

β -catenin protein utilized by Tumour necrosis factor- α in porcine preadipocytes to suppress differentiation

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The Wnt/ β -catenin signaling pathway alters adipocyte differentiation by inhibiting adipogenic gene expression. β -catenin plays a central role in the Wnt/ β -catenin signaling pathway. In this study, we revealed that tumour necrosis factor- α (TNF- α), a potential negative regulator of adipocyte differentiation, inhibits porcine adipogenesis through activation of the Wnt/ β -catenin signaling pathway. Under the optimal concentration of TNF- α , the intracellular β -catenin protein was stabilized. Thus, the intracellular lipid accumulation of porcine preadipocyte was suppressed and the expression of important adipocyte marker genes, including peroxisome proliferator-activated receptor- γ (PPAR γ) and CCAAT/enhancer binding protein- α (C/EBP α), were inhibited. However, a loss of β -catenin in porcine preadipocytes enhanced the adipogenic differentiation and attenuated TNF- α induced anti-adipogenesis. Taken together, this study indicated that TNF- α inhibits adipogenesis through stabilization of β -catenin protein in porcine preadipocytes. [BMB reports 2009; 42(6): 338-343]

INTRODUCTION

The differentiation of preadipocytes is an important process for normal adipose tissue function. The molecular mechanisms of adipocyte differentiation have been widely investigated using adipogenic cell lines, and proved to have sequential activation of various transcription factors involved (1-3). In association with adipocyte differentiation, many transcription factors, including CCAAT/enhancer binding protein (C/EBP) α and peroxisome proliferator-activated receptor (PPAR) γ , are sequentially activated to regulate the adipogenic gene expression such as lipoprotein lipase (LPL). Adipogenesis enters the terminal differentiation stage concurrently (2). However, induction of preadipocyte differentiation is influenced by a balance of positive and negative factors (4).

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One of the negative signaling pathways now known to affect adipogenesis is the Wnt/ β -catenin pathway (5, 6). This signaling pathway is controlled by intracellular β -catenin levels, which plays a central role as a transcriptional co-activator (7). Binding of Wnt ligands to frizzled receptors (Fzs) and low density lipoprotein receptor-related protein (LRP) co-receptors activates Wnt/ β -catenin signaling pathways (8). In this pathway, Wnt signaling inhibits glycogen synthesis kinase (GSK)3 β , resulting in hypophosphorylation and subsequent stabilization of β -catenin in the cytoplasm (9). Subsequently, cytoplasmic β -catenin is translocated into the nucleus where it binds to members of the T-cell factor/lymphoid-enhancing factor (TCF/LEF) family of transcription factors to direct targeted gene expression (10).

The Wnt/ β -catenin signaling pathway has been reported to prevent differentiation of the 3T3-L1 murine preadipocyte cell line by inhibiting expression of the master adipogenic transcription factors C/EBP α and PPAR γ (5, 11). The expression of Wnt10b and β -catenin showed the highest in growing and confluent 3T3-L1 preadipocytes, but decreased upon MDI treatment. Conversely, forced stabilization of β -catenin inhibits adipogenesis (11, 12). Furthermore, transgenic mice expressing Wnt10b under the control of the fat-specific *Fabp4* promoter have reduced body fat content without lipodystrophic diabetes (13). In contrast, inhibiting the Wnt/ β -catenin signaling pathway by over-expressing Axin or dominant-negative TCF4 in 3T3-L1 cell line leads to spontaneous adipogenesis.

TNF- α is a multi-functional cytokine that can regulate many cellular and biological processes such as immune function, proliferation, apoptosis, energy metabolism and especially cell differentiation (14, 15). The expression of TNF- α is elevated in adipose tissue from obese diabetic rodents and is a mediator of obesity-related insulin resistance (16). The actions of TNF- α in adipose tissue have been stimulated through numerous signaling pathways such as c-Jun N-terminal kinase (JNK), extracellular signal-regulated protein kinase (Erk) 1/2 and mitogen-activated protein kinase (MAPK) cascades (17-20). However, these reports were just limited to experimental animal mouse research and the direct signaling pathway in porcine adipose tissue remains to be elucidated.

Animal models of human diseases have always played a central role in biomedical research for the exploration and development of new therapies. Although rat preadipocytes and

adipocytes lines are commonly used in obesity studies, it has been suggested that porcine preadipocytes are a better model for the study of obesity and its related disorders because porcine preadipocytes possess higher lipogenic capacity and similar lipogenic patterns to human adipocytes (21-23). Fernandez reported that guinea pigs could be used as models to study hepatic cholesterol and lipoprotein metabolism (24). However, we found few reports about the effects of TNF- α on porcine preadipocytes differentiation. In the present study, we report that TNF- α induces the accumulation of intracellular β -catenin and activates Wnt/ β -catenin signaling pathway in primary cultured porcine preadipocytes while suppressing adipogenesis by blocking the expression of C/EBP α and PPAR γ 2.

RESULTS

Effects of TNF- α on differentiation of porcine preadipocytes

Adipogenesis is a highly regulatory process. Preadipocyte differentiation into mature adipocytes occurs in a series of stages. As we were interested in the effects of TNF- α on differentiation, preadipocytes were treated with TNF- α and then cultured to evaluate the inhibition of porcine preadipocytes *in vitro*. Afterward, the cells were stained with Oil red O, and the size and number of adipocytes were determined visually by light microscope.

After Oil red O staining, preadipocytes in the control group differentiated into mature adipocytes, which were large, round, and filled with fat droplets. In comparison, there were significantly fewer fat droplets in adipocytes treated with 5 ng/ml and 10 ng/ml TNF- α . Both the size and number of fat cells decreased with TNF- α treatment compared to the control (Fig. 1A-E). Results from Oil red O extraction showed that content notably decreased in the 5 ng/ml and 10 ng/ml TNF- α treatments ($P < 0.05$) (Fig. 1F).

To investigate the molecular mechanism of TNF- α -induced anti-adipogenesis, we used semi-qRT-PCR to analyze the mRNA expression of C/EBP α and PPAR γ 2, which are key adipogenic transcription factors. (25) Compared with control, TNF- α treatment completely prevented the induction of both C/EBP α and PPAR γ 2 (Fig. 1G). And as shown in Fig. 1H, the mRNA expression of C/EBP α and PPAR γ 2 confirmed that TNF- α inhibited their expression in a dose-dependent manner and 5 ng/ml TNF- α greatly inhibited the mRNA expressions of C/EBP α and PPAR γ 2 genes. These data show that TNF- α could decrease the fat content in cells and effectively inhibit preadipocytes differentiation into adipocytes.

TNF- α activates the Wnt/ β -catenin signaling pathway during inhibition of porcine preadipocytes differentiation

We recently demonstrated that the Wnt/ β -catenin signaling pathway inhibited the adipogenic differentiation potential of porcine adipose-derived mesenchymal stem cells (26). In addition, some cytokines could influence the Wnt/ β -catenin signaling pathway in 3T3-L1 preadipocytes (27). Thus, we postulated that TNF- α would activate the Wnt/ β -catenin signaling path-

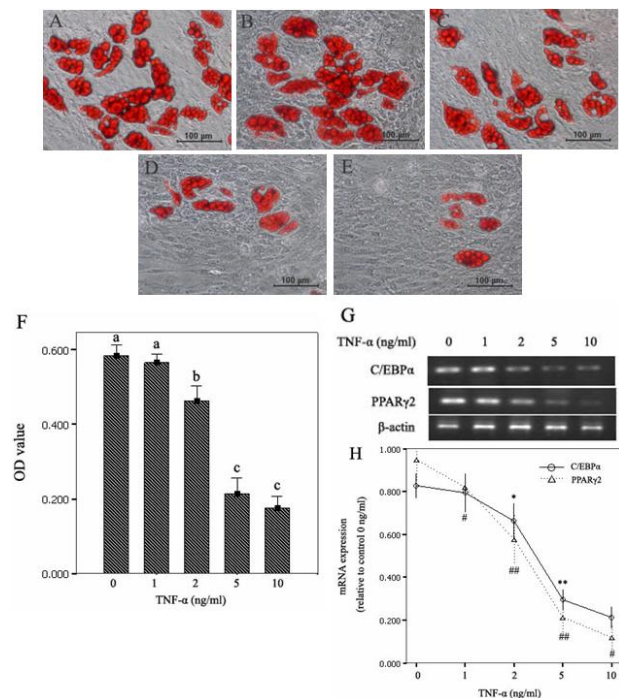


Fig. 1. Effects of TNF- α on porcine preadipocytes differentiation. Confluent porcine preadipocytes were induced to differentiate by the differentiation medium as previously described (37). Cells were treated with different doses of TNF- α during adipocyte differentiation. At 8 days after induction, the extent of adipogenesis was assessed by staining for lipid accumulation with Oil red O. (A) Control group, 0 ng/ml TNF- α , (B) 1 ng/ml, (C) 2 ng/ml, (D) 5 ng/ml, (E) 10 ng/ml. These results were representative of three independent experiments. Scale bars = 100 μ m. (F) TG content was analysed by Oil red O extraction. (G) Semi-qRT-PCR analysis was performed in the presence of the indicated concentrations of TNF- α . The expression of C/EBP α and PPAR γ 2 (H) were measured by PCR products and normalised to β -actin. Data were means \pm S.E. from three experiments. Statistical significance is indicated as follows: *represents C/EBP α and #represents PPAR γ 2. Single symbol indicates a statistical difference ($P < 0.05$). Double symbol indicates a statistical difference ($P < 0.01$).

way during anti-adipogenesis. To test this hypothesis, porcine preadipocytes were stably transfected with TOPFlash, a synthetic TCF/ β -catenin-dependent luciferase reporter. As shown in Fig. 2A, TNF- α up-regulated TOPFlash-dependent luciferase activity at 12 h post-induction of adipogenesis. This data indicated that TNF- α indeed activates the Wnt/ β -catenin signaling pathway during anti-adipogenesis.

Within the Wnt/ β -catenin signaling pathway, TCF transcriptional activation is largely dependent on the level of intracellular β -catenin, which is regulated by an ubiquitin-dependent proteasome pathway (7). To investigate the effect of TNF- α on the level of β -catenin, we analyzed the mRNA and protein expression levels of β -catenin by semi-qRT-PCR and Western blotting. As shown in Fig. 2B and C, TNF- α treatment induced a transient peak in β -catenin mRNA levels, which subsequently

dropped to below preadipocyte levels. Then at later time points (>48 h), β -catenin mRNA levels were maintained in TNF- α treated cells. In addition, the intracellular β -catenin protein level increased by incubating porcine preadipocytes with TNF- α during TNF- α -induced anti-adipogenesis. Compared with β -catenin mRNA levels, the protein level had a dramatic increase (Fig. 2D). These results suggest that TNF- α activates the Wnt/ β -catenin signaling pathway through the up-regulation of β -catenin protein during TNF- α -induced anti-adipogenesis.

Knockdown of β -catenin weakens TNF- α -induced antiadipogenesis and enhances adipogenesis in porcine preadipocytes

To further confirm the roles of β -catenin in TNF- α -induced an-

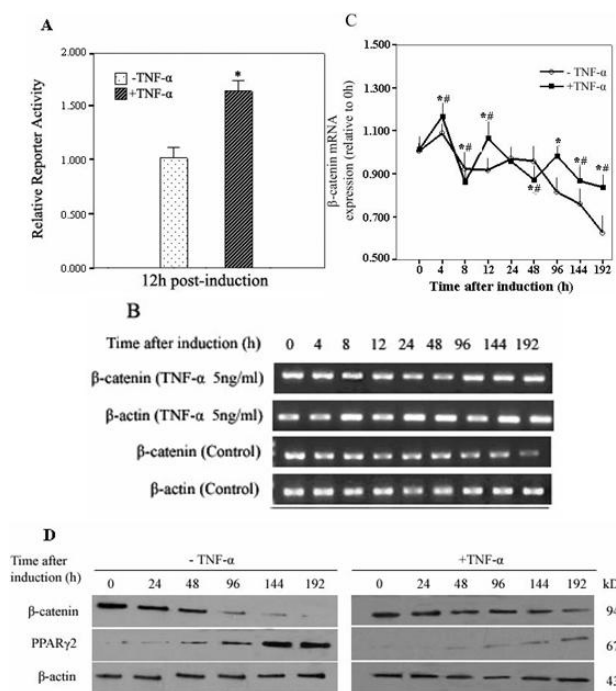


Fig. 2. TNF- α activates the Wnt/ β -catenin signaling pathway during anti-adipogenesis. (A) Porcine preadipocytes were transfected with the TCF reporter TOPFlash as described in Materials and Methods, and were induced to differentiate in the absence or presence of TNF- α (5 ng/ml). Luciferase activity was assayed 12 h post-induction. (B-D) Confluent porcine preadipocytes were induced to differentiate as described in Fig. 1. Cells were treated with or without TNF- α (5 ng/ml) throughout the differentiation procedure. Total RNA and intracellular protein were extracted at the indicated times post-induction. Transcript level of β -catenin (C) was determined by using semi-qRT-PCR and normalized to β -actin (B). Data were means \pm S.E. from three experiments. Statistical significance is indicated as follows: compared to control treatment at the same time point (* P < 0.05); TNF- α treatment compared to time 0 h ($\#P$ < 0.05). (D) Protein expression of β -catenin and PPAR γ 2 were then analyzed by Western blotting. The β -actin was used as a loading control. These results were representative of three independent experiments.

ti-adipogenesis, we performed the knockdown experiments by transfecting the porcine preadipocytes with β -catenin shRNA. In differentiating porcine adipocytes, the expression of β -catenin mRNA and protein were analyzed. By the transfection, the β -catenin mRNA expression was significantly decreased to approximately 60% compared of control (Fig. 3A, B). Moreover, as shown in Fig. 3C, the cells containing β -catenin shRNA had a substantial reduction in β -catenin protein expression, whereas β -catenin protein expression was not affected in control cells. These results indicated that the expression of β -catenin mRNA and protein were remarkably inhibited by the transfection of β -catenin shRNA in porcine preadipocytes.

Subsequently, the effects of β -catenin knockdown on the adipogenesis of porcine preadipocytes were analyzed. The β -catenin shRNA transfected cells were induced to differentiate in the absence or presence of 5 ng/ml TNF- α . The β -catenin knockdown cells exhibited enhanced lipogenesis. Interestingly, TNF- α treatment markedly suppressed lipid accumulation in control cells (transfected by empty vector), but had little effect on lipid accumulation in β -catenin shRNA-expressing cells (Fig. 4A, B). The mRNA expression levels of LPL, PPAR γ 2 and C/EBP α were higher in the β -catenin knockdown cells than those in empty vector transfected cells (Fig. 4C, D). Furthermore, PPAR γ 2 protein levels were also maintained in β -catenin shRNA-expressing cells treated with TNF- α (Fig. 4E). These data indicated that knockdown of β -catenin enhanced adipogenesis, and attenuated TNF- α -induced anti-adipogenesis in porcine preadipocytes.

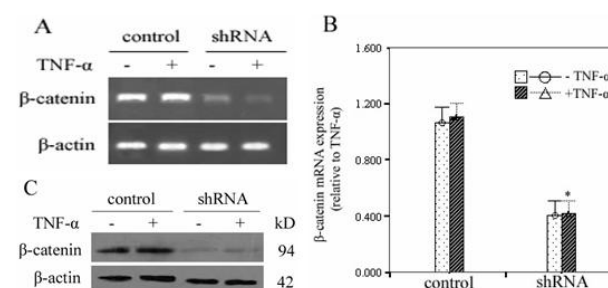


Fig. 3. Analysis of β -catenin expression in porcine preadipocytes transfected with β -catenin-shRNA. Porcine preadipocytes were transfected with an empty vector (control) or a vector encoding for pig β -catenin (shRNA). Three days after transfection, control, and β -catenin shRNA cells were induced to differentiate in the absence or presence of TNF- α (5 ng/ml). (A) Total RNA was extracted at 48 h after induction and expression of β -catenin mRNA was determined by using semi-qRT-PCR. (B) Intensities of PCR products were measured for β -catenin and normalized to β -actin. Data were means \pm S.E. from three experiments. Statistical significance is indicated as follows: compared to control TNF- α treatment at the same time point (* P < 0.05). (C) Intracellular protein was extracted 48 h after induction and the expression of β -catenin was analyzed by Western blotting. The β -actin was used as a loading control. The results in (C) were representative of three independent experiments.

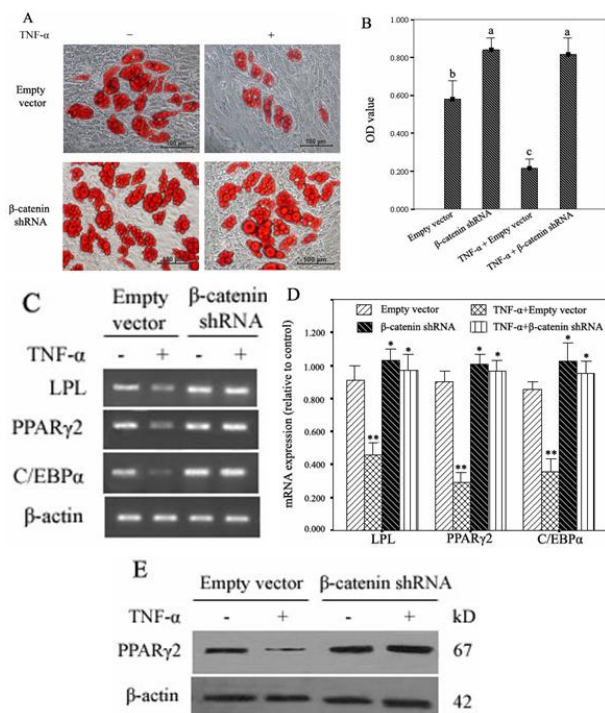


Fig. 4. Knockdown of β -catenin attenuates TNF- α -induced anti-adipogenesis and enhances adipogenesis in porcine preadipocytes. Control and β -catenin shRNA cells were induced to differentiate as described in Fig. 3. (A) Oil red O-stained cells at 8 days after induction of differentiation. Scale bars = 100 μ m. (B) TG content analyzed by Oil red O extraction. The results are represented as means \pm S.E. of three independent runs. Different letters indicated significant differences ($P < 0.05$). (C) Total RNA was extracted at 8 days after induction, and LPL, PPAR γ 2, and C/EBP α mRNA expression was determined by using semi-qRT-PCR. (D) Intensities of PCR products were measured and normalized to β -actin. Data are presented in means \pm S.E. of three experiments. Statistical significance is indicated as described in Fig. 1. (E) Intracellular protein was extracted at 8 days after induction and expression of PPAR γ 2 was analyzed by Western blotting. The β -actin was used as a loading control. These results were representative of three independent experiments.

DISCUSSION

TNF- α is a multi-faceted cytokine that plays an important role on cultured adipocytes (28). Previous studies have focused on the effects of TNF- α in human and rat adipocytes. In 3T3-F442A cells, TNF- α inhibits commitment at concentration as low as 5 ng/ml (29). In humans, TNF- α is more effective at inhibiting differentiation in subcutaneous adipocytes than visceral adipocytes (30). However, as a medical animal model, the effects of TNF- α on porcine adipocytes has rarely been reported. Therefore, we were particularly interested in whether this cytokine had differential effects on cultures of porcine preadipocytes. Our results showed that TNF- α could significantly decrease the lipid accu-

mulation and TG content as measured by Oil red O staining and extraction. At the transcriptional level, we investigated the regulation of transcription factors C/EBP α and PPAR γ 2. We showed that TNF- α inhibited the porcine preadipocyte differentiation in dose-dependent manner and 5 ng/ml TNF- α greatly inhibited the mRNA expressions of C/EBP α and PPAR γ 2 genes. Thus, we used 5 ng/ml of TNF- α as the optimal concentration.

Considerable evidence now suggests that activation of the Wnt/ β -catenin signaling pathway inhibits adipogenesis early in differentiation. Central to this pathway is the level of intracellular β -catenin (7). Based upon previous report that in a columnar intestinal cell model, TNF- α induces c-myc expression which is a target gene of the Wnt/ β -catenin signaling pathway (31), Here, whether TNF- α activated the Wnt/ β -catenin signaling pathway in porcine preadipocytes was analyzed. Our results indicated that TCF transcription increased dramatically when porcine preadipocytes were incubated with TNF- α . In addition, intracellular β -catenin protein was stabilized by TNF- α . Extraordinarily, TNF- α alters β -catenin mRNA levels slightly 48 h post-induction in the present study. Nevertheless, Cho M et al. (32) detected that TNF- α did not alter the expression of β -catenin mRNA throughout the differentiation program in 3T3-L1 preadipocytes. This finding is contrary to our results but it might be related to differences in cell types or species. Compared with the mRNA levels of β -catenin, intracellular β -catenin protein was stabilized by TNF- α .

To address the role of β -catenin in TNF- α -induced anti-adipogenesis, the loss of function experiments were conducted by silencing the β -catenin gene. In analyses, the silencing of the β -catenin gene enhanced C/EBP α , PPAR γ 2, LPL expression and stimulated adipogenesis, while attenuating TNF- α -induced anti-adipogenesis in porcine preadipocytes. In all, these findings indicated that TNF- α induced the up-regulation of TCF transcription and stabilization of intracellular β -catenin, indicating the activation of the Wnt/ β -catenin signalling pathway, and thereby suppressing adipogenesis in porcine preadipocytes. However, multiple pathways regulate the stabilization of intracellular β -catenin, such as the GSK3 β -dependent pathway (33), the Siah-dependent pathway (34) and the PKC pathway (35). Which pathway utilized by TNF- α in porcine adipocytes to induce stabilization intracellular β -catenin protein will be explored in future studies.

In summary, we showed that TNF- α utilizes components of the Wnt/ β -catenin signalling pathway to inhibit porcine preadipocytes differentiation. Knockdown of β -catenin gene attenuates TNF- α -induced anti-adipogenesis. Taken together, our findings will facilitate further study of the molecular mechanism of TNF- α induced anti-adipogenesis in porcine preadipocytes.

MATERIALS AND METHODS

Experimental animals

Three-day-old male crossbred piglets (*Sus scrofa*) (Duroc \times Landrace \times Large-White) were purchased from the experimental farm of Northwest A & F University (Yangling, Shaanxi,

China) and euthanized by electrocution.

Porcine primary preadipocytes *in vitro* culture

Subcutaneous adipose tissue was collected from the neck and back of the piglets. Porcine preadipocytes were obtained as previously described (36, 37). Cells were seeded in culture plates at a density of 5×10^4 cells/cm² and cultured at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed every second day.

Retroviral constructs

The pSuper-puro- β -catenin-shRNA vector, which expresses a short hairpin RNA (shRNA) specific to β -catenin, was previously constructed in our laboratory. Porcine preadipocytes with shRNA-mediated knockdown of β -catenin (or controls) were generated with the pSuper-puro retroviral vector system. The transfections were carried out as previously described (26).

TCF4 reporter assay

To assay for the activation of β -catenin/TCF target genes, porcine preadipocytes were seeded in 24-well plates and grown to confluence. At 2 days post-confluence, cells were transfected with 1 μ g/well of pTOPFlash TCF reporter construct (Upstate Biotechnology, Lake Placid, NY, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To correct for transfection efficiency, 10 ng/well pCMV-RL (Promega, Madison, WI, USA) was co-transfected. Luciferase assays were performed using the Dual Luciferase Assay Kit (Promega, USA).

Oil red O staining and extraction

The cellular lipid contents were determined with Oil red O (Sigma, USA) and the TG contents were determined with Oil red O extraction by the method of Liang Bai et al (36). Cellular morphology was examined and photographed with a light microscope. The OD value of each hole was detected at 500 nm using a UV-2102 PC ultraviolet spectrophotometer (Unico Instrument Co., Ltd., Shanghai, China).

RNA isolation and semi-quantitative RT-PCR (semi-qRT-PCR)

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen, USA). After DNase treatment, RNA was quantified by spectrophotometry at 260 and 280 nm. Quality of the RNA was evaluated by electrophoresis. First strand cDNA was synthesized by following the manufacturer's instruction (Fermentas, RevertAidTM First Strand cDNA Synthesis Kit #K1621). All PCR primers were designed using Primer 5.0 Software and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). β -actin was determined as a control for loading. The parameters of the primers, PCR system and condition were described in a previous paper (26).

Western blotting analysis

Equal amounts of protein were analyzed by Western blotting using antibodies specific for β -catenin (Sigma, USA), PPAR γ 2

and β -actin (Santa Cruz, CA, USA). Protein concentration was determined using the method of Lin et al (38).

Statistical analysis

Data are expressed as means \pm S.E. Data were analyzed by one-way ANOVA (software: SPSS11.5; SPSS, Chicago, IL, USA). Differences were determined to be significant if $P < 0.05$.

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