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Protective effect of metformin on renal injury of C57BL/6J mouse treated with high fat diet

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This study aimed to investigate the protective effect of metformin on renal injury of C57BL/6J mice treated with a high fat diet. High-fat diet for 12 weeks was used to establish the mice model of metabolism syndrome and the intervention of metformin (75 $mg \cdot kg^{-1} \cdot d^{-1}$) for 4 weeks, and plasma biochemical indicator and body weight were used to evaluate the model. Sterol regulatory element-binding protein (SERBP)-1c, TNF-α, NADPH Oxidase (NOX)4 mRNA was determined by real time-PCR. Phospho-AMP-activated protein kinase (P-AMPK)α protein was detected by western blotting. Oil Red O staining, Masson staining and HE staining were for observing renal pathological changes. At the end of 12th week, compared with mice on low fat diet (LFD), body weight (BW), the levels of fasting insulin (FINS), plasma and renal triglyceride (TG) were higher and plasma high density lipoprotein (HDL) and insulin sensitivity index (ISI) were significantly lower, but the levels of fasting blood glycemia (FBG), plasma total cholesterol (TC) and renal TC had no changes; Oil Red O staining revealed renal lipids deposition, Masson staining and HE staining revealed glomerular hypertrophy, matrix increasing, and inflammatory cells infiltration in glomerular; the expression of p-AMPKα protein decreased and the expression of SREBP-1c, TNF-α, NOX4 mRNA increased significantly in mouse treated with high fat diet (HFD). Compared with the HFD group, through metformin interventing, metabolic disorders were significantly improved, renal lipids deposition and other pathological changes were ameliorated, the expression of p-AMPKα protein increased and the expression of SREBP-1c, TNF-α, NOX4 mRNA decreased significantly. Metformin improved metabolic disorders, upregulated activity of renal AMPK, diminished the expression of renal SREBP-1c, TNF-α, NOX4 mRNA, decreased accumulation of renal lipids, and prevened renal injury.

1. Introduction

Obesity is considered as a major promotos of metabolic syndrome (Bagby 2004), and there are several pathophysiologic disturbances including inflammation, oxidative stress, insulin resistance, changes of adipokines, activation of renin–angiotensin–aldosterone system (RAS), macrophage phenotypic switch that contribute to renal injury (Hunley et al. 2010). Dyslipidemia, hyperglycemia, obesity, and hypertension, four of the risk factors for metabolic syndrome, are each independently characterized by increased oxidative stress and a proinflammatory state (Ebenezer et al. 2009).

Systemic lipid overload, "lipotoxicity", is proposed as an important mechanism underlying the metabolic syndrome (Deji et al. 2009). Several studies (Jiang et al. 2005; Kume et al. 2007; Deji et al. 2009) demonstrated that mice on a high fat diet showed renal pathophysiological alterations including renal lipid accumulation and an increased accumulation of type IV collagen in glomeruli. Accumulation of excess lipids in the nonadipose tissues can lead to lipotoxicity and cellular dysfunction, so far as to apoptosis (Schaffer 2003).

AMP-activated protein kinase (AMPK) is a ubiquitously expressed heterotrimeric kinase that acts as a cellular energy sensor. Among several biological processes shown to be regulated by AMPK are glucose and fatty acid uptake, protein synthesis, gene transcription,inflammation, ion transport, and nitric oxide synthesis (Hallows et al. 2010), and AMPK also is considered as a key factor of lipometabolism (Kume et al. 2007). Several upstream AMPK kinases (LKB1 and CaMKK) (Hallows et al. 2010) and mang drugs like (metformin (Zhou et al. 2001), esrosiglitazone (Ceolotto et al. 2007)) can activate AMPK, thus causing a series of effects. In this study, we established the model of metabolic syndrome, then treated with metformin for activating AMPK, and observed the changes of renal lipid accumulation, inflammation and oxidative stress, and the protective effect of metformin for renal injury.

2. Investigations and results

2.1. Alterations of BW, biochemical indicator, urine protein and renal lipids

The body weight was significantly heavier, the levels of plasma TG and FINS were obviously higher, the levels of ISI and plasma HDL were significantly lower, and abundance of urine protein increased distinctly in the mice on HFD compared with those in mice on LFD at weeks 12 of the experimental period. After metformin intervention for 4 weeks, these metabolic disturbances were improved significantly (Table 1). In the kidney sections

Table 1: Comparison of body weight, plasma biochemical indicator, ISI and urine protein between 3 groups at the end of 12 weeks

	BW (g)	PBG (mmol/L)	FINS (mU/L)	ISI ($\times 10^{-3}$)	TG (mmol/L)	TC (mmol/L)	HDL (mmol/L)	urine protein (mg/24 h)
LFD	25.4 ± 0.68	11.35 ± 1.74	8.92 ± 3.31	11.23 ± 3.90	0.42 ± 0.04	2.18 ± 0.16	1.03 ± 0.08	0.98 ± 0.27
HFD	32.8 ± 1.69**	11.46 ± 2.30	15.37 ± 5.10*	6.56 ± 2.97*	0.84 ± 0.41*	2.43 ± 0.31	0.89 ± 0.09*	2.02 ± 0.68*
M	29.0 ± 1.71▲	11.06 ± 2.01	9.31 ± 3.62▲	11.00 ± 3.14▲	0.54 ± 0.13	2.03 ± 0.41	1.01 ± 0.10▲	1.65 ± 0.47

* $P < 0.05$, versus C57BL/6J mice with LFD; ** $P < 0.01$, versus C57BL/6J mice with LFD; ▲ $P < 0.05$, versus C57BL/6J mice with HFD; ▲▲ $P < 0.01$, versus C57BL/6J mice with HFD.

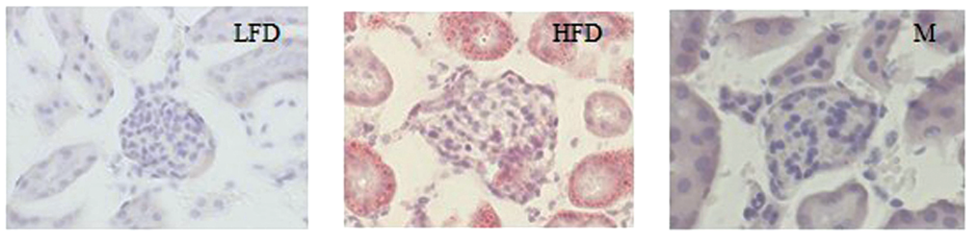

Fig. 1: Changes of the deposition of neutral lipid in kidney from mice in each group under microscope (Oil Red O staining, 400 ×).

Table 2: Comparison of accumulation of renal lipid between 3 groups at the end of 12 weeks

	TG (mmol/L)	TC (mmol/L)
LFD	0.73 ± 0.24	0.57 ± 0.18
HFD	1.35 ± 0.38**	0.70 ± 0.17
M	0.79 ± 0.26▲▲	0.58 ± 0.12

** $P < 0.01$, versus C57BL/6J mice with LFD; ▲▲ $P < 0.01$, versus C57BL/6J mice with HFD.

from mice examined by oil red O staining, the accumulation of neutral lipid was detected in the glomeruli and renal tubules of the mice on a HFD, but not in the mice on the LFD, and compared with the mice on the HFD, the deposition of neutral lipid in kidney in the mice treated with metformin was alleviated distinctly (Fig. 1). The level of renal TG at week 12 was significantly higher in mice on the HFD than these on the LFD, but renal TC was not changed. Whereas, metformin intervention makely reduced the abundance of renal TG (Table 2).

2.2. *Renal pathogenetic changes in mice in each group*

To evaluate the effects of treating with HFD and metformin intervention on renal histopathology, we performed the Masson staining and HE staining on kidney sections. Compared with the mice on LFD, renal pathogenetic changes were obversed in the mice on HFD, concrete shows were glomerular hypertrophy, matrix increasing and inflammatory cells infiltration in glomerular, and these changes were alleviated after metformin intervention (Fig. 2).

2.3. *Alterations of the expression of TNF-α, NOX4, SREBP-1c mRNA and p-AMPKα protein in kidney*

We measured the mRNA abundance of two key factors, TNF-α and NOX4, to evaluate inflammation and oxidative stress induced by treating with HFD. Compared with the mice under LFD, in those mice with HFD TNF-α and NOX4 mRNA abundance, significantly increased and abundance of TNF-α and NOX4 mRNA were obviously alleviated by treating with metformin (Fig. 3).

SREBP-1 has been considered as key regulators of fatty acid metabolism. Compared with the mice under LFD, mRNA abundance of SREBP-1c increased significantly, and this trend was reversed by treating with metformin (Fig. 4). Correlation analysis revealed that mRNA abundance of SREBP-1c positively correlated with abundance of renal TG ($r = 0.846$, $p < 0.01$).

AMPK is a heterotrimer consisting of a catalytic α-subunit and regulatory β- and γ-subunits. Phosphorylation at αThr^{172} is

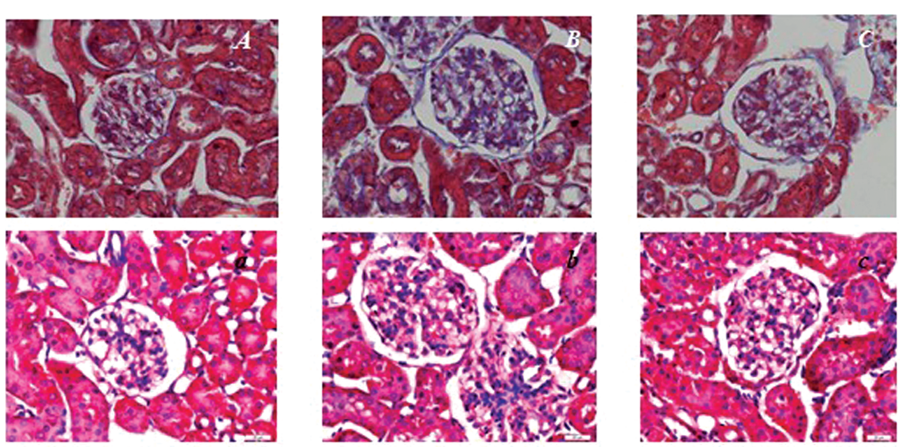

Fig. 2: Renal pathological changes under microscope at week 12 of the experimental period for a mouse on a LFD (A,a), HFD (B,b), and HFD and metformin (C,c). (A, B, C are Masson staining and a, b, c are HE staining, 400 ×).

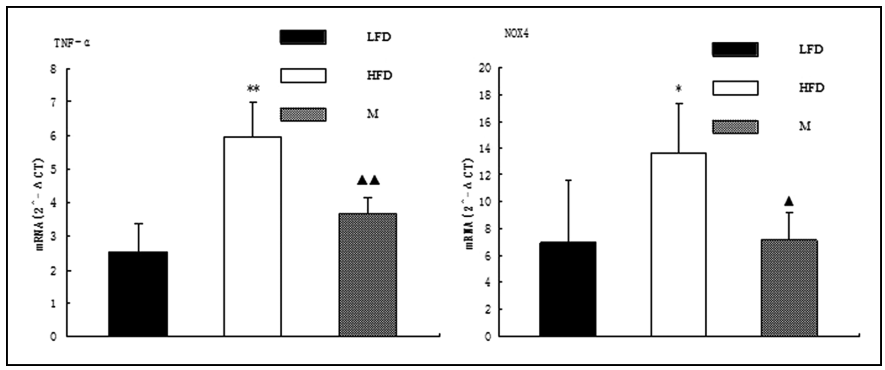

Fig. 3: (1). Comparison of TNF- α mRNA between 3 groups at the end of 12 weeks. ** $P < 0.01$, versus C57BL/6J mice with LFD; ▲$P < 0.05$, versus C57BL/6J mice with HFD. (2). Comparison of NOX4 mRNA between 3 groups at the end of 12 weeks. ** $P < 0.01$, versus C57BL/6J mice with LFD; ▲$P < 0.05$, versus C57BL/6J mice with HFD.

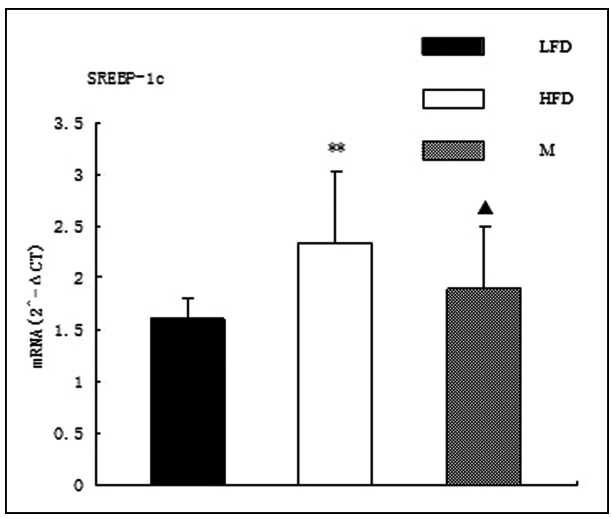

Fig. 4: Comparison of SREBP-1c mRNA between 3 groups at the end of 12 weeks. ** $P < 0.01$, versus C57BL/6J mice with LFD; ▲$P < 0.05$, versus C57BL/6J mice with HFD.

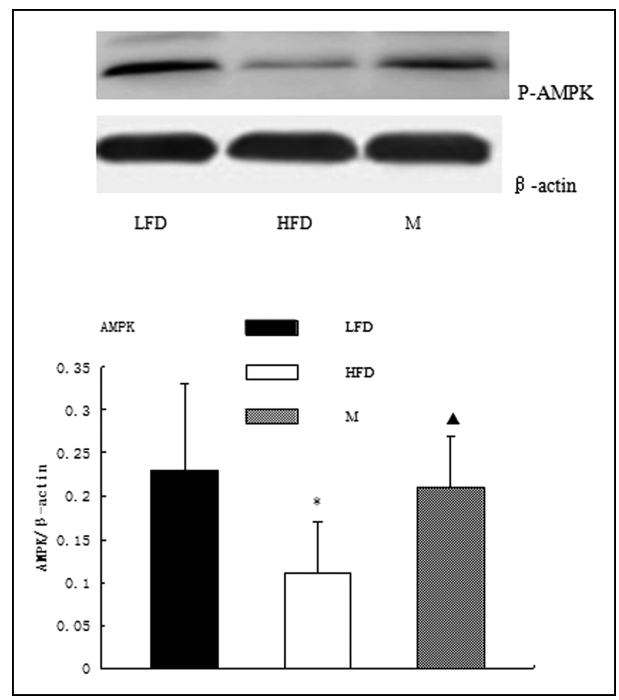

Fig. 5: Comparison of AMPK activity in kidney between 3 groups at the end of 12 weeks. * $P < 0.05$, versus C57BL/6J mice with LFD; ▲$P < 0.05$, versus C57BL/6J mice with HFD.

essential for activation of AMPK, so we evaluated the activity of AMPK by measuring abundance of p-AMPKα protein. The abundance of renal p-AMPKα protein increased obviously in the mice on HFD compared with those in mice on the LFD at weeks 12 of the experimental period. With metformin intervention for 4 weeks, its abundance decreased significantly (Fig. 5). Correlation analysis revealed that the abundance of p-AMPKα protein negatively correlate with mRNA abundance of SREBP-1c, TNF-α, NOX4 in kidney section, and abundance of renal TG ($r = -0.815$, $p < 0.01$; $r = -0.665$, $p < 0.05$; $r = -0.714$, $p < 0.05$; $r = -0.910$, $p < 0.01$).

3. Discussion

Treated with high fat diet for 12 weeks, C57BL/6J mice showed conditions of metabolic syndrome and renal structural and functional injury, including obesity. The level of plasma triglycesides was higher, the concentration of plasma high density lipoprotein and insulin sensitivity index decreased significantly, and glomerular hypertrophy, matrix increasing, renal lipid deposition and urinary protein increased. These indicates that long-term high fat diet can lead to systemic metabolic disturbance and renal injury.

Oxidative stress occurs when the levels of reactive oxygen species (ROS) overcome the antioxidant capability of the tissue. ROS have been considered as candidates for eliciting endothelial dysfunction and renal injury in obesity and insulin resistance (Knight and Imig 2007). NADPH oxidase (NOX) can lead to the increased generation of ROS, by up-regulating components of tissue RAS in metabolic syndrome (Ebenezer et al. 2009). Levels of NOX have been reported to be elevated in animal models of obesity, and up-regulation of NOX has also been linked to the development of glomerulosclerosis (Knight and Imig 2007). NOX4 is a recently described nonphagocytic NAPDH oxidase that is highly expressed in the kidney (Sharma et al. 2008). The study implemented by Sharma et al. (2008) demonstrated that systemic adiponectin deficiency led to up-regulation of NOX4 in the kidney and podocytes, providing another critical link to obesity, insulin resistance, and oxidant stress. In keeping with above, our results showed that compared with mice on LFD, the expression of NOX4 mRNA increased evidently, and its expression decreased remarkably after metformin intervention, indicating that metformin could ameliorate renal oxidative stress induced by treating with high fat diet. Activation of AMPK has been reported to suppress expression of NOX4 protein, thus improving histiocytic injury (Sharma et al. 2008), and our study demonstrated that through metformin interventing, renal AMPK activity was up-regulated significantly, and negatively correlated with abundance of NOX4 mRNA of renal cortex. Therefore we infer that metformin meliorates renal oxidative stress induced by high fat diet at least partially *via* activating AMPK.

Fat distribution, particularly visceral adiposity, is a key determinant of renal dysfunction. Visceral fat secretes bioactive substances, that promote a low-grade chronic inflammatory state (Mathieu et al. 2009). TNF-α has been shown to be a key inflammatory factor leading to insulin resistance (Borst 2004). It has apoptotic and pro-inflammatory properties, elicits a direct negative effect on kidney function, and glomerular injury and albuminuria were exacerbated after TNFα infusion in rats (Knight and Imig 2007). Glomeruli of patients with obesity-related glomerulopathy (ORG) contained not only elevated protein levels of TNF-α but also elevated TNF-α mRNA, indicating that TNF-α is an important factor involved in the development of ORG. This also implies that glomerular cells themselves could act as active responders to the abnormal ambience of elevated levels of inflammatory factors, thus accelerating the process of glomerular injury (Wu et al. 2006). Data from our study showed expression of TNF-α mRNA of renal cortex from C57BL/6J mice treated with HFD was elevated, and HE staining revealed inflammatory cell infiltration in glomeruli, indicating that renal parenchyma cells interact with inflammatory cells and contribute to renal injury (Clarke and Hardie 1990; Zang et al. 2004; Awazawa et al. 2009; Kim et al. 2009; Kim et al. 2009; Yuan et al. 2010). Several studies (Sag et al. 2008; Jeong et al. 2009; Lu et al. 2010) demonstrate that up-regulation of AMPK activity could improve histiocytic inflammatory injury. However, Hallows et al. (2010) pointed out that activation of AMPK has anti-inflammatory effects in stimulated macrophages and MRL/lpr mesangial cells, yet the role of AMPK in inflammatory diseases of the kidney is not clear. In the present study, we observed that expression of TNF-α mRNA decreased remarkably after metformin intervention, and abundance of TNF-α mRNA of renal cortex negatively correlated with renal AMPK activity. From above, we infer that metformin improves renal inflammatory injury induced by high fat diet at least partially by up-regulating the activity of AMPK.

Accumulation of excess lipids in the nonadipose tissues can lead to lipotoxicity and cellular dysfunction (Hallows et al. 2010). Deposition of lipids in the kidney has been implicated in the progression of glomerular and tubulointerstitial lesions in metabolic syndrome and chronic glomerulopathies. Cellular lipid homeostasis is regulated by influx, synthesis, catabolism, and efflux of lipids, and an imbalance in these processes can lead to conversion of macrophages, mesangial cells, and vascular smooth muscle cells into foam cells (Kim et al. 2009). Several studies (Jiang et al. 2005; Wu et al. 2006; Kume et al. 2007) about obesity and metabolic syndrome, discovered that expression of factors about lipid synthesis increased significantly in kidney, such as sterol regulatory element-binding proteins (SREBPs), fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC). SREBPs have been considered as key regulators of both fatty acid and cholesterol metabolism. Three SREBP isoforms, SREBP-1a, SREBP-1c, and SREBP-2, have been identified, and SREBP-1 preferentially activates genes involved in fatty acid synthesis, thus SREBP-2 preferentially activates genes involved in cholesterol synthesis (Jiang et al. 2005). In SREBP-1c$^{-/-}$ mice, the increased mRNA levels of molecules related to glomerulosclerosis, such as plasminogen activator inhibitor-1, vascular endothelial growth factor, and two ECM proteins, type IV collagen and fibronectin induced by HFD in wild-type mice were significantly attenuated (Jiang et al. 2005). Data from our study showed that expression of SREBP-1c mRNA of renal cortex from C57BL/6J mice treated with HFD was obviously much higher than that in LFD-fed mice. This was in agreement with famous studies (Jiang et al. 2005; Kume et al. 2007; Deji et al. 2009). In our study, compared with LFD group mice, abundance of renal triglycerides in HFD-fed animals increased significantly, and positively correlate with abundance of SREBP-1c mRNA of the renal cortex. These data indicated that renal lipids synthesis is increased in metabolic syndrome.

AMPK regulates abundance of cellular lipids in several ways, but study results about changes of AMPK activity in obesity were not identical. Compared with LFD-fed mice, renal AMPK activity of C57BL/6J animals treated with HFD decreased significantly in our study, indicating that HFD could induce down-regulation of renal AMPK activity. Studies (Clarke and Hardie 1990; Zhou et al. 2001; Awazawa et al. 2009; Kim et al. 2009; Yuan et al. 2010) indicate that persistently activating AMPK with drugs or other methods could down-regulate factors involved in lipids synthesis, such as SREBP-1, FAS, and up-regulate enzymes involved in fatty acid oxidation, such as acetyl-CoA oxidase (ACO), medium chain acyl-CoA dehydrogenase (mCAD), carnitine palmitoyl transferase-1 (CTP-1), peroxisome proliferator-activated receptor-γcoactivator-1α (PGC-1α), uncoupling protein 2 (UCP2), peroxisome proliferator-activated receptor-α (PPAR-α), and inactivating HMG-CoA reductases. The present study revealed that expression of p-AMPKα protein negatively correlates with abundance of SREBP-1c mRNA and triglycerides in renal cortex, indicating that AMPK influences renal lipidic abundance possibly by regulating downstream target genes. KS renal AMPK activity was increased distinctly by treating with metformin, we infer that metformin could improve accumulation of renal lipids at least partially by up-regulating the activity of AMPK.

In summary, metformin improves metabolic disorders, up-regulates renal activity of AMPK, diminishes the expression of SREBP-1c, TNF-α, NOX4 mRNA, decreased accumulation of renal lipid, and essentially prevents renal injury.

4. Experimental

4.1. Materials

Antibody against P-AMPKα was obtained from CST. The antibody for β-actin and peroxidase-conjugated affinipure goat anti-rabbit IgG were purchased from ZSGB-BIO. Oil red O was purchased from Solarbio. Kit for triglyceride, cholesterol, high density lipoprotein was purchased from the Nanjing Jiancheng Bioengineering Institute. Insulin radioimmunoassay kit was purchased from HTA Co., Ltd. SERBP-1c, TNF-α, NOX4 prime and Real time-PCR kit were obtained from TaKaRa Biotechnology. Metformin was purchased from Pacific Pharmaceutical Technology Group.

4.2. Animals

The 6 weeks male C57BL/6J mouse were purchased from China medical university and were housed in colony cages, maintained on a 12-h light/12-h dark cycle. All mice were randomly divided into three groups: group I: mouse fed with low fat diet (LFD, 10% of total calories from fat); group II: mouse fed by high-fat diet (HFD, 59% of total calories from fat); and group III: mouse fed by high-fat and metformin ($75\,mg\,kg^{-1}\cdot d^{-1}$) for 4 weeks. High-fat diet for 12 weeks was used to establish the mice model of metabolic syndrome. At the end of the 12th week, mice were placed in metabolic balance cages for urine collection to measure protein. Mice were then anesthetized by intraperitoneal injection of pentobarbital followed by blood draw for biochemical assays and kidney removal for RNA, protein, and lipid extraction. The kidneys were then processed for hematoxylin and eosin (HE), Oil Red O and Masson staining.

4.3. Oil red O staining, masson staining, and HE staining

Frozen sections were used for Oil Red O staining to determine renal accumulation of neutral fats. Paraffin sections were used for Masson staining, and HE staining to observe the renal structural changes.

4.4. Lipid extraction and lipid composition measurement

Total lipids were extracted from 100 mg of tissue by the method of Folch. In the first step, the lipids were extracted by homogenizing the tissue with 2:1 chloroform-mathanol (v/v), then shaking for 20 min in room temperature, and centrifugating under 2000 r/min for 10 min. Supernatant was taked and

mixed with 5-fold its volume of water, shaking for seconds and centrifugating, abnegates supernatant, swab-offing the rest liquid, remaining substance dissolved in 1 ml alcohol. Lipidic detection was implemented according to kit introduce.

4.5. RNA extraction and real-time PCR

Total RNA was isolated from the renal cortex using TRIzol from Invitrogen, and cDNA was synthesized using reverse transcript reagents from Takara. iQSYBR Green Supermix was used for realtime PCR (ABI Prime TM 7500 Sequence Detection System). The levels of mRNA expression were quantified using the method of 2^{-ct} . The sequence of primers were the following: SREBP-1cR: 5'TGGAGACATCGCAAACAAG3', SREBP-1cF: 5'GGTAGACAACAGCCGCATC3'; TNFαR: 5'GGCAGGTCTACTTTGGAG3', TNFαF: 5'GGAAAGCCCATTTGAGT3'; NOX4R: 5'CATTGGGCGTCCTCGG3', NOX4F: 5'CAACAGCGTGCGTCTAAC3'.

4.6. Western blot analysis

An equal abundance of protein (80 μg) from each sample was separated on SDS-PAGE gels and transferred to PVDF membranes. Blots were blocked with 5% BSA in Tris-buffered saline with 0.1% Tween-20 at room temperature for 60 min, followed by overnight incubation with primary antibodies at 4 °C. After being washed with TBST three times, the blots were hybridized with secondary antibodies conjugated with horseradish peroxidase in 5% BSA dissolved in TBST at room temperature for 2 h and washed three times with TBST. The membranes were then incubated with enhanced-chemiluminescence reagents for ECL. Image-Pro Plus software was used for analysis.

4.7. Statistical analyses

Results are expressed as means ± SEM. Oneway ANOVA followed by Scheffe′'s test was used to determine the significance of differences among three independent groups. Statistical significance was accepted at the $p < 0.05$ level.

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Conflict of interest: The authors have declared no conflict of interest.

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