Systemic overexpression of the 11β-HSD1 promotes endoplasmic reticulum stress in multiple tissues and the development of metabolic syndrome in mice

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Abstract. Glucocorticoids are associated with lipid metabolism and their abnormal expression has an important function in the development of metabolic syndrome. The 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) is a metabolic enzyme of glucocorticoids and may be a potential drug target for the treatment of metabolic syndrome. However, the association between the systemic expression of 11β-HSD1 and metabolic syndrome remains to be elucidated. The present study used a cytomegalovirus promoter to obtain mice that systemically overexpressed the 11β-HSD1 gene. The transgenic mice and negative control groups received a high-fat diet at the age of 10 weeks in order to induce metabolic syndrome and this diet was continued for 12 weeks. Several indicators, including body weight, blood glucose, glucose tolerance and insulin resistance, were monitored *in vivo*. In addition, the protein expression levels of 11β-HSD1 and DNA damage inducible transcript 3 were detected and the histopathology of important tissues for metabolic syndrome were analyzed. The current findings revealed that the body weights of transgenic mice were significantly higher compared with the control group before and during the periods of high fat diet induction. Transgenic mice also exhibited significantly impaired glucose tolerance, insulin resistance, endoplasmic reticulum stress and increased metabolic syndrome-associated biochemical indicators in the blood and severely impaired liver and kidney functions. The present study successfully established a 11β-HSD1 systemic overexpression mouse model that exhibited typical characteristics of metabolic syndrome and may be useful for future studies of metabolic syndrome.

Introduction

Metabolic syndrome consists of a group of complex metabolic disorders associated with the metabolism of carbohydrates, lipids and proteins. According to the definition of the International Diabetes Federation, patients who meet at least three of the following criteria are diagnosed with metabolic syndrome: i) Abdominal obesity; ii) hypertension, hyperlipemia, hypercholesterolemia; and iii) reduced high-density lipoprotein cholesterol levels (1). In 2005, >25% the total world population was diagnosed with metabolic syndrome (2). In 2012, >1/3 of the adult population and half of the population above 60 years of age have been diagnosed with metabolic syndrome in the United States (3). The negative impact of metabolic syndrome is primarily due to its influence on other diseases. In patients with metabolic syndrome, the risk of diabetes mellitus is increased 5-fold, the risk of cardiovascular diseases 3-fold, the risk of cardiovascular death 2-fold and the overall risk of death is increased 1.5-fold (4,5).

Glucocorticoids are involved in the regulation of carbohydrate, lipid, protein, water and salt metabolism and have important physiological functions (6-8). Previous studies have confirmed that excessive glucocorticoids may induce insulin resistance, inhibit glucose absorption and reduce insulin release, thus increasing the possibility developing metabolic syndrome (6). In patients with metabolic syndrome, the glucocorticoid levels in blood circulation are normal; however, the glucocorticoid levels in local tissues are increased. 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) is a metabolic reductase and dehydrogenase that converts glucocorticoids between active cortisol and inactive cortisone (7). Therefore, 11β-HSD1 is a local amplifier of glucocorticoid functions (9). Alberts *et al* (8) confirmed that the 11β-HSD1-specific inhibitor BTV2733 reduced the levels of fasting blood glucose, insulin, the concentrations of blood cholesterol, free fatty acids and triglycerides in mice. Additionally, 11β-HSD1 knockout mice exhibited weakened insulin resistance and gluconeogenesis reaction, enhanced

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glucose tolerance and improved lipid distribution (10). Masuzaki et al (11) and Paterson et al (12) already confirmed that the tissue-specific overexpression of 11β-HSD1 in the liver and fat induced metabolic syndrome characteristics in mice. However, these two transgenic models could not reflect the overall effect of the systemic expression of the 11β-HSD1 gene in patients with metabolic syndrome. Therefore, the present study established transgenic mice that systemically expressed the 11β-HSD1 gene and fed these mice with a high-fat diet to induce metabolic syndrome. The current findings revealed that transgenic mice exhibited metabolic syndrome characteristics and pathological features in key tissues.

Materials and methods

Experimental animals. The porcine 11β-HSD1 gene was ligated into the T vector and then ligated into the pcDNA3.1 plasmid (donated by Dr Li Li, Institute of Zoology, Chinese Academy of Sciences) using double-enzyme digestion. The digestion sites were *Nhe*I and *Pme*I. The constructed vector plasmid was injected into 10 C57BL/6L mice (Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences; Beijing, China) via pronuclear microinjection to obtain transgenic mice. This was conducted at the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. Matched C57 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The offspring of transgenic founders were divided into the transgenic group and the negative control group and each group consisted of 5 mice. High-fat diets were purchased from Beijing Biopike Biotechnology Co., Ltd. (Beijing, China) with fat content of 58%. All mice received humane care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China.' The procedures were approved by the Institutional Animal Care and Use Committee of Chinese Academy of Agricultural Sciences. The mice were housed under a 12-h light/dark cycle with access to food and water *ad libitum*.

Following the termination of high-fat diet feeding, the mice in the two groups were anaesthetized by intraperitoneal injection of sodium pentobarbital (Sigma-Aldrich, Shanghai, China) at a dose of 50 mg/kg body weight and subsequently decapitated. Blood samples were collected and pancreatic, liver, lipid, kidney and muscle tissues were immediately removed, rinsed with cold physiological saline, frozen in liquid nitrogen and stored at -80°C. Pieces of liver about ~10 mm diameter were fixed in 4% paraformaldehyde at 4°C for histopathological studies.

Polymerase chain reaction (PCR) detection. At 2 weeks of age, the tail tips of the mice were cut to extract genomic DNA using the phenol-chloroform method (13). The PCR product was 598 bp long for the following primers: F 5'-CCCATA GTAACGCCAATA-3' and R, 5'-CTACTGCTATTCCGC AAA-3'. The reaction was conducted in a total volume of 20 μl and performed for 35 cycles under the following conditions: Pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec and a final extension at 72°C for 5 min. All reagents

were obtained from Takara Biotechnology Co., Ltd. (Dalian, China). Following PCR, 1% agarose gel electrophoresis (Biowest Regular Agarose G-10; Biowest, Hong Kong, China). was performed.

Body weight, glucose and insulin tolerance test. During the high-fat diet feeding period, the body weight of the mice was measured once every other week. At the end of the experiment, a glucose tolerance test was performed. Before the test, the mice were fasted for 12 h and an intraperitoneal injection of 20% glucose at a dose of 2 g/kg body weight was then administered. Blood samples (2 μl) were collected from the tail tips prior to injection and at 15, 30, 60, 90 and 120 min after injection to quantify the blood glucose levels. For the insulin tolerance test, the mice were fasted for 6 h prior to an intraperitoneal injection of insulin at a dose of 1 IU/kg body weight. Blood samples (2 μl) were collected from tail tips prior to the injection and 15, 30, 60 and 90 min after the injection to measure the blood glucose levels. Glucose was purchased from Mallinckrodt Pharmaceuticals Ltd. (Staines-upon-Thames, UK) and insulin (Novolin) was purchased from Novo Nordisk (Bagsværd, Denmark). A Onetouch Ultra blood glucose meter and blood glucose test strips were obtained from LifeScan, Inc. (Milpitas, CA, USA).

Serological quantification. Biochemical parameters were analyzed using a AU480 automatic biochemistry analyzer (Olympus Corporation, Tokyo, Japan). Assay kits were purchased from InTec Products, Inc. (Xiamen, China). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and uric acid were analyzed using the dehydrogenase enzyme method, triglyceride and creatinine were analyzed using the oxidase enzyme method, high-density lipoprotein-cholesterol and low-density lipoprotein-cholesterol were analyzed using direct method and total cholesterol was analyzed using the enzyme method as previously described (14).

Western blotting. Total protein was extracted from tissues using a tissue total protein extraction reagent kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the total protein concentration was quantified using an enzyme-linked immunosorbent assay (ELISA) using a microplate reader (Spectra Max M5, Molecular Devices, LLC, Sunnyvale, CA, USA). Subsequently, the proteins were denatured and stored at -20°C. For each sample, 30 μg of protein was separated using SDS-PAGE and the proteins were then transferred onto a nitrocellulose (NC) membrane. The NC membrane was blocked in 5% non-fat milk. To quantitatively detect the 11β-HSD1 protein, a primary 11β-HSD1 antibody (cat. no. ab83522; 1:1,000; Abcam, Cambridge, UK) was used at room temperature for 2 h and a horseradish peroxidase-labeled secondary goat anti-rabbit antibody (cat. no. ab6721; 1:5,000; Abcam) was added at room temperature for 40 min. To assess endoplasmic reticulum stress, a DNA damage inducible transcript 3 primary antibody (DDIT3; cat. no. ab11419; 1:1,000; Abcam) was used for incubation at room temperature for 2 h and a secondary goat anti-mouse antibody (cat. no. ab6789; 1:5,000; Abcam) were added for incubation at room temperature for 40 min. Following washing, Super Signal West Pico

Chemiluminescent substrate (Thermo Fisher Scientific, Inc.) was used to detect the immunoblots.

Hematoxylin and eosin (HE) staining. Tissues were sectioned at a thickness of 4 μm, and the sectioned slides were warmed at 60°C for 1 h, deparaffinized using xylene, rehydrated, stained with hematoxylin at room temperature for 2 min, washed, differentiated, blued, stained with eosin at room temperature for 1 min, and washed with water. The stained sections were dehydrated in 70, 80, 90, 95 and 100% ethanol (2 min for each concentration), followed by clearing in xylene 1 and xylene 2 (5 min each), the sections were subsequently mounted in resin.

Statistical analysis. The differences of data between the two study groups were statistically analyzed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance and the least significant difference post-hoc test were used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference. Data are presented as the mean ± standard deviation.

Results

Detection of positive transgenic mice. The founders of 11β-HSD1 transgenic mice and their offspring were detected using PCR. The positive F0 individuals had the band of 598 bp (Fig. 1).

Significant increase in obesity of transgenic mice. The average body weight of transgenic mice was 32.07±2.43 g prior to feeding with high-fat diet and the negative control group weight was 26.29±1.09 g (P<0.05). From week 2 of high-fat diet induction until the end of the induction experiment, transgenic mice were significantly more obese compared with control mice. In addition, the body weights of transgenic mice consistently increased, whereas the body weights of control mice plateaued at week 20 and subsequently decelerated (Fig. 2). Following induction, the average body weight of the transgenic group was 1.56-fold higher compared with the control group, 54.22±5.90 g and 40.46±3.23 g (P<0.05).

Biochemical parameters. Concentration of biochemical parameters was increased, indicating metabolic syndrome and damage to key organs in the transgenic group. In the transgenic group, the values of the triglyceride, total cholesterol, and low-density lipoprotein cholesterol significantly increased and were 2.13, 1.68, and 2.08-fold higher compared with the control group (Table I). These values were indicative of metabolic syndrome in the transgenic group. The values of ALT and AST, which reflect liver function, significantly increased and were 2.94 and 1.43-fold higher compared with the control group, respectively (Table I). The levels of uric acid and creatinine, which reflect kidney function, significantly increased and were 1.48 and 2.25-fold higher compared with the control group values, respectively (Table I). These findings revealed that transgenic mice exhibited characteristics of metabolic syndrome and that important organs involved in metabolic syndrome were damaged.
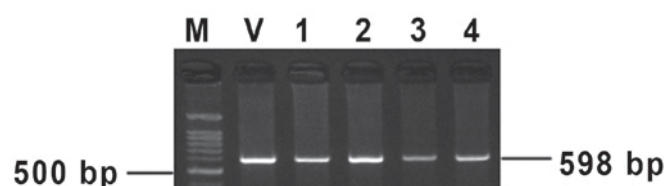

Figure 1. Identification of transgenic mice. M, DNA Ladder 100 marker; V, plasmid; lanes 1-4, transgenic mice samples.
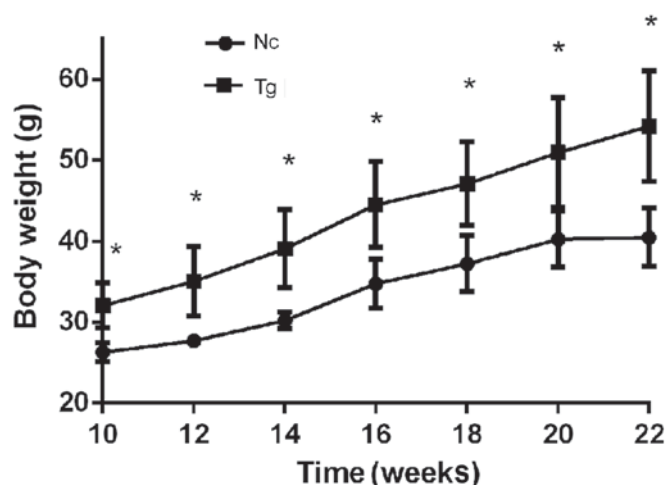

Figure 2. Average body weight of the 11β-HSD1 Tg and Nc groups with high-fat diet during 12-weeks. *P<0.05. 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; Tg, transgenic mice; Nc, negative control.

Severely impaired glucose tolerance in transgenic mice. Fasting glucose tolerance is an important indicator that comprehensively reflects insulin release and insulin resistance in an organism. Following high-fat diet induction, the intraperitoneal glucose tolerance was quantified in the transgenic and the control groups (Fig. 3). The peak glucose of the control group appeared at 15 min after injection, whereas the peak value of the transgenic group appeared at 30 min. The peak glucose of the control group and the transgenic group were 20.35±1.65 mmol/l and 27.03±1.49 mmol/l, respectively (P<0.01). Following the peak, the blood glucose levels in these two groups began to decrease. At 4 detection points between 30 and 120 min, the blood glucose levels in the transgenic group were all significantly higher compared with the in the control group. After 2 h, the blood glucose level in the transgenic group remained at 14.9 mmol/l, whereas the blood glucose level in the control group decreased to 8.58 mmol/l. Therefore, after 12 weeks of high-fat diet feeding, the transgenic group exhibited severely impaired glucose tolerance. This finding is supported by the area under the glucose tolerance curve (Fig. 4), which was 1.58-fold higher for the transgenic group compared with the control group (P<0.01).

Significant insulin resistance in the transgenic group. Prior to feeding with a high-fat diet, the 6 h fasting glucose value was 10.7 mmol/l in the transgenic group and 8.28 mmol/l in the control group (P<0.01). Following an insulin injection, the glucose of these two groups rapidly reduced and reached a minimum after 30 min at 6.25 mmol/l in the transgenic group

Table I. Biochemical parameters of the Tg and Nc groups.

Biochemical parameters	Tg	Nc	P-value
ALT, IU/l	211.33±13.47	70.4±30.71	<0.01
AST, IU/l	254.67±23.80	178±8.88	0.04
Triglyceride, μmol/l	0.81±0.06	0.38±0.17	<0.01
Total cholesterol, μmol/l	5.57±0.64	3.31±0.85	0.02
HDL-C, μmol/l	2.23±0.13	1.72±0.40	0.04
LDL-C, μmol/l	0.59±0.09	0.28±0.05	0.03
Uric acid, μmol/l	269.7±10.50	182.72±18.27	<0.01
Creatinine, μmol/l	10.33±1.25	4.6±1.85	<0.01

Tg, transgenic mice; Nc, negative control; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.
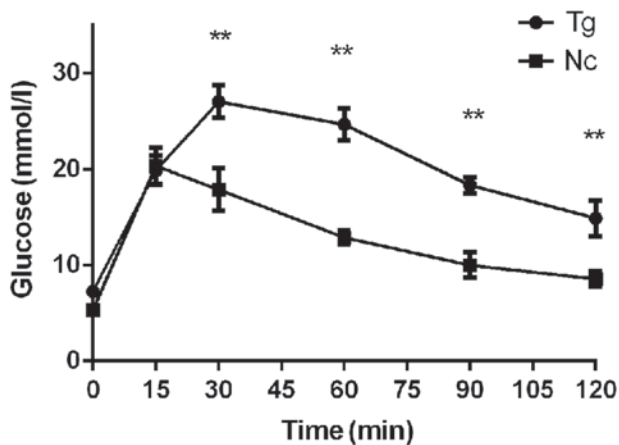

Figure 3. Intraperitoneal glucose tolerance test for the 11β-HSD1 Tg and Nc groups; **P<0.01. Tg, transgenic mice; Nc, negative control.

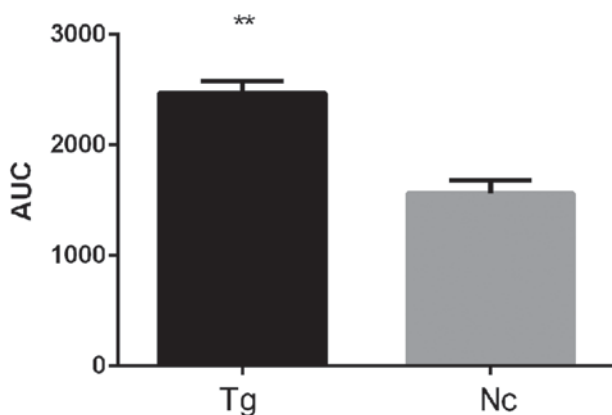Figure 4. Glucose tolerance AUC for the 11β-HSD1 Tg and Nc groups. **P<0.01. AUC, area under the curve; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; Tg, transgenic mice; Nc, negative control.

Figure 5. Insulin resistance for the 11β-HSD1 Tg and Nc groups. **P<0.01. 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; Tg, transgenic mice; Nc, negative control.

These findings revealed that the transgenic group exhibited reduced insulin sensitivity and severe insulin resistance (Fig. 5).

Tissue expression profiles of 11β-HSD1 in transgenic mice. The expression of 11β-HSD1 was the highest in the liver, followed by the kidney and muscle. The pancreases did not express evident quantity of 11β-HSD1 protein. The sizes of 11β-HSD1 protein differed by tissue, and expression levels in the positive individuals were higher compared with the negative individuals (Fig. 6). The percentage of 11β-HSD1 protein levels increased in transgenic mouse compared with the control group is presented in Fig. 7.

Severe endoplasmic reticulum stress associated with the expression of 11β-HSD1 in the transgenic group. Endoplasmic reticulum stress is central to the development of metabolic syndrome induced by insulin resistance (15). Therefore, the present study assessed endoplasmic reticulum stress in the mice. The muscle and liver in the transgenic group exhibited

and 3.73 mmol/l in the control group a significant difference was identified between these values. After 90 min, the blood glucose levels began to increase again and reached 6.23 and 4.03 mmol/l in the transgenic group and the control group, respectively, the former was 1.55 times higher than the latter.
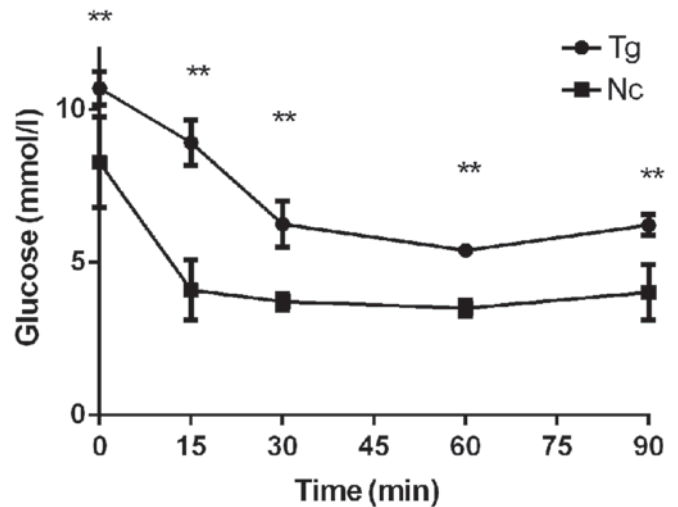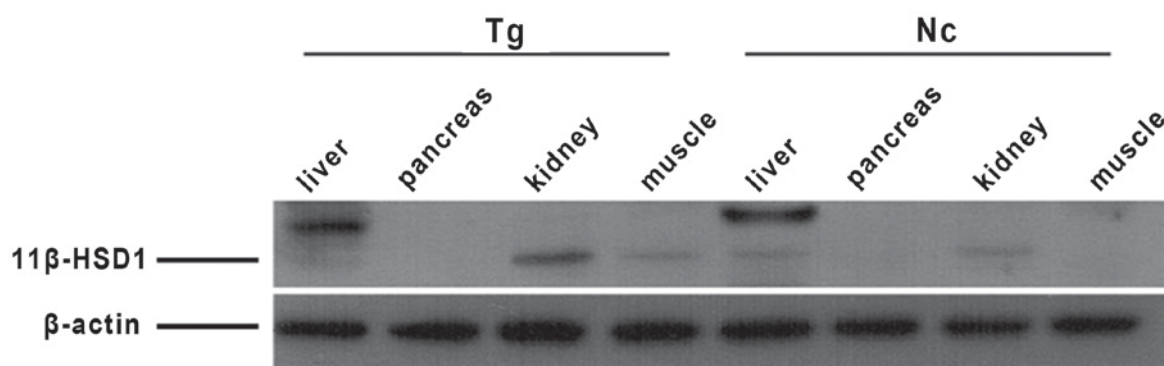
Figure 6. Tissue expression profiles of 11β-HSD1 protein in the Tg and Nc groups. The four tissue types examined include liver, pancreas, kidney and muscle. 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; Tg, transgenic mice; Nc, negative control.

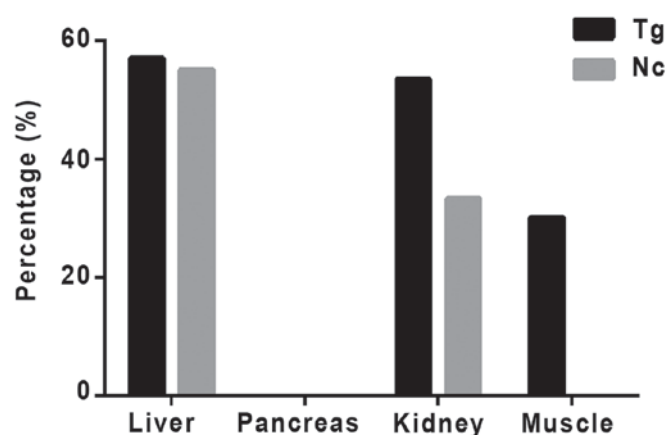Figure 7. 11β-HSD1 protein levels were higher in the Tg group compared with the Nc group. 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; Tg, transgenic mice; Nc, negative control.

severe endoplasmic reticulum stress due to high DDIT3 expression levels, and the kidney also revealed some endoplasmic reticulum stress (Fig. 8).

Hepatic lipidosis and hepatocyte injury in transgenic mice. The liver is the largest detoxification organ in the human body and impaired liver detoxification due to hepatic lipidosis and impaired liver function are important elements in the development of metabolic syndrome, with almost two thirds of patients with type 2 diabetic having hepatic steatosis (16). Therefore, the mice liver tissues in these two groups were stained with . The it was determined that transgenic mice suffered from severe hepatic lipidosis, with lipid droplets being diffused throughout the liver and liver cells exhibiting necrosis and lysis. The sections exhibited inflammatory foci and infiltration of inflammatory cells. Conversely, the control group exhibited only mild hepatic lipidosis, hepatocytes were intact and inflammatory cell infiltration was absent (Fig. 9).

Discussion

Glucocorticoids are closely associated with metabolic syndrome, primarily by promoting gluconeogenesis, reducing insulin sensitivity in peripheral tissues, inhibiting insulin secretion and promoting islet β cell apoptosis (6). 11β-HSD1 is a metabolic enzyme of glucocorticoids it may activate glucocorticoids and amplify their function locally (9). The current study established transgenic mice that systemically overexpress the 11β-HSD1 gene in order to simulate the development of metabolic syndrome. After 12 weeks of high-fat diet, mice that overexpressed the 11β-HSD1 gene exhibited obesity and significantly impaired glucose tolerance and insulin resistance. Additionally, the levels of triglycerides, total cholesterol, and high- and low-density lipoprotein cholesterol significantly increased in the transgenic mice, indicating that mice overexpressing 11β-HSD1 exhibited characteristics of metabolic syndrome. In addition, serology measurements indicated that liver and kidney function in transgenic mice was impaired. Masuzaki *et al* (11) determined that the specific overexpression of 11β-HSD1 in adipose tissues may lead to increased corticosteroid levels in adipose tissues. Paterson *et al* (12) established transgenic mice that specifically overexpressed 11β-HSD1 in the liver. These mice exhibited mild insulin resistance, hepatic lipidosis and dyslipidemia, which was accompanied by increased hepatic lipid synthesis/efflux, elevated liver X receptor and peroxisome proliferator-activated receptor mRNA expression levels, impaired hepatic lipid clearance and significant transgenic dose-dependent hypertension combined with increased expression levels of hepatic vascular angiotensinogen (12). The findings of the present study were consistent with these previous findings.

The current study revealed that the endoplasmic reticulum stress was associated with the expression of 11β-HSD1 in the tissues of transgenic mice. Additionally, Kim *et al* (17) quantified the expression of the endoplasmic reticulum stress-associated marker genes X-box binding protein 1, activating transcription factor 4 (ATF4), ATF6 and DDIT3 (also termed CHOP) to confirm that the 11β-HSD1 inhibitor carbenoxolone attenuated tunicamycin-induced endoplasmic reticulum stress and neuronal apoptosis in the hypothalamus (17). These findings indicated that 11β-HSD1 directly induced endoplasmic reticulum stress in hypothalamic neurons and the subsequent apoptosis, which also indirectly confirmed the association between 11β-HSD1 and endoplasmic reticulum stress (17). Endoplasmic reticulum stress induced by overexpression of 11β-HSD1 activates unfolded protein response, which activates c-Jun N-terminal kinase (JNK) via inositol-requiring enzyme-1, activated JNK may induce the serine phosphorylation of insulin receptor substrates and inhibits the normal tyrosine phosphorylation of insulin receptor substrate, which
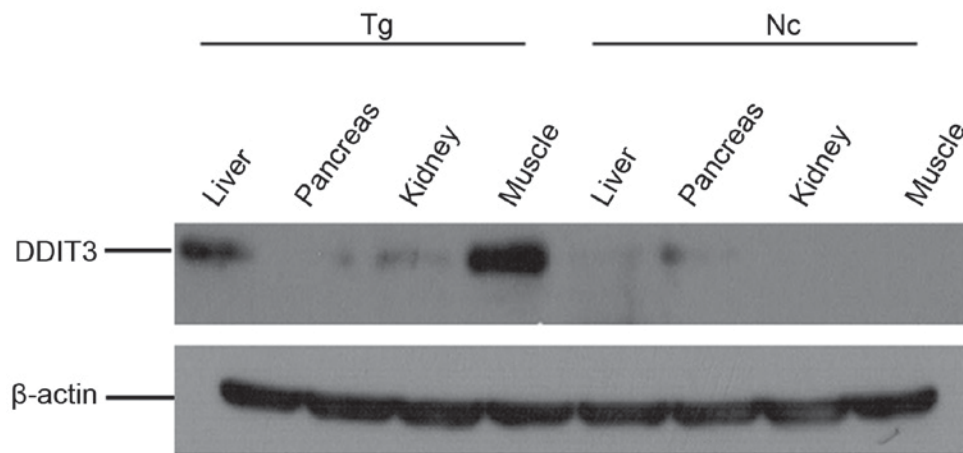
Figure 8. Tissue expression profiles of DDIT3 protein in the Tg and Nc groups. The four tissue types examined include liver, pancreas, kidney and muscle. DDIT3, DNA damage inducible transcript 3; Tg, transgenic mice; Nc, negative control.
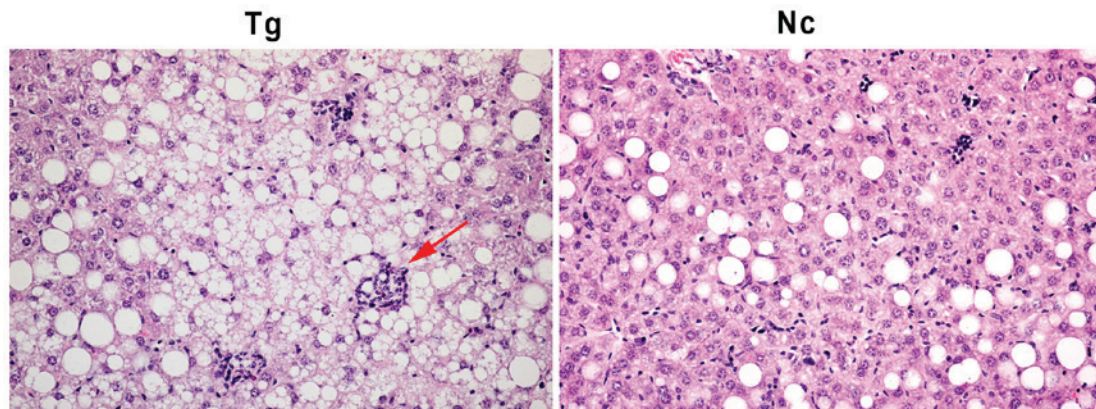

Figure 9. Histopathological changes in the mice liver of the 11β-HSD1 Tg and Nc groups; magnification, x40. Red arrow indicates inflammatory foci. 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; Tg, transgenic mice; Nc, negative control.

in turn inhibit the transduction of insulin, ultimately leading to insulin resistance (15).

The present study assessed endoplasmic reticulum stress in important metabolic syndrome-associated organs. The current findings revealed that the liver and muscle tissues of mice overexpressing 11β-HSD1 has severe endoplasmic reticulum stress and that the kidney also exhibited some endoplasmic reticulum stress. The liver is the largest detoxification organ in the human body. Impaired liver detoxification due to hepatic lipidosis and impaired liver function is an important element in the development of metabolic syndrome (18). It is possible that that the transgenic mice in the present study overexpressing 11β-HSD1 suffered from liver lesions due to their metabolic syndrome phenotype.

The quantification of liver function-associated indicators in mice revealed that the level of ALT, the most sensitive indicator of liver function, significantly increased. Additionally, the level of AST also significantly increased and was higher than the ALT level, indicating that the mouse liver parenchyma in the transgenic group was severely injured and that liver function was severely impaired. These findings were consistent with the significant endoplasmic reticulum stress observed in the liver. Histological evaluation identified severe fat deposition, lipid droplets that had diffused throughout the liver, necrotic and lysed hepatocytes, many inflammatory foci and significant inflammatory cell infiltration in the livers of transgenic mice. However, endoplasmic reticulum stress-induced hepatocyte apoptosis may be directly caused by high 11β-HSD1 expression in the liver or indirectly caused by 11β-HSD1-induced lipid deposition in the liver and this mechanism requires further investigation.

In conclusion, the present study successfully established transgenic mice that systemically overexpressed 11β-HSD1. These mice exhibited obvious characteristics of metabolic syndrome, such as severe hepatic lipidosis, hepatocyte necrosis, impaired liver function, and endoplasmic reticulum stress in muscle, liver and kidney. These findings indicate that the systemic overexpression of 11β-HSD1 in mice produces pathological changes that approximate to the clinical symptoms of human metabolic syndrome. Therefore, these mice may be used as a model for future studies of metabolic syndrome.

Acknowledgements

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