Deficiency of LRP1 in Mature Adipocytes Promotes Diet-induced Inflammation and Atherosclerosis – Brief Report

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Online Supplement

Materials and Methods

Animals

Adipocyte-specific LRP1 knockout (*adLrp1*$^{-/-}$) mice in C57BL/6 background were generated by mating C57BL/6 *Lrp1*$^{flox/flox}$ mice with C57BL/6 *aP2-cre* transgenic mice.[1] The resulting *adLrp1*$^{-/-}$ mice lack LRP1 expression specifically in mature white and brown adipocytes with no impairment of LRP1 expression in other cell types including the macrophages.[1] The *Ldlr*$^{-/-}$ mice in C57BL/6 background were obtained from Jackson Laboratories (stock #2207) and maintained as a breeding colony. Mice were fed a basal rodent chow 5058 PicoLab Mouse Diet 20 (9% fat by weight; LabDiet) with free access to food and water during the maintenance period. For experiments, age-matched male *adLrp1*$^{-/-}$ and *adLrp1*$^{+/+}$ mice were fed a Western type diet containing 21.1% fat (42% kcal) and 0.15% cholesterol by weight (TD88137; Harlan-Teklad, Madison, WI, USA) for 16 weeks when fat mass as percent of body weight reached a new steady state in both *adLrp1*$^{+/+}$ and *adLrp1*$^{-/-}$ mice.[1] The animals were euthanized at the end of the feeding period to harvest adipose tissues for characterization. To determine the influence of adipocyte-expressed LRP1 in atherosclerosis, 2 mg of white perivascular adipose tissues (PVAT) were obtained from the inferior margin of the lesser curvature of the thoracic aorta near the aortic arch of age-matched male *adLrp1*$^{+/+}$ and *adLrp1*$^{-/-}$ mice and then transplanted to the left common carotid arteries of *Ldlr*$^{-/-}$ mice according to the procedure described previously.[2] The recipient mice were allowed to recover for 4 weeks on standard mouse chow and then placed on the Western diet for an additional 8 weeks prior to sacrifice for tissue harvesting. All procedures and animal care were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Adipose tissue characterization

Epididymal and perivascular adipose tissues were harvested from Western diet-fed *adLrp1*$^{+/+}$ and *adLrp1*$^{-/-}$ mice and fixed in isotonic neutral 4% paraformaldehyde solutions prior to embedding in paraffin. Three 5-µm sections from different levels of the tissue from each mouse were analyzed for adipocyte size after staining with hematoxylin and eosin. Crown-like structures were identified based on aggregates of nucleated cells surrounding individual adipocytes, and then quantified based on counting the total number compared with total number of adipocytes (at least 500 adipocytes per mouse) in each section as described previously.[3] PVAT sections were also used for immunofluorescent staining with anti-CD68 antibodies (1:200 dilution; Abcam, catalog number ab955) and anti-rat secondary antibodies conjugated to Alexa488. All of the immunofluorescence sections were counterstained with 4,6-diamidino-2-phenylindole.

Atherosclerosis lesion analysis

The *Ldlr*$^{-/-}$ mice were fed the Western diet for 8 weeks after transplantation of PVAT from *adLrp1*$^{+/+}$ and *adLrp1*$^{-/-}$ donor mice as described above. At the end of the feeding period, the

mice were anesthetized and perfused with buffered saline solution followed by perfusion with 4% paraformaldehyde. Both the right and left carotid arteries were harvested from the animals after perfusion and then processed for frozen section preparation.[4] Cryosections of 10-μm thickness were stained with Oil Red O and counter-stained with hematoxylin. The sections were examined with an Olympus BX61 microscope and mean lesion area was calculated from ImageJ (NIH) analysis of digitalized images obtained from 14 sections per mouse with 13 mice in each group.

RNA quantification

Total RNA was extracted from adipose tissues with TRIzol reagent (Invitrogen) and treated with Turbo DNase (Applied Biosystems/Ambion, Austin, TX) prior to use as template for cDNA synthesis with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time PCR was performed on a StepOnePlus Fast Thermocycler using Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) with primer sequences as shown in the Table in online supplement. The mRNA expression levels relative to the expression of the cyclophilin housekeeping gene were calculated using the ΔCT qPCR data analysis method. Results were reported based on comparison to the mean expression levels of each mRNA in corresponding $adLrp1^{+/+}$ tissues.

Statistics

All results are expressed as means ± standard error with sample size of N=6 in each group for adipose tissue inflammation experiments and N=13 in each group for atherosclerosis analysis. Sample size was chosen based on previous results indicating that N=6 is sufficient to yield statistical significant differences in inflammation gene expression between adipose tissues from mice with different genotypes,[3] whereas a minimum of 8-10 mice in each group are required for statistical evaluation of carotid lesions.[5] Statistical analysis was performed using SigmaPlot version 13.0 software (SysStat Software, San Jose, CA). Normality was examined using the Shapiro-Wilk's test. Data with equal variance based on Levene's analysis were evaluated by Student's *t* test and data with unequal variance were evaluated using the Mann-Whitney test. Differences at $P < 0.05$ were considered statistically significant.

REFERENCES

1. Hofmann SM, Zhou L, Perez-Tilve D, Greer T, Grant E, Wancata L, Thomas A, Pfluger PT, Basford JE, Gilham D, Herz J, Tschop MH, Hui DY. Adipocyte LDL receptor-related protein-1 expression modulates postprandial lipid transport and glucose homeostasis in mice. *J Clin Invest*. 2007;117:3271-3282
2. Manka D, Chatterjee TK, Stoll LL, Basford JE, Konaniah ES, Srinivasan R, Bogdanov VY, Tang Y, Blomkalns AL, Hui DY, Weintraub NL. Transplanted perivascular adipose tissue accelerates injury-induced neointimal hyperplasia. Role of monocyte chemoattractant protein-1. *Arterioscler Thromb Vasc Biol*. 2014;34:1723-1730
3. Kuhel DG, Konaniah ES, Basford JE, McVey C, Goodin CT, Chatterjee TK, Weintraub NL, Hui DY. Apolipoprotein E2 accentuates postprandial inflammation and diet-induced obesity to promote hyperinsulinemia in mice. *Diabetes*. 2013;62:382-391
4. Waltmann MD, Basford JE, Konaniah ES, Weintraub NL, Hui DY. Apolipoprotein E receptor-2 deficiency enhances macrophage susceptibility to lipid accumulation and cell death to augment atherosclerotic plaque progression and necrosis. *Biochim Biophys Acta*. 2014;1842:1395-1405
5. Zhu B, Reardon CA, Getz GS, Hui DY. Both apolipoprotein E and immune deficiency exacerbate neointimal hyperplasia after vascular injury in mice. *Arterioscler. Thromb. Vasc. Biol.* 2002;22:450-455

Table

Primer sequences used for quantitative real-time PCR analysis of RNA

Gene Name	Sense Primer Sequence	Antisense Primer Sequence
Leptin	GCAGCACACGATGGAAGCACTTAT	TTGGGCAGACCCATCAATAGGATT
Adiponectin	GTTGCAAGCTCTCCTGTTCC	CCAAGAAGACCTGCATCTCC
Resistin	AGACTGCTGTGCCTTCTGGG	CCCTCCTTTTCCTTTTCTTCCTTG
MCP1/CCL2	CTTCCTCCACCACCATGCA	CCAGCCGGCAACTGTGA
CD68	TTTCTCCAGCTGTTCACCTTGA	CCCGAAGTGTCCCTTGTCA
IL6	TTCCATCCAGTTGCCTTCTTG	GGGAGTGGTATCCTCTGTGAAGTC
TNFα	ATCCGCGACGTGGAACTG	ACCGCCTGGAGTTCTGGAA
Cyclophilin A	TCATGTGCCAGGGTGGTGAC	CCATTCAGTCTTGGCAGTGC