LECT2 promotes inflammation and insulin resistance in adipocytes via P38 pathways

Tae Woo Jung¹, Yoon Hee Chung², Hyoung-Chun Kim³, A M Abd El-Aty⁴,⁵ and Ji Hoon Jeong⁶

¹Research Administration Team, Seoul National University Bundang Hospital, Gyeonggi, Republic of Korea
²Department of Anatomy, College of Medicine, Chung-Ang University, Seoul, Republic of Korea
³Neuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University, Chunchon, Republic of Korea
⁴Department of Pharmacology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
⁵Department of Medical Pharmacology, Medical Faculty, Ataturk University, Erzurum, Turkey
⁶Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul, Republic of Korea

Correspondence should be addressed to J H Jeong or A M Abd El-Aty: jhjeong3@cau.ac.kr or abdelaty44@hotmail.com

Abstract

Leukocyte cell-derived chemotaxin 2 (LECT2) is a recently identified novel hepatokine that causes insulin resistance in skeletal muscle by activating c-Jun N-terminal kinase (JNK), thereby driving atherosclerotic inflammation. However, the role of LECT2 in inflammation and insulin resistance in adipocytes has not been investigated. In this study, we report that LECT2 treatment of differentiated 3T3-L1 cells stimulates P38 phosphorylation in a dose-dependent manner. LECT2 also enhanced inflammation markers such as IκB phosphorylation, nuclear factor kappa beta (NF-κB) phosphorylation and IL-6 expression. Moreover, LECT2 treatment impaired insulin signaling in differentiated 3T3-L1 cells, as evidenced by the decreased levels of insulin receptor substrate (IRS-1) and Akt phosphorylation and reduced insulin-stimulated glucose uptake. Furthermore, LECT2 augmented lipid accumulation during 3T3-L1 cell differentiation by activating SREBP1c-mediated signaling. All these effects were significantly abrogated by siRNA-mediated silencing of P38, CD209 expression or a JNK inhibitor. Our findings suggest that LECT2 stimulates inflammation and insulin resistance in adipocytes via activation of a CD209/P38-dependent pathway. Thus, these results suggest effective therapeutic targets for treating inflammation-mediated insulin resistance.

Introduction

The main features of adipose tissue dysfunction in obesity and metabolic syndrome are chronic low-grade inflammation in adipose tissue and macrophage infiltration (Hajer et al. 2008, Bluher 2009). The large numbers of macrophages and T cells that infiltrate adipose tissue stimulate inflammation, which is thought to alter adipose tissue function, leading to metabolic disorders and systemic insulin resistance (Olefsky & Glass 2010, Sun et al. 2012). Various proinflammatory cytokines secreted by adipocytes and infiltrated macrophages have been shown to cause insulin resistance (Xu et al. 2003), suggesting that infiltrated macrophages in adipose tissue play an important role in obesity and that macrophage-mediated inflammatory responses may induce the development of obesity-related insulin resistance.

Leukocyte cell-derived chemotaxin 2 (LECT2) is a secretory protein that was discovered in a screen for novel neutrophil chemotactic factors (Yamagoe et al. 1996). LECT2...
is mainly produced by the liver, where it is released into the bloodstream (Yamagoe et al. 1998). LECT2-knockout mice demonstrated impaired homeostasis of hepatic natural killer T cells, resulting in severe hepatitis (Saito et al. 2004). Furthermore, LECT2 has shown anti-inflammatory and tumor-suppressive effects against β-catenin-induced hepatic carcinogenesis (Anson et al. 2012) and has been reported to attenuate activation of macrophage-induced bacterial sepsis via the CD209 receptor (Lu et al. 2013). More recently, however, LECT2 has been documented to promote atherosclerotic inflammatory processes through CD209-mediated signaling in human endothelial cells (Hwang et al. 2015a), implying that LECT2 also has pro-inflammatory properties. Lan et al. reported that LECT2 knockout increased insulin sensitivity in the muscles of mice. They also demonstrated that treatment of C2C12 cells with recombinant LECT2 impaired insulin signaling by acting on c-Jun NH2-terminal kinase (Lan et al. 2014). Although LECT2 has been demonstrated to attenuate fatty changes and insulin resistance in the liver, the effects of LECT2 on inflammation and insulin signaling in adipocytes remain to be elucidated (Hwang et al. 2015b).

The MAP kinase P38 stimulates adipocyte inflammation in β-adrenergic receptor-activated adipocytes through the hormone-sensitive lipase-mediated lipolytic pathway with proinflammatory cytokines (Mottillo et al. 2010). In addition, P38-ERK1/2-mediated signaling has been documented to play an essential role in IL-1β-induced insulin resistance in mouse adipocytes (Jager et al. 2007). Furthermore, P38 activation promotes visceral adiposity-related whole-body insulin resistance (Bluher et al. 2009). These findings suggest that P38 plays a crucial role in inflammation and insulin resistance in adipocytes. Therefore, we hypothesized that LECT2 causes inflammation and insulin resistance in adipocytes.

Here, we investigated (1) the effect of LECT2 on inflammation and insulin resistance in differentiated 3T3-L1 cells and (2) the mechanisms of LECT2-mediated stimulation of inflammation and insulin resistance through CD209/P38/NF-κB-dependent signaling.

Materials and methods

Cell culture, reagents and antibodies

3T3-L1 mouse preadipocytes (ATCC) were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen). Cells were cultured in a humidified atmosphere of 5% CO2 at 37°C. Differentiation was induced 48 h after reaching confluence (day 2) by cultivation in culture medium supplemented with 1 μM insulin, 0.5 mM IBMX (Sigma) and 0.5 μg/mL dexamethasone for 2 days and thereafter in DMEM supplemented with 1 μM insulin for another couple of days. Subsequently, cells were maintained in and refed every 2 days with fresh culture medium (6 days after initiation of differentiation). The differentiated 3T3-L1 cells were treated with 0–500 ng/mL LECT2 (Abcam) and 10 μM SP600125 (Abcam) for 24 h. Insulin (10 nM) was used to stimulate insulin signaling (insulin receptor substrate (IRS-1)) and Akt for 3 min. Anti-phospho IRS-1 (1:1000), anti-IRS-1 (1:1000), anti-phospho Akt (1:1000), anti-Akt (1:1000), anti-phospho P38 (1:1000), anti-P38 (1:1000), anti-phospho NF-κB p65, anti-NF-κB p65 (1:2500), anti-phospho IκB (1:1000), anti-IκB (1:1000), anti-phospho JNK (1:1000) and anti-JNK (1:1000) antibodies were purchased from Cell Signaling Technology. Anti-IL-6 (1:2000), anti-CD209 (1:1000) and anti-β-actin (1:5000) antibodies were obtained from Santa Cruz Biotechnology.

Western blot analysis

Differentiated 3T3-L1 cells were harvested and lysed with PRO-PREP buffer (Intron Biotechnology, Seoul, Republic of Korea) for 60 min at 4°C to generate protein extracts. Proteins (30 μg) were resolved by SDS-PAGE on 12% gels and transferred to nitrocellulose membranes (Amersham Bioscience). The membranes were probed with the indicated primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Immunoreactive signals were detected using an ECL kit (Amersham Bioscience).

RNA extraction and quantitative real-time PCR

Total RNA was isolated from harvested hepatocytes using TRIzol reagent (Invitrogen). Gene expression was measured by quantitative real-time PCR (qPCR) using a fluorescent TaqMan 5’-nuclease assay with an Applied Biosystems 7000 sequence detection system. The qPCR reactions included cDNA, 2× TaqMan Master Mix and 20× premade TaqMan gene expression assays (Applied Biosystems). The thermocycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR primer mixes for mouse SCD1 (MP201188; Sino Biological) were used. Mouse SREBP1c mRNA was quantified using the following primers: 5’-cgaagctgctccccgttag-3’
and 5'-gtttgtgatagctggagca-3'. Mouse β-actin mRNA was quantified as an endogenous control, using the following primers: 5’-CGATGCTCCCGGGCTGTAT-3’ and 5’-TGGGTACTTCCAGGGTACC-3’.

**Transfection with siRNAs for gene silencing in cells**

siRNA oligonucleotides (20nM) specific for P38 (a; SC-29434, b; SC-39117, c; SC-39014) and CD209 (a; SC-42857, b; SC-42858, c; SC-142186) were purchased from Santa Cruz Biotechnology. To suppress gene expression, cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief, 3T3-L1 preadipocytes were differentiated, grown to 60–70% confluence, and then serum starved for 12 h. The cells were then transfected with validated siRNA or scrambled siRNA at a final concentration of 20nM in the presence of the transfection reagent. At 24 h after transfection, the cells were harvested for protein extraction and additional analyses. We performed transfection twice using DeliverX Plus (Panomics, Freemont, CA, USA) to examine the effect of LECT2 on lipid accumulation and lipogenic genes mRNA expression during differentiation period (Supplementary Fig. 1, see section on supplementary data given at the end of this article). In brief, 3T3-L1 preadipocytes were cultured in 2% bovine calf serum (BCS) for 6h before transfection. The cells were then transfected with validated siRNA or scrambled siRNA at a final concentration of 20nM in the presence of the transfection reagent (day 1). At 72 h after the first transfection, the cells were transfected with siRNA at a final concentration of 10nM in the presence of the transfection reagent again (day 2). At 72–96 h after second transfection, differentiated 3T3-L1 cells were harvested for protein and mRNA extraction and stained with Oil red-O (day 6).

**Measurement of glucose uptake and acetyl-CoA and ATP content**

Glucose uptake levels were measured using the Glucose Uptake Assay Kit (Abcam). Briefly, proliferating and differentiating 3T3-L1 cells were seeded at 5 × 10⁵ cells/well in black-walled clear bottom 96-well plates (Corning) in DMEM containing 10% FBS. Upon reaching a confluency of 95%, differentiation was induced with differentiating media. After 48h, media was changed to media containing 0–500 ng/mL LECT2 for 24 h. Following treatment, media was removed from wells and cells were treated with 10 nM insulin and 1 mM 2-deoxyglucose (2-DG) for 30 min. Afterward, plates were centrifuged for 1 min at 30 g and incubated for 1 h at room temperature. After 2-DG taken up by the cells was extracted using the Glucose Uptake Assay Kit extraction buffer, 2-DG uptake levels were measured at a wavelength of OD 412 nm on a BioTek Synergy HT plate reader (BioTek Instruments). Intracellular levels of acetyl-CoA were measured in differentiated 3T3-L1 cells using a PicoProbe Acetyl CoA Assay Kit (Abcam), while intracellular ATP levels were measured using an ATP Assay Kit (Abcam) according to the manufacturer's instructions.

**Oil red-O staining and triglyceride (TG) assay**

Differentiated 3T3-L1 cells were stained using the Oil Red-O method to measure the accumulated cellular neutral lipids, including TG. After fixation with 10% formalin for 40 min, adipocytes were stained with the Oil Red-O solution (Sigma) for 1 h at 37°C. Oil Red-O stained TG content was quantified by adding isopropanol to each sample. The mixtures were gently agitated at 25°C for 8 min. At last, 100 µL isopropanol-extracted samples were analyzed by a spectrophotometer at 510 nm.

**Statistical analysis**

Results are presented as the fold of the highest values (mean±s.e.m.). All experiments were performed at least three times. Student's t test or two-way ANOVA was used for statistical analysis. All analyses were performed using the SPSS/PC statistical program (version 12.0 for Windows; SPSS).

**Results**

**LECT2 induces inflammation in differentiated 3T3-L1 cells through a CD209/P38-dependent pathway**

Since P38 is a key regulator of the pro-inflammatory response (Schieven 2005, Olson et al. 2007, Ulivi et al. 2008), we next evaluated the effect of LECT2 on P38 phosphorylation in differentiated 3T3-L1 cells. We found that LECT2 stimulated P38 phosphorylation in a dose-dependent manner (Fig. 1A). LECT2 increases inflammatory markers, such as phosphorylation of NF-κB and IκB and TNFα and MCP-1 release from differentiated 3T3-L1 cells (Figs 1B and 2A). We then investigated whether LECT2-induced P38 phosphorylation is associated with the stimulation of inflammation in differentiated 3T3-L1 cells. The receptor CD209 is required for LECT2 to elicit

---

References:
- Schieven 2005
- Olson et al. 2007
- Ulivi et al. 2008
a pro-inflammatory response (Lu et al. 2013, Hwang et al. 2015a, Shen et al. 2016). Suppression of CD209 by siRNA abrogated the effect of LECT2 on P38 phosphorylation (Fig. 1A). siRNA-mediated suppression of both P38 and CD209 abrogated the effects of LECT2 on inflammation (Figs 1B and 2A). To exclude the off target effects, we also tested different P38 and CD209 siRNAs and demonstrated same results (Fig. 2B).

**LECT2 impairs insulin signaling and insulin-stimulated glucose uptake in differentiated 3T3-L1 cells through a CD209/P38-mediated pathway**

LECT2 treatment impaired insulin-stimulated IRS-1 and Akt phosphorylation (Fig. 3A) and also decreased glucose uptake (Fig. 3B) in differentiated 3T3-L1 cells. We also examined whether LECT2-induced P38 phosphorylation affected the development of insulin resistance in differentiated 3T3-L1 cells. As shown in Fig. 3, the effects of LECT2 on insulin resistance were significantly blocked by siRNA-mediated silencing of P38 and CD209. To exclude the off target effects, we also tested different P38 and CD209 siRNAs and demonstrated same results (Fig. 3C).

**LECT2 stimulates lipogenesis in 3T3-L1 pre-adipocytes via CD209/p38-dependent signaling**

LECT2 has been reported to be associated with SREBP1-mediated hepatic lipogenesis (Hwang et al. 2015b). Therefore, we examined the effect of LECT2 on the differentiation of 3T3-L1 cells. Microscopic evaluation demonstrated that treatment of 3T3-L1 cells with recombinant mouse LECT2 markedly enhanced lipid accumulation (Fig. 4A) and upregulated the mRNA expression of lipogenesis-related genes such as SREBP1c and SCD1 (Fig. 4B). Since P38 has been reported to play an essential role in the differentiation of 3T3-L1 cells (Engelman et al. 1998), we next evaluated the roles of P38 and its receptor CD209 in LECT2-induced lipid accumulation. siRNAs targeting P38 or CD209 significantly reversed these changes (Fig. 4).

**LECT2 induces inflammation and insulin resistance through JNK-mediated pathway**

It has been reported that JNK contributes to LECT2-induced inflammation and insulin signaling impairment (Lan et al. 2014, Hwang et al. 2015a). Therefore, we next...
examined the effects of LECT2 on JNK phosphorylation in differentiated 3T3-L1 cells. LECT2 stimulated JNK phosphorylation in a dose-dependent manner (Fig. 5A). Furthermore, suppression of JNK by SP600125, a specific JNK inhibitor reversed LECT2-induced NF-κB and IκB phosphorylation (Fig. 5B) and impairment of insulin-stimulated IRS-1 and Akt phosphorylation (Fig. 5C).

**LECT2 does not decrease fatty acid oxidation**

It has been reported that incomplete fatty acid oxidation causes insulin resistance (Zhang et al. 2010). Therefore, we examined the effect of LECT2 on fatty acid oxidation. As shown in Fig. 6, LECT2 did not affect intracellular acetyl-CoA and intracellular ATP levels in differentiated 3T3-L1 cells (Fig. 6).
Discussion

Obesity induces a low-grade chronic inflammatory response and stimulates systemic inflammation by elevating proinflammatory cytokine expression. Obesity is also associated with a low-grade chronic inflammatory condition in adipose tissue that is accompanied by infiltration of immune cells such as neutrophils and macrophages (Chawla et al. 2011, Huh et al. 2014). Adipose tissue and invasive macrophages secrete a variety of proinflammatory cytokines that affect further immune cell infiltration, leading to impaired liver and skeletal metabolic homeostasis and ultimately systemic insulin resistance. (Hotamisligil et al. 1993, Weisberg et al. 2003, Xu et al. 2003). Furthermore, adipose tissue dysfunction caused by chronic inflammation increases the release of free fatty acids (FFAs), thus elevating their serum levels. This is important because FFAs are known to contribute to insulin resistance by interfering with insulin signaling (Boden 1997). Impaired insulin signaling leading to insulin resistance is a main characteristic of metabolic disorders and type 2 diabetes.

LECT2 has been previously documented to show potent pro-inflammatory effects in macrophages (Lu et al. 2013, Shen et al. 2016), endothelial cells (Hwang et al. 2015a) and hepatocytes (Anson et al. 2012). Moreover, LECT2 has been shown to induce insulin resistance in the liver (Hwang et al. 2015b) and in skeletal muscle (Lan et al. 2014). The aim of this study was to investigate the effects of LECT2 on inflammation and insulin resistance in adipocytes. We found that treatment of differentiated 3T3-L1 cells with LECT2 resulted in an NF-κB-mediated pro-inflammatory response. We also found that LECT2 treatment significantly induced the pro-inflammatory cytokines TNFα and MCP-1. These results suggest that the pro-inflammatory effects of LECT2 in adipocytes are likely mediated by stimulation of NF-κB-mediated signaling and activation of downstream molecules.

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway plays a central role in the regulation of various processes...
Lect2 promotes inflammation and insulin resistance

In conclusion, we investigated the effects of lect2 on fully differentiated 3T3-L1 cells to determine whether lect2 promotes the inflammatory response in adipocytes and whether it is involved in obesity-induced low-grade chronic inflammation in adipose tissue. This is the first report that lect2 induces pro-inflammatory processes and decreases insulin sensitivity through CD209/P38 and JNK-mediated activation of NF-kB-dependent signaling. Furthermore, we found that lect2 stimulates lipogenesis in 3T3-L1 cells. All these effects of lect2 on inflammation and insulin resistance are regardless of the impairment of fatty acid oxidation (Fig. 7). Suppression of pro-inflammatory cytokines such as IL-6, TNFα and IL-1β, which are secreted by immune cells (Weisberg et al. 2003, Lumeng et al. 2008) and adipocytes (Mohamed-Ali et al. 1997, de Luca & Olefsky 2008), may have therapeutic role in the regulation of energy homeostasis in skeletal muscle (Puigserver et al. 2001), hepatocytes (Cao et al. 2005) and adipocytes (Cao et al. 2001). Since P38 has been reported to suppress SREBP1, a central lipogenic gene in hepatocytes (Foretz et al. 1999), we further examined the involvement of P38 in lect2-induced lipogenesis in 3T3-L1 adipocytes. Unexpectedly, lect2 upregulated the mRNA expression of lipogenic genes in a CD209/P38-dependent fashion. Further investigation is required to characterize the underlying mechanisms by which lect2 induces differentiation of 3T3-L1 cells through P38-dependent pathways.

In the present study, we suggest CD209/P38-dependent pathway as a novel mechanism of the effects of lect2 on inflammation and insulin resistance in differentiated adipocytes. In addition, lect2 has been reported to induce activation of NF-kB in macrophages (Shen et al. 2016) and pro-inflammatory cytokines through CD209 receptor-dependent JNK pathway in human endothelial cells (Hwang et al. 2015a). Lect2 also impairs insulin signaling in hepatocytes (Hwang et al. 2015b) and skeletal muscle through JNK-mediated pathway (Lan et al. 2014). Therefore, in the current study, we investigated the effects of lect2 on JNK phosphorylation in differentiated adipocytes. We found that lect2 stimulated JNK phosphorylation. Moreover, suppression of JNK activity by a specific JNK inhibitor abrogated the effects of lect2 on inflammation and insulin resistance. These results suggest that JNK plays an important role in lect2-induced inflammation and insulin resistance. These results rely on intracellular signaling such as cell survival, proliferation and differentiation (Sinor & Lillien 2004). Akt is suppressed under obese conditions because serine residues in IRS-1 are phosphorylated, thereby impairing the IRS-1-induced PI3K-mediated pathway (Gao et al. 2002) and inhibiting IRS-1-mediated Akt activation (Zick 2005, Guo 2013). Phosphorylation of Akt on Ser473 has been associated with a stronger insulin response in the skeletal muscle of lect2-knockout mice compared to wild-type mice (Lan et al. 2014). In the current study, we found that lect2 treatment significantly suppressed insulin signaling and insulin-stimulated glucose uptake. Furthermore, siRNA-mediated suppression of P38 and CD209 markedly abrogated these changes. These results imply that lect2 impairs insulin sensitivity in adipocytes through a CD209/P38-mediated pathway.

In this study, we found that lect2 treatment increased the mRNA expression of lipogenic genes (SREBP1c and SCD1), accompanied by increased lipid accumulation in pre-adipocytes. These results suggest that lect2 may cause obesity and are consistent with a previous study that reported body weight loss in lect2-knockout mice (Lan et al. 2014). P38-mediated signaling plays a crucial role in the regulation of energy homeostasis in skeletal muscle (Puigserver et al. 2001), hepatocytes (Cao et al. 2005) and adipocytes (Cao et al. 2001). Since P38 has been reported to suppress SREBP1, a central lipogenic gene in hepatocytes (Foretz et al. 1999), we further examined the involvement of P38 in lect2-induced lipogenesis in 3T3-L1 adipocytes. Unexpectedly, lect2 upregulated the mRNA expression of lipogenic genes in a CD209/P38-dependent fashion. Further investigation is required to characterize the underlying mechanisms by which lect2 induces differentiation of 3T3-L1 cells through P38-dependent pathways.
effects on systemic low-grade chronic inflammation related to metabolic syndrome. Therefore, our findings suggest that LECT2 may be an effective therapeutic target for obesity-associated metabolic disorders, including insulin resistance.

**Supplementary data**
This is linked to the online version of the paper at https://doi.org/10.1530/JME-17-0267.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**
This work was supported by a grant from the National Research Foundation (NRF), which is funded by the Ministry of Science, ICT and Future Planning (2016R1C1B2012674), Republic of Korea.

**Author contribution statement**
T W J, Y H J, H C K, A A E, and J H J: substantial contribution to conception and design; T W J and J H J: acquisition of data, analysis and interpretation of data; H C K: drafting and revising of the manuscript. All authors approved the final version of the manuscript. T W J and J H J are responsible for the integrity of the work as a whole.

**References**


Boden G 1997 Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* **46** 3–10. (https://doi.org/10.2337/ diab.46.1.3)


Motillo EF, Shen XJ & Granneman JG 2010 beta3-adrenergic receptor induction of adipocyte inflammation requires lipolytic activation of...


Received in final form 20 March 2018
Accepted 12 April 2018
Accepted Preprint published online 12 April 2018