3)	
Forward	TCTACAATGAGCTGCGTGTG
Reverse	GGTGAGGATCTTCATGAGGT
TNF- α (NM 013693.2)	
Forward	CCACCACGCTCTTCTGTCTAC
Reverse	TGGGCTACAGGCTTGTCAT
IL-6 (NM 031168.1)	
Forward	CCACTTCACAAGTCGGAGGC TTA
Reverse	GCAAGTGCATCATCGTTGTTC ATAC
PGC-1 α (NM 008904.2)	
Forward	GTGTTCCCGATCACCATATTCC
Reverse	CGGTGTCTGTAGTGGCTTGA TTC
PEPCK (NM 011044.2)	
Forward	ATCTTTGGTGGCCGTAGACCT
Reverse	GCCAGTGGGCCAGGTATTT
PDX-1 (NM 008814.3)	
Forward	GATGAAATCCACCAAAGCTCA
Reverse	AGAATTCCTTCTCCAGCTCCA
PCNA (NM 011045.2)	
Forward	TCGACACATACCGCTGCGACC
Reverse	GCAAATTCACCCGACGGCATC
UCP-2 (NM 011671.4)	
Forward	GCATTGGCCTCTACGACTCT
Reverse	CTGGAAGCGGACCTTTACC
iNOS1 (NM 010927.3)	
Forward	AATGGCAACATCAGGTCGGC CATCACT
Reverse	GCTGTGTGTACAGAAGTCTC GAACTC

RIP140, receptor interacting protein 140; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; PGC-1 α , pepsinogen C; PEPCK, phosphoenolpyruvate carboxykinase; PDX-1, pancreatic and duodenal homeobox 1; PCNA, proliferating cell nuclear antigen; UCP-2, uncoupling protein-2; iNOS1, nitric-oxide synthase 1.

promotes TNF- α and IL-6 expression in macrophages in high palmitate conditions.

Conditioned media from macrophages modifies cell viability. Conditioned media collected from palmitate-treated RAW264.7 macrophages ($500 \mu\text{M}$ palmitate) was added to MIN6 pancreatic beta cells and cell viability was measured by an MTT assay. It was observed that cell viability was significantly reduced by

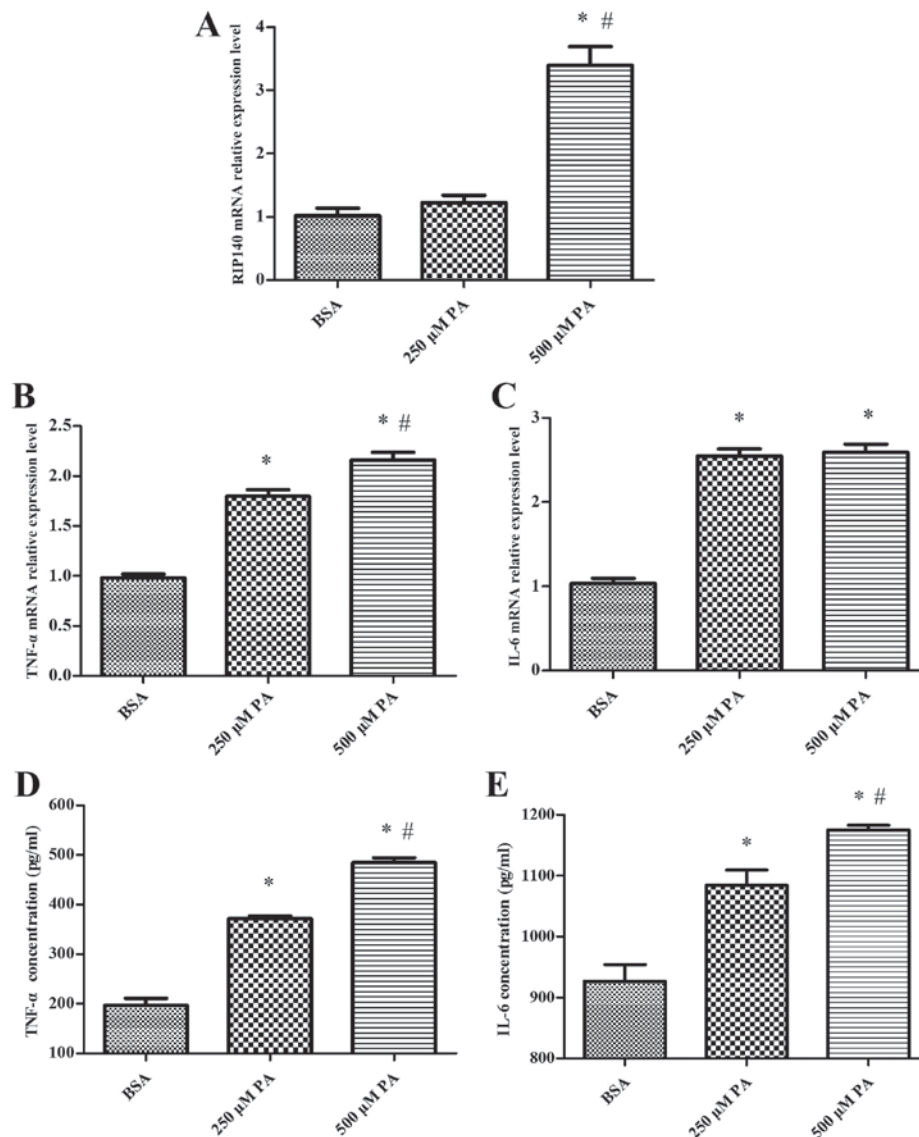


Figure 1. Palmitate induced the expression of RIP140 and inflammatory cytokines in RAW264.7 macrophages. RAW 264.7 cells were treated with BSA, 250 or 500 μ M palmitate for 24 h and the levels of (A) RIP140, (B) TNF- α and (C) IL-6 mRNA expression were determined by semiquantitative reverse transcription polymerase chain reaction. The concentrations of (D) TNF- α and (E) IL-6 in the RAW264.7 cell culture supernatant were determined by ELISA. Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$ vs. BSA, # $P < 0.05$ vs. 250 μ M PA. BSA, bovine serum albumin; PA, palmitate; RIP140, receptor-interacting protein 140; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6.

conditioned media from palmitate-treated RAW264.7 cells at 24 and 48 h post-media treatment, relative to media from control BSA-treated RAW264.7 cells ($P < 0.05$; Fig. 4). It was also demonstrated that the viability of MIN6 cells was significantly decreased by conditioned media from palmitate-treated RAW264.7 cells transfected with RIP140 overexpression plasmid at 24 and 48 h post-treatment, relative to media of palmitate-treated RAW264.7 cells transfected with pEGFP-N1 plasmid alone ($P < 0.01$; Fig. 4). By contrast, conditioned media of palmitate-treated RAW264.7 cells transfected with RIP140 siRNA significantly increased MIN6 cell viability at 24 and 48 h post-media treatment, relative to media from control palmitate-treated RAW264.7 cells transfected with scramble siRNA ($P < 0.01$; Fig. 4).

Conditioned media modifies insulin secretion in MIN6 cells. Levels of GSIS and BIS in MIN6 cells treated with

conditioned media from RAW264.7 macrophages prior to palmitate treatment were measured by ELISA. As depicted in Fig. 5, it was observed that GSIS was significantly decreased by conditioned media from palmitate-treated RAW264.7 cells, relative to the media of control RAW264.7 cells ($P < 0.01$; Fig. 5A). However, the insulin secretion index (ISI; defined as GSIS/BIS) did not differ significantly ($P = 0.121$; Fig. 5B). This suggests that pancreatic beta cells may increase insulin secretion with short-term increases in levels of free fatty acids by a compensatory mechanism.

It was also demonstrated that GSIS and ISI in MIN6 cells were significantly decreased by conditioned media from RAW264.7 cells transfected with RIP140 overexpression plasmid, relative to media of RAW264.7 cells transfected with pEGFP-N1 plasmid alone ($P < 0.01$; Fig. 5). By contrast, GSIS and ISI were significantly increased by conditioned media from RIP140-knockdown RAW264.7 cells ($P < 0.01$ and

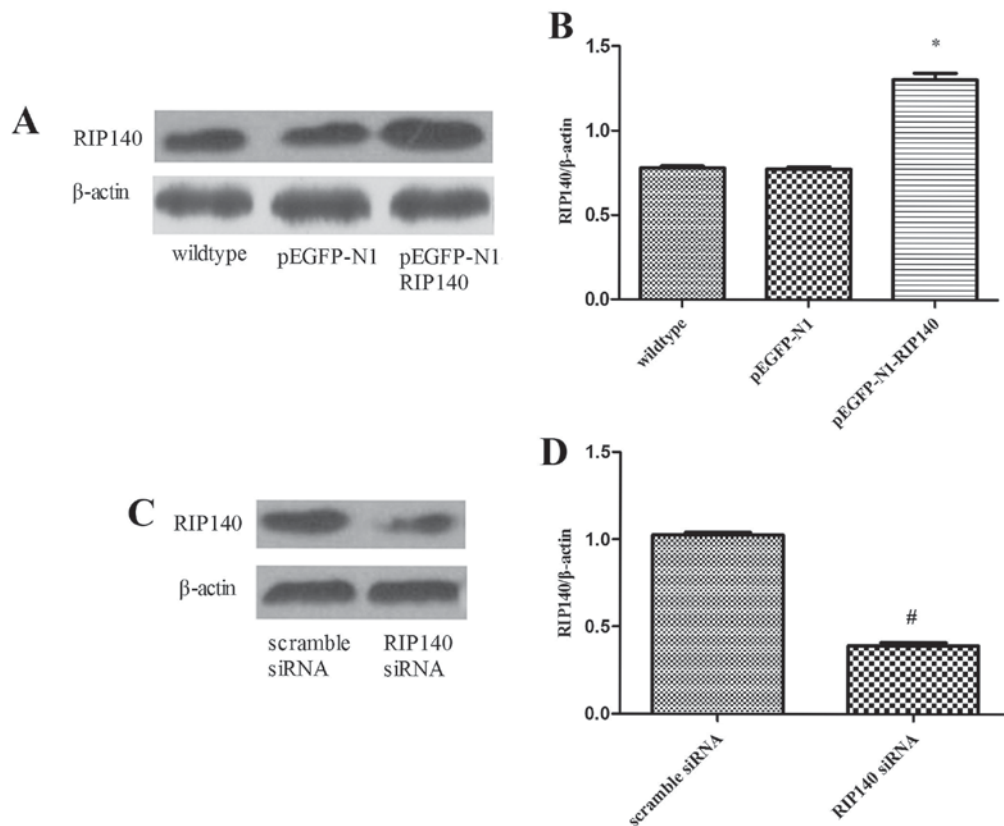


Figure 2. Level of RIP140 protein expression in RAW264.7 macrophages. RAW264.7 cells were transfected with (A and B) RIP140 overexpression plasmid or (C and D) RIP140 siRNA for 48 h and the level of RIP140 protein expression was determined by western blotting. β -actin was used as an internal control. Levels of target protein bands were quantified by AlphaEaseFC 4.0 software. Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$ vs. pEGFP-N1, # $P < 0.05$ vs. scramble siRNA. RIP140, receptor-interacting protein 140; siRNA, small interfering RNA; pEGFP-N1-RIP140; RIP140 overexpression plasmid; pEGFP-N1, control plasmid.

$P < 0.05$, respectively), relative to media of RAW264.7 cells transfected with scramble siRNA (Fig. 5).

Conditioned media modifies specific gene expression in MIN6 cells. To identify the effects of the conditioned media from palmitate-treated RAW264.7 macrophages on pancreatic beta cell function, the levels of mRNA for oxidative stress-related genes, namely PPAR γ coactivator 1 α (PGC-1 α), uncoupling protein 2 (UCP-2), nitric-oxide synthase 1 (iNOS1), insulin secretion related gene pancreatic and duodenal homeobox 1 (PDX-1), hepatic gluconeogenesis-related phosphoenolpyruvate carboxykinase (PEPCK) and proliferating cell nuclear antigen (PCNA) were determined by semiquantitative RT-PCR. It was observed that treatment with conditioned media from palmitate-treated RAW264.7 cells overexpressing RIP140 led to a significant downregulation in the levels of PGC-1 α ($P < 0.05$), PEPCK ($P < 0.05$) and PCNA ($P < 0.01$) mRNA in MIN6 cells, while significant upregulation in the mRNA of UCP-2 ($P < 0.01$), iNOS1 ($P < 0.01$) and PDX-1 ($P < 0.05$) was observed. However, in MIN6 cells treated with media from palmitate-treated, RIP140-knockdown RAW264.7 cells, significant decreases were observed in the levels of PGC-1 α ($P < 0.05$), PEPCK ($P < 0.05$) and PCNA ($P < 0.01$) mRNA were significantly increased, while levels of UCP-2 ($P < 0.01$), iNOS1 ($P < 0.05$) and PDX-1 ($P < 0.05$) mRNA (Fig. 6). These results suggest that conditioned media from palmitate-treated macrophages

influences the transcription of genes associated with oxidative stress, insulin secretion and glucose metabolism in pancreatic beta cells.

Conditioned media modifies JNK and ERK1/2 phosphorylation in MIN6 cells. Levels of p-JNK and p-ERK1/2 in MIN6 cells treated with conditioned media were measured by western blotting. As depicted in Fig. 7, it was demonstrated that conditioned media from palmitate-treated macrophages stimulated a significant increase in JNK and ERK1/2 phosphorylation, relative to conditioned media from control RAW264.7 macrophages ($P < 0.01$). It was also demonstrated that levels of p-JNK and p-ERK 1/2 were significantly increased in MIN6 cells by the conditioned media of palmitate-treated RAW264.7 cells transfected with RIP140 overexpression plasmid, relative to the media of RAW264.7 cells transfected with pEGFP-N1 plasmid alone ($P < 0.01$; Fig. 7). By contrast, levels of p-JNK and p-ERK 1/2 were significantly decreased by the conditioned media of palmitate-treated, RIP140-knockdown RAW264.7 cells, relative to the media of control RAW264.7 cells transfected with scramble siRNA ($P < 0.01$; Fig. 7).

Macrophages chemotax towards MIN6 cells. Chemotaxis of murine macrophages (RAW264.7 and peritoneal) towards MIN6 cells following treatment with conditioned media from palmitate-treated MIN6 cells (500 μ M palmitate), was determined by Transwell assays (Fig. 8A and B). For RAW264.7

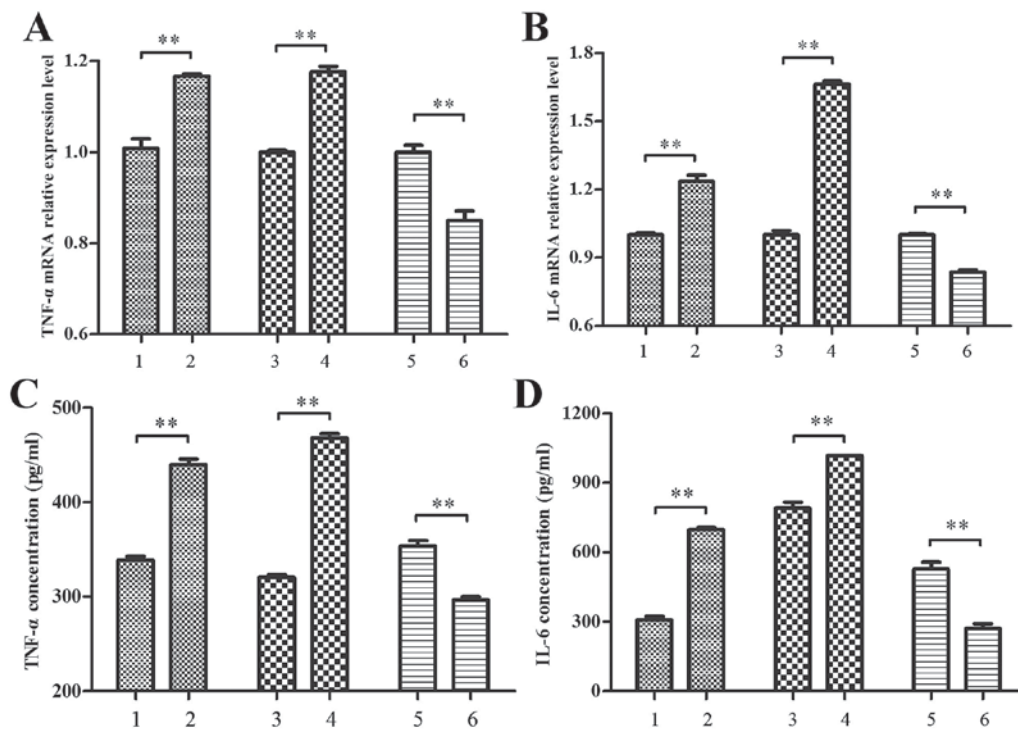


Figure 3. Palmitate promotes inflammatory cytokine expression via RIP140 in RAW264.7 macrophages. Levels of (A) TNF- α and (B) IL-6 mRNA expression were measured by semiquantitative reverse transcription polymerase chain reaction and the concentrations of (C) TNF- α and (D) IL-6 in RAW264.7 cell culture supernatant were determined by ELISA. The data represents the following experimental groups: i) RAW264.7 cells treated with bovine serum albumin, ii) RAW264.7 cells treated with 500 μ M palmitate, iii) RAW264.7 cells transfected with pEGFP-N1 control plasmid, iv) RAW264.7 cells transfected with pEGFP-N1-RIP140 plasmid, v) RAW264.7 cells transfected with scramble siRNA and vi) RAW264.7 cells transfected with RIP140 siRNA. Groups 3-6 were treated with 500 μ M palmitate post-transfection. Data are presented as the mean \pm standard deviation of three independent experiments. **P<0.01. RIP140, receptor-interacting protein 140; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6 siRNA, small interfering RNA.

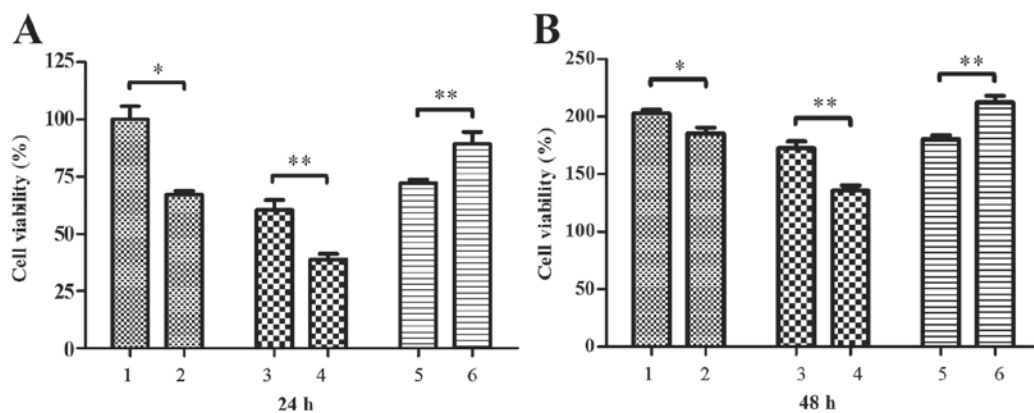


Figure 4. Conditioned media altered macrophage viability. MIN6 cells were incubated with conditioned media from RAW264.7 macrophage cultures and cell viability at (A) 24 h and (B) 48 h was determined by an MTT assay. Cell viability was measured relative to that of MIN6 cells 24 h after incubation with conditioned media from control macrophages treated with BSA. MIN6 cells were incubated with conditioned media obtained from the following: i) RAW264.7 cells treated with BSA, ii) RAW264.7 cells treated with 500 μ M palmitate, iii) RAW264.7 cells transfected with pEGFP-N1 control plasmid, iv) RAW264.7 cells transfected with pEGFP-N1-RIP140 plasmid, v) RAW264.7 cells transfected with scramble siRNA and vi) RAW264.7 cells transfected with RIP140 siRNA. Groups 3-6 were treated with 500 μ M palmitate post-transfection. Data are presented as the mean \pm standard deviation of three independent experiments. *P<0.05, **P<0.01. MIN6, murine pancreatic beta cell line; BSA, bovine serum albumin; siRNA, small interfering RNA.

macrophages, it was observed that cell chemotaxis towards MIN6 cells was significantly increased by conditioned media from palmitate-treated MIN6 cells, relative to media from control BSA-treated MIN6 cells (P<0.01; Fig. 8C). It was also demonstrated that RAW264.7 cell chemotaxis was significantly increased by conditioned media from palmitate-treated MIN6 cells transfected with RIP140 overexpression plasmid,

relative to media from palmitate-treated MIN6 cells transfected with pEGFP-N1 plasmid alone (P<0.01; Fig. 8C). By contrast, RAW264.7 cell chemotaxis was significantly decreased by the conditioned media of palmitate-treated, RIP140 siRNA-knockdown MIN6 cells, relative to the media of palmitate-treated MIN6 cells transfected with scramble siRNA (P<0.01; Fig. 8C).

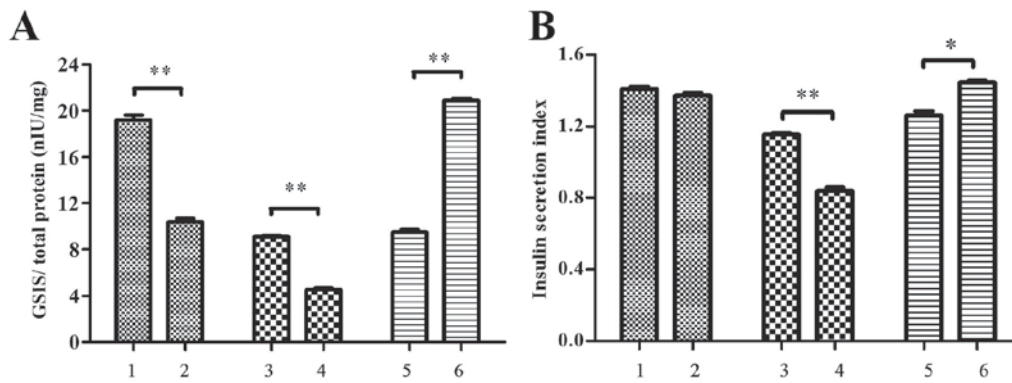


Figure 5. Conditioned media altered insulin secretion in MIN6 cells. MIN6 cells were incubated with conditioned media from RAW264.7 macrophage cultures and the level of insulin secretion (nIU/ml) in response to 2.8 and 25 mM glucose (BIS and GSIS, respectively) was determined by ELISA. Total protein concentration (mg/ml) was used as a control. The ISI was derived as GSIS/BIS. Measurements of (A) GSIS and (B) ISI are shown. MIN6 cells were incubated with conditioned media obtained from the following: i) RAW264.7 cells treated with bovine serum albumin, ii) RAW264.7 cells treated with 500 μ M palmitate, iii) RAW264.7 cells transfected with pEGFP-N1 control plasmid, iv) RAW264.7 cells transfected with pEGFP-N1-RIP140, v) RAW264.7 cells transfected with scramble siRNA and vi) RAW264.7 cells transfected with RIP140 siRNA. Groups 3-6 were treated with 500 μ M palmitate post-transfection. Data are presented as the mean \pm standard deviation of three independent experiments. * P <0.05, ** P <0.01. MIN6 cells, murine pancreatic beta cell line; BIS, basal insulin secretion; GSIS, glucose-stimulated insulin secretion; ISI, insulin secretion index; siRNA, small interfering RNA.

Similar results were observed for the peritoneal macrophages isolated from c57BL/6 mice. Cell chemotaxis towards MIN6 cells was significantly increased by conditioned media from palmitate-treated MIN6 cells, with and without RIP140 overexpression, while chemotaxis was significantly decreased by conditioned media from palmitate-treated MIN6 cells with knockdown of RIP140, relative to their respective control groups (P <0.01; Fig. 8D).

TNF- α and IL-6 concentration in the supernatant of MIN6 cells were also measured by ELISA following 500 μ M palmitate treatment. It was observed that levels of TNF- α and IL-6 were significantly increased in the supernatant of palmitate-treated MIN6 cells (P <0.01 and P <0.05, respectively) and in that of palmitate-treated cells overexpressing RIP140 (both P <0.05), relative to their respective control groups. By contrast, TNF- α and IL-6 were significantly decreased in the supernatant of palmitate-treated, RIP140-knockdown MIN6 cells, relative to control cells (P <0.05 and P <0.01, respectively; Fig. 8E and F).

Discussion

Inflammation is involved in insulin resistance within adipose and muscular tissue, thus promoting the onset of type II diabetes (26). In addition, pancreatic beta cell dysfunction serves a key role in pathophysiology of type II diabetes (27). However, whether crosstalk between macrophages and beta cells, with involvement of nuclear receptor cofactor RIP140, contributes to the onset of diabetes remains unknown. Previous results suggest that loss of RIP140 impairs inflammatory responses mediated by TLRs. RIP140 is considered to provide a platform for the stabilization of the NF- κ B cofactors, RelA, and CBP, thus regulating the expression of pro-inflammatory cytokines, including TNF- α , IL-1 β and IL-6 (19). In turn, high levels of inflammatory cytokines may disturb beta cell function and proliferation (28). The results of the present study suggested that the level of TNF- α and IL-6 expression in macrophages was increased by upregulation of RIP140 in the presence of palmitate, and that higher levels of TNF- α and IL-6 triggered an inflammatory response, thus contributing

to decreased cell viability and insulin secretion of beta cells. These findings indicate the effects of macrophage-derived cytokines on beta cell function and the involvement of RIP140. Specifically, RIP140 may promote the release of inflammatory cytokines under lipotoxic conditions, thus accelerating beta cell failure.

In the present study, under high palmitate conditions, conditioned media derived from macrophages with and without RIP140 overexpression were able to activate JNK and ERK1/2 signaling. Phosphorylation of JNK and ERK1/2 serve important roles in cell signal transduction events, including gene transcription, proliferation, apoptosis, differentiation and insulin signaling (29). In addition, it has been observed that palmitate activates the phosphorylation of ERK1/2 and p38 mitogen-activated protein kinase pathways and endoplasmic reticulum stress in cell such as skeletal muscle and mesenchymal stem cells (30). A number of inflammatory cytokines, including IL-1 β , IFN- γ and TNF- α , are considered to be activators of JNK and ERK1/2 signaling, which may lead to an increase in the levels of reactive oxygen species and compensatory generation of nitric oxide (31). Collectively, these data suggest that the high levels of inflammatory mediators identified in the conditioned media of palmitate-treated and RIP140-upregulated macrophages may have disturbed beta cell signal transduction and proliferation, thus contributing to a decrease in insulin secretion. However, the mechanistic involvement of JNK and ERK1/2 is not fully understood and studies evaluating their pharmacological inhibition are warranted.

High levels of inflammatory cytokines may also affect the transcription of specific genes, including PGC-1 α , UCP-2, iNOS1, PEPCK, PDX-1 and PCNA. As a transcriptional activator, PGC-1 α interacts with RIP140 and upregulates the expression of genes associated with mitochondrial metabolism (32). UCP-2 is a ubiquitously expressed mitochondrial carrier protein that uncouples the respiratory chain from ATP synthesis (33), with increases in UCP-2 possibly involved in reduced insulin secretion during oxidative stress (34). iNOS1 stimulates L-arginine to generate

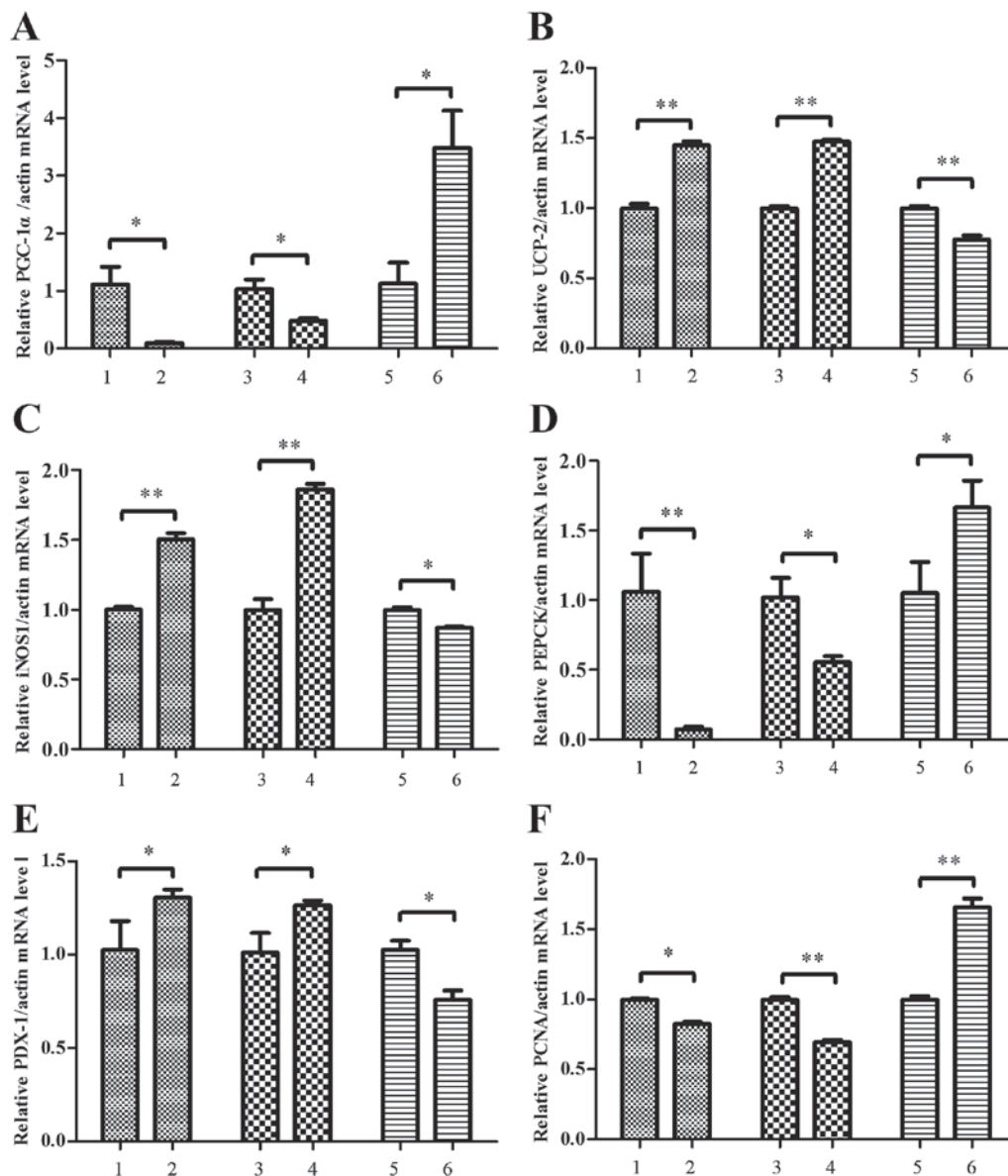


Figure 6. Conditioned media altered the expression of oxidative-stress related genes in MIN6 cells. MIN6 cells were treated with conditioned media from RAW264.7 macrophage cultures and the levels of (A) PGC-1 α , (B) UCP-2, (C) iNOS-1, (D) PEPCK, (E) PDX-1 and (F) PCNA mRNA expression in MIN6 cells were determined by semiquantitative reverse transcription polymerase chain reaction. MIN6 cells were incubated with conditioned media obtained from the following: i) RAW264.7 cells treated with bovine serum albumin, ii) RAW264.7 cells treated with 500 μ M palmitate, iii) RAW264.7 cells transfected with pEGFP-N1 control plasmid, iv) RAW264.7 cells transfected with pEGFP-N1-RIP140 plasmid, v) RAW264.7 cells transfected with scramble siRNA and vi) RAW264.7 cells transfected with RIP140 siRNA. Groups 3-6 were treated with 500 μ M palmitate post-transfection. Data are presented as the mean \pm standard deviation of three independent experiments. *P<0.05, **P<0.01. MIN6, murine pancreatic beta cell line; PPAR γ coactivator 1 α ; UCP-2, uncoupling protein 2; iNOS1, nitric-oxide synthase 1; PEPCK, phosphoenolpyruvate carboxykinase; PDX-1, pancreatic and duodenal homeobox 1; PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA.

NO, inducing an inflammatory reaction (35). In the present study, it was demonstrated that conditioned media from palmitate-treated and RIP140-upregulated macrophages, consisting of high levels of TNF- α and IL-6, induced a significant decrease in the level of PGC-1 α mRNA and a significant increase in the levels of UCP-2 and iNOS1 mRNA. These results indicate that RIP140 in macrophages induces cytokine secretion, which may then alter beta cell function and increase oxidative reaction stress. It has been demonstrated that RIP140 is necessary for LXR-dependent expression of PEPCK (16), as a rate-limiting enzyme in gluconeogenesis. In the current study, conditioned media containing high TNF- α and IL-6 was found to decrease PEPCK expression,

suggesting that inflammatory cytokines may contribute to carbohydrate metabolism disorder. Moore *et al* (36) demonstrated that palmitate reduces insulin secretion by downregulating the expression of the insulin transcription factors PDX-1 and MafA. In addition, it has been observed that LPS represses PDX-1 expression and insulin secretion by pathways involving TLR4 and NF- κ B (37). In the present study, conditioned media from macrophages overexpressing RIP140 prior to palmitate exposure significantly decreased GSIS and ISI, by upregulating PDX-1 mRNA expression. Fontes *et al* (38) suggested that Per-ARNT-Sim kinase may be independent from the ERK1/2 pathway and have no effect on MafA expression, suggesting that ≥ 3 independent signaling

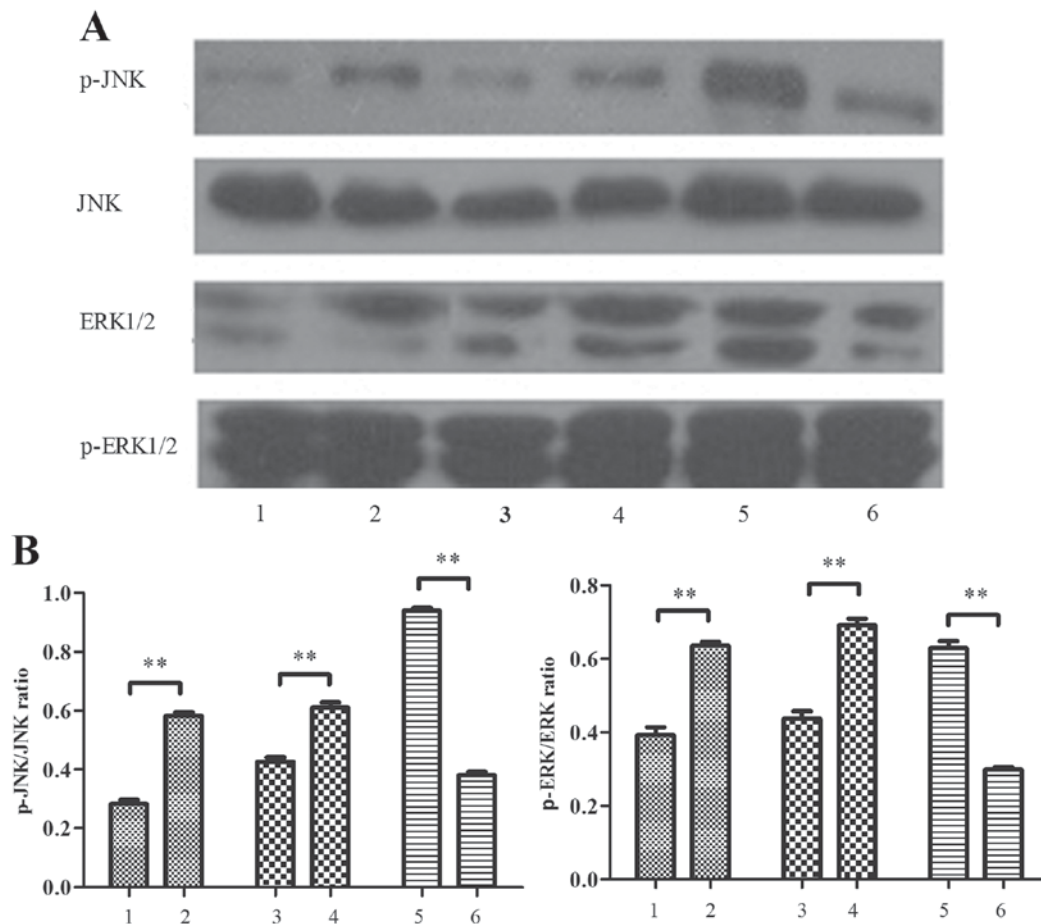


Figure 7. Conditioned media altered the levels of p-JNK and p-ERK1/2 in MIN6 cells. (A) Levels of p-JNK and p-ERK1/2 in MIN6 cells treated with conditioned media from RAW264.7 macrophages were determined by western blotting. (B) Relative protein levels were measured using AlphaEaseFC 4.0 software. MIN6 cells were incubated with conditioned media obtained from the following: i) RAW264.7 cells treated with BSA, ii) RAW264.7 cells treated with 500 μ M palmitate, iii) RAW264.7 cells transfected with pEGFP-N1 control plasmid, iv) RAW264.7 cells transfected with pEGFP-N1-RIP140 plasmid, v) RAW264.7 cells transfected with scramble siRNA and vi) RAW264.7 cells transfected RIP140 siRNA. Groups 3-6 were treated with 500 μ M palmitate post-transfection. Data are presented as the mean \pm standard deviation of three independent experiments. ** P <0.01. JNK, c-Jun N-terminal kinases; ERK, extracellular signal-regulated kinase; p-, phosphorylated; MIN6, murine pancreatic beta cell line; siRNA, small interfering RNA.

pathways may contribute to reduced insulin gene expression. Inflammatory cytokines may also affect PDX-1 expression by other pathways. In the current study, increasing levels of PDX-1 expression may have been a compensatory mechanism that occurred during stressful conditions, though insulin secretion did not increase accordingly and further study is warranted to verify this.

Macrophage infiltration of adipose and muscular tissue and hepatocytes may lead to peripheral insulin resistance (7-9). In the present study, chemotaxis assays were performed using Transwell plates. The conditioned media from MIN6 beta cells was added to the lower chamber and murine macrophages (peritoneal or RAW264.7) were added to the upper chamber. In assays of each cell type, cells migrated across the Transwell membrane, possibly due to the presence of a chemoattractant gradient. This is in accordance with the results of a previous study, in which the effects of a chemoattractant gradient were demonstrated by counting the number of macrophages on the lower surface of a Transwell membrane (39). In the present study, levels of TNF- α and IL-6 in the supernatant of MIN6 beta cells were significantly decreased by downregulation of RIP140 under

high palmitate conditions, and the chemotaxis of RAW264.7 macrophages towards beta cells was reduced. By contrast, upregulation of RIP140 in beta cells under high palmitate conditions led to enhanced RAW264.7 macrophage chemotaxis and levels of TNF- α and IL-6 in the supernatant were elevated. These results were analogous for peritoneal macrophages. Collectively, these findings suggest that RIP140 mediates inflammatory cytokine secretion in the presence of palmitate, which subsequently increases macrophage chemotaxis towards beta cells. However, macrophages are divided into two phenotypic categories: i) Anti-inflammatory (M2), which produce anti-inflammatory cytokines such as IL-10 and ii) pro-inflammatory (M1), which produce inflammatory cytokines such as IL-6 and TNF- α (40). Therefore, a range of pro- and anti-inflammatory cytokines may be secreted *in vivo*. Therefore, the cumulative effects of inflammatory cytokines *in vivo* warrant further study.

In conclusion, the present study indicated that in high concentrations of palmitate, expression of RIP140 in macrophages stimulates the release of inflammatory cytokines, which may subsequently suppress beta cell viability and insulin secretion. The underlying mechanism remains

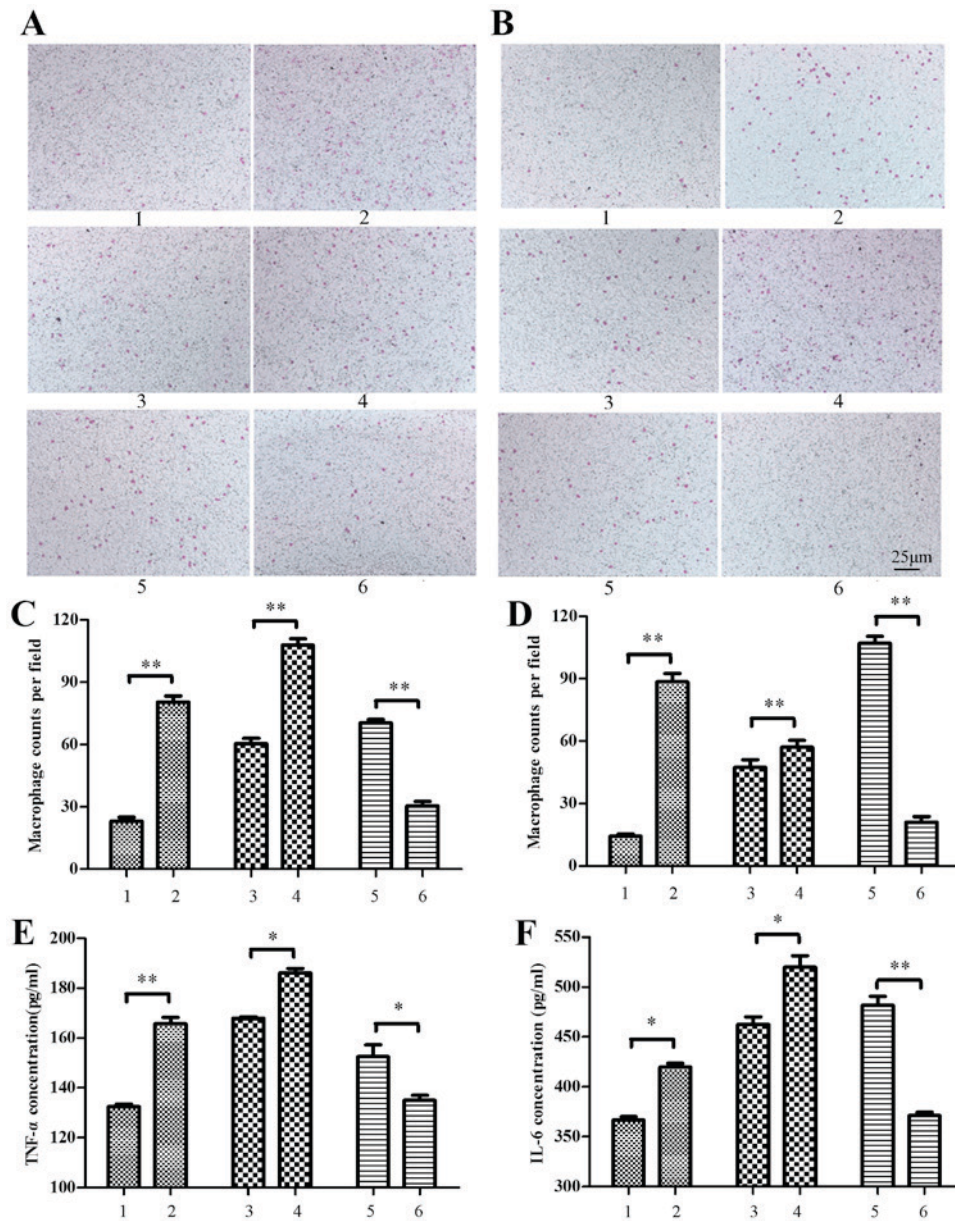


Figure 8. Macrophages chemotax towards MIN6 cells. Murine macrophages were treated with MIN6 cell culture supernatant for chemotaxis assays. (A) The RAW264.7 murine macrophage cell line and (B) peritoneal macrophages isolated from c57BL/6 mice were stained with crystal violet and viewed under a light microscope (magnification, x200). (C) RAW264.7 and (D) peritoneal macrophages were counted in six randomly selected fields. The concentration of (E) TNF- α and (F) IL-6 in the culture supernatant was also measured by ELISA. Supernatant for macrophage treatment was obtained from the following: i) MIN6 cells treated with bovine serum albumin, ii) MIN6 cells treated with 500 μ M palmitate, iii) MIN6 cells transfected with pEGFP-N1 control plasmid, iv) MIN6 cells transfected with pEGFP-N1-RIP140 plasmid, v) MIN6 cells transfected with scramble siRNA and vi) MIN6 cells transfected with RIP140 siRNA. Groups 3-6 were treated with 500 μ M palmitate post-transfection. Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.01$. MIN6, murine pancreatic beta cell line; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; siRNA, small interfering RNA.

unknown, though possibly involves regulation of specific gene expression and activation of JNK and ERK signaling. The present results also suggest that RIP140-mediated cytokine secretion under lipotoxic conditions may modulate macrophage chemotaxis towards beta cells. This may promote macrophage infiltration and local inflammation, ultimately leading to beta cell dysfunction.

Acknowledgements

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