SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 17: 5958-5963, 2018

Toll-like receptor 2 mediates deposition of collagen I in adipose tissue of high fat diet-induced obese mice

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Received March 4, 2017; Accepted November 23, 2017

DOI: 10.3892/mmr.2018.8590

Abstract. Obesity is marked by deposition of collagen I in adipose tissue. Toll like receptor (TLR)2 is involved in lipid metabolism, however the association between TLR2 and collagen I remains unclear. The present study was designed to investigate the effect of TLR2 knockout on collagen I in adipose tissue in obese mice. TLR2 knockout and C57BL/6J mice (aged 4 weeks) were fed normal chow or a high-fat-diet for 16 weeks. Compared with adipose tissue from lean controls, that from C57BL/6J mice fed a high-fat diet had increased levels of collagen I, TIMP1 and TGFβ1 and lower levels of MMP1. However, adipose tissue from TLR2 knockout mice fed a high-fat diet revealed decreased levels of collagen I, TIMP metallopeptidase inhibitor (TIMP)1, and transforming growth factor (TGF)β1, in addition to increased levels of matrix metallopeptidase (MMP)1. These findings suggest that, in the adipose tissue of obese mice, TLR2 is involved in the metabolism of collagen I and may exhibit a role in the metabolism of MMP1, TIMP1 and TGFβ1.

Introduction

The rates of overweight and obesity have increased over recent decades (1-3) with the incidences of pre-diabetes and diabetes (4,5). Obesity is characterized by hyperlipidemia, proliferation and hypertrophy in adipocytes with the deposition of collagen I in adipose tissue (6-9). Deposition of collagen I in adipose tissue is associated with insulin sensitivity (10). Previous reports have demonstrated an inverse correlation between adipocyte size and collagen I deposition, which is a hallmark of fibrosis (11-13). These findings suggest a role for fibrosis in negatively regulating adipocyte hypertrophy.

Over-expression of collagen I adipose tissue in obesity has been well studied; however, the effect of TLR2 on collagen I remains unclear. TLR2, along with TLR1 and TLR6, recognizes a wide variety of pathogen-associated molecules including lipoproteins, peptidoglycans, lipophilic acids, zymosan, mannan, and free fatty acid (FFA) (14). Plasma FFA could activate TLR2 (15) and downstream inflammatory factors, which regulate MMPs and TIMPs (8). MMPs and TIMPs regulate the deposition of collagen I, which is one of the most important components of the extracellular matrix (ECM) (16,17). ECM levels in adipose tissue are also increased in human and murine obesity (10,13,18,19). In the pesent study, the role of TLR2 in the deposition of collagen I in adipose tissue was explored in TLR2 gene knockout mice fed a high-fat diet.

Materials and methods

Animals and groups. Male C57BL/6J mice [SCXK (Jing) 2009-0015] and age-matched TLR2 gene knockout mice (022507) were purchased from HFK (Beijing HFK Bioscience Co., Ltd., Beijing, China) and Jackson Laboratory (Bar Harbor, ME, USA), respectively. Mice were divided in 4 groups: Normal control mice (C57BL/6J) fed common chow (NC), TLR2 knockout mice fed common chow (TK), C57BL/6J mice fed a high-fat (60%) diet to model obesity (OB), and TLR2 knockout mice fed a high-fat diet to model obesity (TO). To monitor weight gain, mice were weighed every 2 weeks, especially the following 3 important time points. Prior to the start of the experiment (4 weeks of age), after the 4th week of the experiment (8 weeks of age) and at the end of the experiment (20 weeks of age). All animal experiments were approved by the Ethics Committee for Experimental Research, Jinzhou Medical University.

Biochemical index measurements. Blood samples were collected from hearts prior to sacrifice. Fasting plasma glucose (FPG) and levels of triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HD), low-density lipoprotein (LDL) and FFA were measured.

Hematoxylin & eosin and Masson staining. Adipose tissue was collected from fresh sacrificed mice and fixed with 4%

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Key words: obesity, collagen I, Toll-like receptor 2, inflammation factors, Matrix metalloproteinase 1, Tissue inhibitor of metalloproteinase 1

paraformaldehyde for 48 h before paraffin sections were obtained. H&E and Masson staining were conducted according to the manufacturer's protocol (Wanleibio Co., Ltd., Shenyang, China).

Immunohistochemistry. Section, 5 μm in thickness, were incubated with rabbit-anti-mouse collagen I (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) primary antibody overnight at 4˚C. Then slides were washed and incubated with 1:5,000 goat-anti-rabbit secondary antibody (Absci, Nanjing, China) for 4 h. A DAB kit (OriGene Technologies, Inc., Beijing, China) was used to bind secondary antibody, according to the manufacturer's protocol.

Western blotting. Total proteins in adipose tissue were extracted with RIPA (with 1% PMSF). BCA assay was used to detect protein concentrations in middle extracting solution. Targeting proteins were separated by SDS-PAGE, transferred to PVDF membranes and blocked with skim milk for 2 h at room temperature. Rabbit anti-mouse primary antibodies to collagen I (Wanleibio Co., Ltd.), MMP2 (Wanleibio Co., Ltd.), TIMP1 (Wanleibio Co., Ltd.), TLR2 (Beijing Biosynthesis Biotechnology Co., Ltd.), myd88 (Beijing Biosynthesis Biotechnology Co., Ltd.), TGFβ1 (Wanleibio Co., Ltd.), p38 MAPK (Bisson, Beijing, China), P-P38MAPK (Wanleibio Co., Ltd.), and β-actin (Absci) were used to bind targeting proteins. An ECL-sensitive kit (Wanleibio Co., Ltd.) was used to detect goat anti-rabbit secondary antibody binding.

RNA extraction and reverse transcription. Total RNA was extracted from adipose tissue using TRIzol reagent, then dissolved in RNase free water with RNase inhibitor. Prior to cDNA synthesis, RNA was monitored by Nandrop2000, and DEPC water was added to a concentration of 100 μg/μl. Then DNA was removed in a mix (10 μl) with RNA (1 μl), 5X gDNA eraser buffer (2 μl), gDNA eraser (1 μl), and RNase free dH2O (6 μl). cDNA was synthesized in a mix (20 μl) with primer script RT enzyme mix (1 μl), RT primer mix (1 μl), 5X primer script buffer (4 μl), RNase free dH2O (4 μl), and the mix (10 μl) from the previous step. Reverse transcription was performed in 20-μl reactions at 25˚C for 10 min, followed by 37˚C for 15 min, and finally denaturation at 85˚C for 5 min. cDNA was stored at -80˚C until further use. All experiments involving the PrimeScript™ RT reagent kit with gDNA eraser (Takara Bio, Inc., Otsu, Japan) were performed according to the manufacturer's.

Polymerase chain reaction (PCR). Expression levels of IL-6 *mRNA*, TNF-α *mRNA* collagen I α1 *mRNA*, and collagen I α2 *mRNA* were measured in a 20-μl System with 2XGreenStar Master mix (10 μl), forward primer (1 μl, 10 pmol/μl), reverse primer (1 μl, 10 pmol/μl), DEPC water (6 μl), and template DNA (2 μl) obtained by reverse transcription. Primers were designed and synthesized by Sangon Biotech (Sangon Biotech Co., Ltd, Shanghai, China) (Table I). All reactions were performed in a 20-μl volume for 30 sec at 95˚C, followed by 45 cycles of 95˚C for 5 sec, and 60˚C for 34 sec. Relative quantification of gene expression was performed using the comparative $2^{-\Delta\Delta Ct}$ method. GAPDH was used as a validated reference gene. Real-time PCR experiments were performed according to the protocol provided by the manufacturer of the AccuPower 2xGreenStar qPCR Master Mix kit with gDNA eraser (Bioneer Corporation, Pangyo R&D Center, Republic of Korea).

Table I. Primers of collagen I α1, collagen I α2, IL6, TNFα and GAPDH.

Genes	Primer sequences (5'-3')
Collagen I α1	
Forward	GACGGCTGAGTAGGGAACAC
Reverse	CTGACTGGAAGAGCGGAGAG
Collagen I α2	
Forward	ACCCCTGTGCCCTTTATCAC
Reverse	GGTTCTGTTGGTCCTGTTGG
IL6	
Forward	ATGAAGTTCCTCTCTGCAAGAGACT
Reverse	CACTAGGTTTGCCGAGTAGATCTC
TNFα	
Forward	TGTCTCAGCCTCTTCTCATT
Reverse	AGATGATCTGAGTGTGAGGG
GAPDH	
Forward	TTGTCAAGCTCATTTCCTGGTATG
Reverse	GGATAGGGCCTCTCTTGCTCA

IL, interleukin; TNF-α, tumor necrosis factor-α.

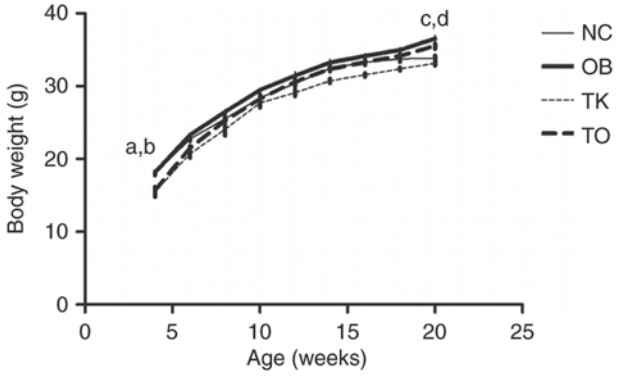

Figure 1. Body weights of mice. C57BL/6J mice and TLR2 gene knockout mice were weighed before the experiment. C57 BL/6J mice weighed more than TLR2 gene knockout mice [a, TK vs. NC (P=0.045); b, TO vs. OB (P=0.002)]. At the end of the experiment, we measured an increased body weight in OB group vs. NC group (P=0.000) and TO group vs. TK group (P=0.000). Although not significant, mice were heaver in OB group than TO group (P=0.066).

Statistical analysis. Data are presented as means ± standard error (S.E.M.). Differences between groups were analyzed by one-way ANOVA with SPSS 20.0 (IBM Corp., Armonk, NY, USA). When appropriate, differences between groups were evaluated by LSD. Interaction effects were analyzed by analysis of variance of factorial design. $P<0.05$ was considered to indicate a statistically significant difference.

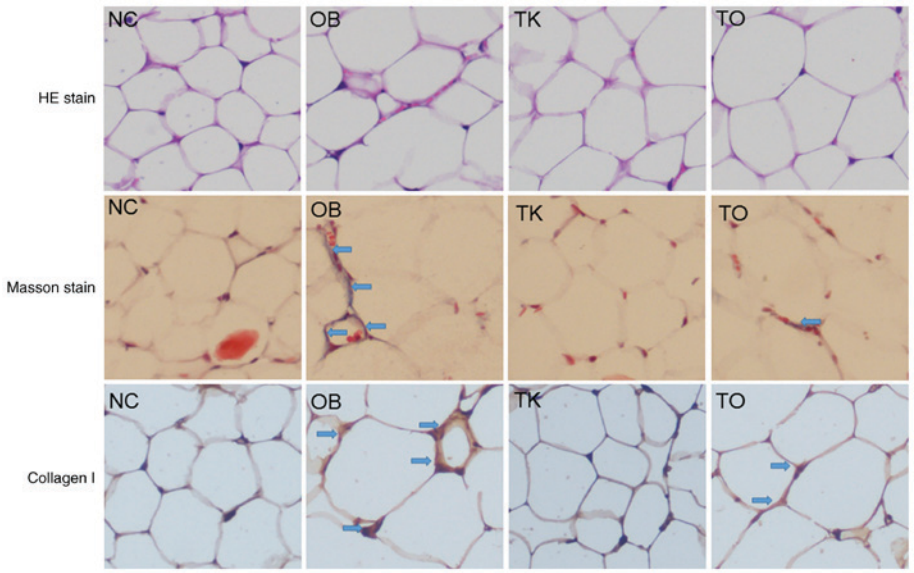

Figure 2. H&E stain, Masson stain and collagen I immunohistochemistry. HE stain showed larger adipocytes in OB and TO compared with NC and TK mice. Blue arrows highlight total collagen staining. Collagen is significantly increased in the OB group compared with the TO group. Brown areas show collagen I.

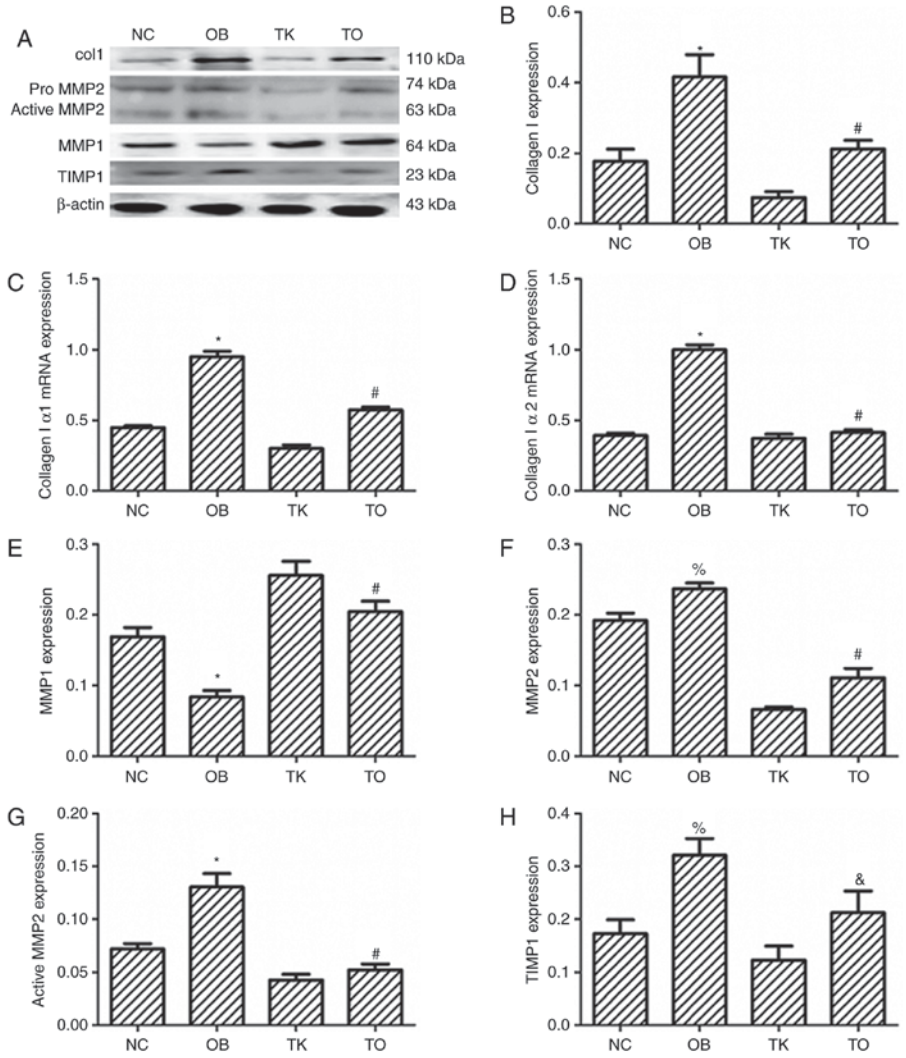

Figure 3. (A) Western blotting and RT-PCR to measure levels of (B) collagen I, (C) collagen I α1 *mRNA*, (D) collagen I α2 *mRNA*, (E) MMP1, (F) MMP2, (G) active MMP2, and (H) TIMP1. Increased collagen I, collagen I α1 *mRNA*, and collagen I α2 *mRNA* in the OB group. Decreased collagen I, collagen I α1 *mRNA*, and collagen I α2 *mRNA* in the TO group (*P<0.01 vs. NC; $^{\#}$P<0.01 vs. OB). Increased levels of MMP2, active MMP2, and TIMP1 and decreased levels of MMP1 in the OB group. Decreased levels of MMP2, active MMP2, and TIMP1 and increased levels of MMP1 were observed in the TO group (*P<0.01, $^{\%}$P<0.05 vs. NC; $^{\#}$P<0.01, P<0.05 vs. OB).

Table II. Levels of FPG, TC, TG, HDL, LDL and FFA in plasma of mice.

Variable	NC	OB	TK	TO
FPG (mmol/l)	6.78±0.43	9.79±0.32[a]	6.37±0.46[b]	6.88±0.33[c,e]
TC (mmol/l)	0.26±0.02	0.37±0.03[a]	0.08±0.01[a]	0.12±0.02[a,c,e]
TG (mmol/l)	2.64±0.06	3.03±0.07[a]	2.30±0.06[b]	2.36±0.06[a,c]
HDL (mmol/l)	1.14±0.05	1.05±0.02[a]	0.98±0.03[b]	0.94±0.02[a,c,f]
LDL (mmol/l)	1.46±0.02	1.65±0.07[a]	1.19±0.03[b]	1.2675+0.04[a,c,e]
FFA (mEq/l)	670.40±22.10	1124.89±36.37[a]	552.85±23.08[b]	563.73+26.37[a,c]

[a]P<0.01, [b]P<0.05 vs. NC; [c]P<0.01, [d]P<0.05 vs. OB; [e]P<0.01, [f]P<0.05 vs. TK.

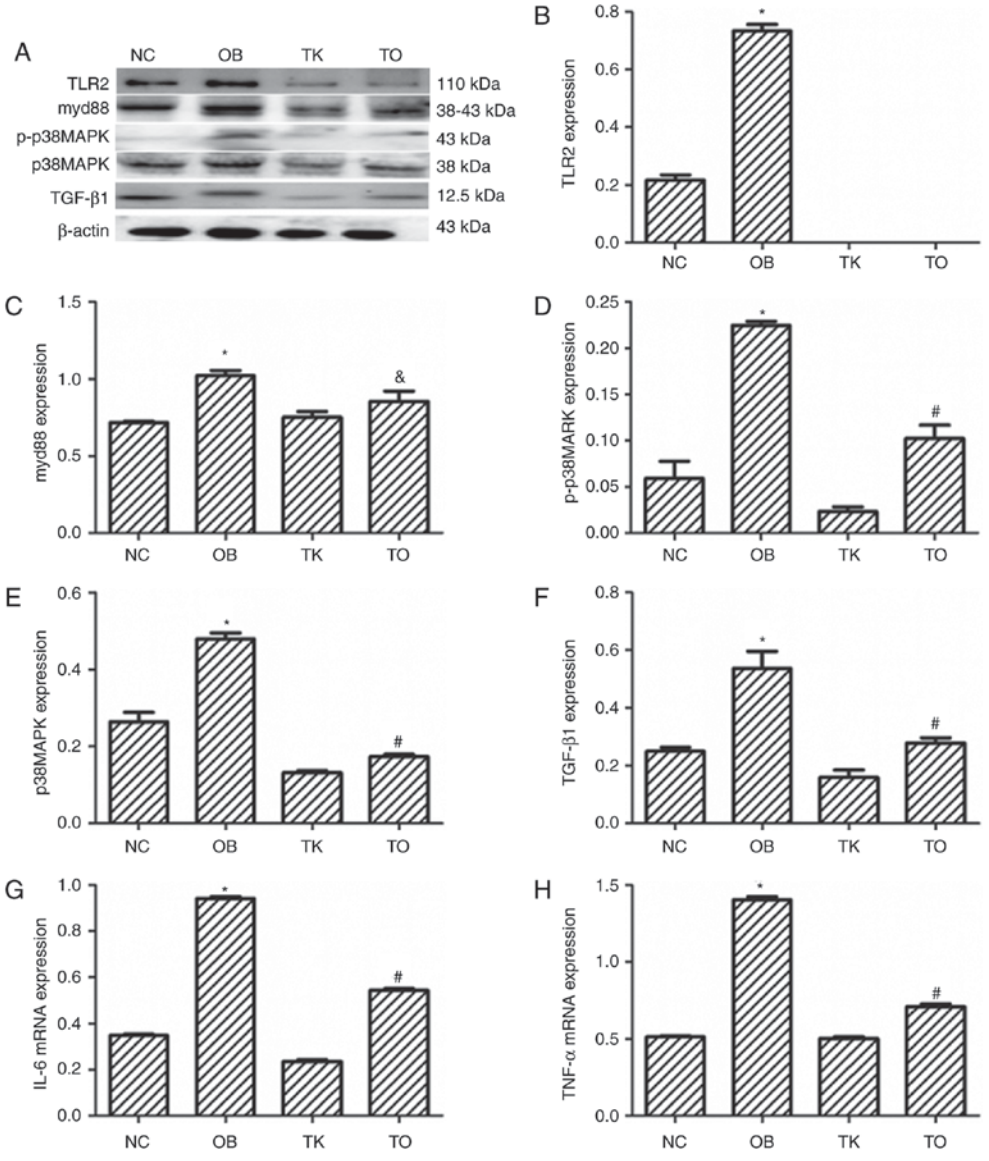

Figure 4. (A) Western blotting and RT-PCR to measure levels of (B) TLR2, (C) myd88, (D) p-p38MAPK, (E) p38 MAPK, (F) TGF-β1, (G) IL6 mRNA and (H) TNF-α mRNA. Increased levels of TLR2, myd88, p-p38MAPK, p38MAPK, TGFβ1, IL6 *mRNA*, and TNFα *mRNA* levels in the NC group (*P<0.01 vs. NC). myd88, p-p38MAPK, p38MAPK, TGFβ1, IL6 *mRNA*, and TNFα *mRNA* levels in the TO group (#P<0.01, P<0.05 vs. OB).

Results

At the start of the experiment, C57BL/6J mice (NC and OB mice) were heavier than TLR2 gene knockout mice (TK and TO mice). However, there was no significant difference between the NC and OB as well as TK and TO mice groups. At 4 weeks, there was no significant difference among these 4 groups in weight. At 16 weeks, Weight was increased in the

OB group as compared to the NC group, and decreased in the TO group compared to the OB group (Fig. 1).

Levels of TC, TG, LDL and FFA were higher in the OB group compared with the NC group. HLD level was lower in the OB group compared with the NC group. Levels of TC, TG, LDL, HDL and FFA were lower in the TO group compared with the OB group (Table II).

Masson stain revealed more collagen in adipose tissue in the OB group compared with the NC group. Levels of total collagen, collagen I, collagen I α1 *mRNA*, and collagen I α2 *mRNA* increased in adipose tissue of OB compared with NC mice but decreased in TO compared with OB mice (Figs. 2 and 3).

Levels of active MMP2, MMP2, and TIMP1 were higher in OB compared with NC mice. Expression of MMP1 in adipose tissue was lower in OB compared with NC mice. Expression of active MMP2, MMP2, and TIMP1 was lower in TO compared with OB mice. Expression of MMP1 in adipose tissue was higher in TO compared with OB mice (Fig. 3).

Levels of TLR2, myd88, p38MAPK, p-p38MAPK and TGF-β1 in adipose tissue were higher in OB compared with NC mice. Levels of TLR2, myd88, p38MAPK, p-p38MAPK, and TGF-β1 in adipose tissue were lower in TO compared with OB mice (Fig. 4).

Expression levels of IL-6 *mRNA* and TNF-α *mRNA* were up-regulated in adipose tissue of OB compared with NC mice, but down-regulated in TO compared with OB mice (Fig. 4).

Discussion

Our findings of increased total collagen and collagen I in adipose tissue of obese mice support previous reports (20). Levels of MMP2 and TIMP1, which regulate collagen I, were also increased in obese mice (16,17). The increase in levels of MMP2 and TIMP1 was reported previously (8). Levels of MMP1, which also regulates collagen deposition, were observed to decrease in this study. The results presented here suggest that MMP1 and TIMP1 mediate the deposition of collagen I.

FFA activates TLR2, which in turn triggers the overexpression of proinflammatory factors (15). The adipose tissue of obese individuals is therefore characterized by chronic inflammation, which may be controlled by TLR2 (21). Such chronic inflammation increases the expression of MMPs and TIMPs (8). We believe that knocking out TLR2 reduced the expression of MMP1, TIMP1, and other inflammatory factors, reducing the deposition of collagen I in mice fed a high-fat diet. In this study, increased levels of myd88, p38 MAPK, p-p38 MAPK, IL-6, and TNF-α (inflammatory factors downstream of TLR2) were observed in the adipose tissue of OB compared with NC mice; levels of these factors were increased in the OB compared with the TO group. Compared with OB mice, TO mice had lower levels of collagen I and TIMP1 and higher levels of MMP1. TLR2 knockout appears to have increased the expression of MMP1 and decreased the expression of TIMP1. These factors likely reduced the level of collagen I deposition. These findings support the previous studies (22,23). Genes knockout resulting in decreased levels fibrosis factors are reported to have decreased expression of inflammatory factors.

To further explore deposition of collagen I, collagen I α1 *mRNA* and collagen I α2 *mRNA* expression were measured in adipose tissue. OB mice showed up-regulation of collagen I α1 mRNA and collagen I α2 mRNA compared with NC mice. However, down-regulation of collagen I α1 mRNA and collagen I α2 mRNA were detected in TO mice compared with OB mice (Fig. 3). Statistical analysis revealed an interaction effect on collagen I α1 and collagen I α2 mRNA between high fat diet and TLR2 knocking out in adipose tissue of mice. These findings suggest that the transcription and translation of collagen I were affected by TLR2 gene knockout and ingestion of a high-fat diet.

Higher expression of TGFβ1 was observed in OB compared with NC mice. TGFβ1 expression was lower in TO compared with OB mice. Elevated levels of cytokines such as TGFβ1 (13,21,24) trigger SMAD and non-SMAD signaling cascades that contribute to obesity (25-29) and fibrosis (30). Gene knockouts resulting in decreased levels of TGFβ1 are reported to have decreased expression of inflammatory factors (22,23). TLR2 gene knockout appears to have had similar effects, resulting in fibrosis and the deposition of collagen I in adipose tissue.

In conclusion, the present results showed that TLR2 gene knockout may reduce collagen I expression in adipose tissue of mice with obesity. We hypothesize that this effect is mediated by a balance in pro -vs. anti-inflammatory factors and downstream MMP1, TIMP1, and TGFβ1.

Acknowledgements

The present study was supported by the Scientific Foundation for Doctoral Research (20131067) and Natural Scientific Foundation (201602308) at the Liaoning Science and Technology Administration Bureau. Professor Zheng and Professor Zhai in the Department of Brain and Spinal Cord injury in Liaoning province contributed substantially to this study.

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