



Effect of Bilirubin on Triglyceride Synthesis in Streptozotocin-Induced Diabetic Nephropathy

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We aimed to elucidate the effect of bilirubin on dyslipidemia and nephropathy in a diabetes mellitus (DM) type I animal model. Sprague-Dawley rats were separated into control, DM, and bilirubin-treated DM (Bil) groups. The Bil group was injected intraperitoneally with 60 mg/kg bilirubin 3 times per week and hepatoma cells were cultured with bilirubin at a concentration of 0.3 mg/dL. The Bil group showed lower serum creatinine levels 5 weeks after diabetes onset. Bilirubin treatment also decreased the amount of mesangial matrix, lowered the expression of renal collagen IV and transforming growth factor (TGF)- β 1, and reduced the level of apoptosis in the kidney, compared to the DM group. These changes were accompanied by decreased tissue levels of hydrogen superoxide and NADPH oxidase subunit proteins. Bilirubin decreased serum total cholesterol, high-density lipoprotein cholesterol (HDL-C), free fatty acids, and triglycerides (TGs), as well as the TG content in the liver tissues. Bilirubin suppressed protein expression of LXR α , SREBP-1, SCD-1, and FAS, factors involved in TG synthesis that were elevated in the livers of DM rats and hepatoma cells under high-glucose conditions. In conclusion, bilirubin attenuates renal dysfunction and dyslipidemia in diabetes by suppressing LXR α and SREBP-1 expression and oxidative stress.

Keywords: Bilirubin; Diabetes-Related Complications; Triglyceride; Fibrosis; Transforming Growth Factor beta

INTRODUCTION

Bilirubin is an end product of heme metabolism; heme oxygenase cleaves the heme ring, producing carbon monoxide, iron, and biliverdin, which is converted by biliverdin reductase into bilirubin (1). Recent studies have indicated that bilirubin, at mildly elevated levels, acts as a cytoprotectant in diabetes, diabetic complications, and in diseases of the cardiovascular and renal systems, mainly through its antioxidative and anti-inflammatory properties (2). By inhibiting oxidative stress and down-regulating NADPH oxidase, bilirubin attenuates pancreatic damage (3) and renal injury (4) in hyperbilirubinemic Gunn rats compared to genetic controls with streptozotocin (STZ)-induced type I diabetes. The beneficial effects of bilirubin are also reported in human diseases including metabolic syndrome (5-7), diabetes (8, 9), diabetic nephropathy (9, 10), hypertension (11), IgA nephropathy (12), and cardiovascular disease (13). Bilirubin is inversely related with insulin resistance, as estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) (5-7) and the concentration of triglycerides (TGs) in human sera (5-9). Studies have also reported a relationship between serum bilirubin and the concentration of total cholesterol (5), low-density lipoprotein cholesterol (LDL-C) (5), rem-

nant lipoprotein cholesterol (14), and high-density lipoprotein cholesterol (HDL-C) (7,8), although the significances of these relationships varied depending on the experimental conditions. Bilirubin is evidently closely related to lipid metabolism and insulin resistance; however, few studies have evaluated the mechanisms by which bilirubin regulates dyslipidemia. In 1979, Shepherd et al. (15) reported that a high concentration of bilirubin (0.45 mM) inhibited lipolysis and stimulated glucose oxidation in rat fat cells in the presence of an equimolar concentration of bovine serum albumin. To the best of our knowledge, this is the only study in which the influence of bilirubin on lipid metabolism was examined. Insulin resistance is a potentially important mechanism leading to the development of dyslipidemia, especially in increasing TG and decreasing HDL-C levels (8-12). However, the inverse relationship between serum TG concentration and bilirubin was evident in Korean women after adjusting for HOMA-IR, body mass index, C-reactive protein, and menopausal status (7). Therefore, we postulate that bilirubin regulates TGs through a direct pathway. We examined the effects of bilirubin on renal function and serum lipid levels, and investigated possible mechanisms by which bilirubin regulates lipogenesis in rats with STZ-induced type I diabetes.

MATERIALS AND METHODS

Animals

Eighteen male Sprague-Dawley (SD) rats (Orient Bio Inc., Seongnam, Korea) weighing 200-250 g were housed in a temperature-controlled animal facility at Seoul National University Bundang Hospital. Animals were exposed to a daily 12-hr light/dark cycle. The animals were provided with standard rat chow and sufficient water and were adapted to the environment for 1 week. The rats were assigned to the experiment groups as follows: control (n = 5), diabetes control (DM; n = 8), and DM rats treated with bilirubin (Bil; n = 5). Twenty-four-hour urine volumes were measured before and 5 weeks after inducing diabetes by injecting STZ intraperitoneally. After 8 hr of fasting, STZ (Sigma, MO, USA) at a dose of 65 mg/kg body weight was injected into the peritoneums of rats belonging to the DM and Bil groups. Bilirubin (Sigma, #B4125), dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 20 mg/mL, was administered to the Bil group by intraperitoneal (IP) injection 3 times per week (60 mg/kg) after the diabetic status of these rats had been confirmed. DMSO was injected into the control and DM groups as vehicle. Five weeks after diabetes induction, rats were anesthetized using zolazepam and xylazine, blood was sampled, and then kidney and liver tissues were collected and fixed in 10% formalin for 24 hr for morphological analyses. This study was approved by and conducted in accordance with the guidelines of the Seoul National University Bundang Hospital Committee of Animal Experiments.

Physiologic measurements

Glucose levels in sera collected from rat tail veins 6 hr after fasting were measured using a HemoCue B-glucose kit (HemoCue AB, Angelholm, Sweden) before and 3 days after STZ injection. Fasting glucose levels greater than 300 mg/dL confirmed the induction of diabetes. Thereafter, serum glucose levels were measured three times a week. Long-acting insulin (Lantus, Aventis, Paris, France) was administered subcutaneously (2-4 IU/injection/2 days) into the diabetic rats, maintaining their non-fasting glucose level at approximately 400 mg/dL (3). Glycated hemoglobin (HbA1c) levels were measured at baseline and at the time of sacrifice by using the DCA 2000+ HbA1c kit (Bayer, IN, USA). The 24-hr urine volume was measured and the urine albumin concentration was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Exocell, Philadelphia, PA, USA). Creatinine levels in sera (Bioassay Systems, Hayward, CA, USA) and urine (Exocell) were also measured using ELISA kits. We measured serum TG, HDL-C, free fatty acid, and total cholesterol by using an automatic analyzer (ADIVA 2400, Siemens, USA).

Detection of ROS generation

Oxidation-sensitive 2',7'-dichlorofluorescein diacetate (H₂D-

CF-DA; Sigma, MO, USA) was used to measure the intracellular production of ROS. Kidney lysates were incubated with 10 μ M H₂DCF-DA at 37°C for 30 min. The fluorescence intensity was measured using a fluorescence spectrophotometer at excitation and emission wavelengths of 490 nm and 526 nm, respectively.

Liver tissue TG measurement

One hundred milligrams of liver tissue per animal was collected and the amount of TG per gram of tissue was measured using a TG quantification kit (ab65336; Abcam, CA, USA) according to the manufacturer's instructions.

Histologic analyses

Kidney sections, 3 μ m in thickness, were stained with periodic acid-Schiff (PAS). A computer-assisted image analysis system was used to quantify the mesangial expansion in glomeruli. The ratios of mesangial area to glomerulus area were determined for 30 glomeruli from each rat. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed on kidney paraffin sections by using the In Situ Cell Death Detection Kit (Fluorescein, Roche Diagnostics, Mannheim, Germany) to detect apoptotic cells in glomeruli. The positive rate of TUNEL staining was examined in 30 glomeruli per kidney section and the mean value for each group was compared.

Hepatoma and mesangial cell culture

Hepatoma cells (ATCC CRL-1830) were cultured in Dulbecco's modified Eagle's medium (Gibco, Life Technologies, USA) in the presence of normal glucose (NG; 100 mg/dL D-glucose) or high glucose (HG; 450 mg/dL D-Glucose) and supplemented with 10% heat-inactivated fetal calf serum and 1% antibiotics (10 mg/mL streptomycin and 10,000 IU/mL penicillin G) in a humidified environment with 5% CO₂ at 37°C. NG, HG, and Bil cells were initially grown in NG medium. The HG and Bil groups were transferred to HG once they had reached 70%-80% cell confluence in NG medium. The Bil group was treated with 0.3 mg/dL bilirubin (Sigma #B4125, MO, USA) dissolved in DMSO prior to being transferred to HG medium. The cells were harvested for protein extraction after 8 hr of culturing in HG medium. The same experimental procedure was repeated using mesangial cells obtained from C57B6 mice (3).

Transfection of small interfering RNA (siRNA)

A 6-well tissue culture plate was seeded with 2×10^5 hepatoma cells/well in HG medium (450 mg/dL D-glucose). Cells were incubated at 37°C in a CO₂ incubator overnight until they reached 60%-80% confluence and cells were then treated with bilirubin (0.3 mg/dL) or vehicle (DMSO). Transfection of LXR α siRNA (Labome TF711433, NJ, USA) was performed 8 hr after bilirubin treatment. The cells were divided into the following groups:

untreated HC control cells (no bilirubin or siRNA treatment), HL cells transfected with LXR α siRNA, and BilC cells treated with bilirubin but not transfected with siRNA.

Protein extraction and Western blotting

Tissues and cells were homogenized in 10 mM Tris-buffered saline (20 mM Tris-HCl pH 7.4, 0.25 M sucrose, and 1 mM EDTA) in the presence of a protease inhibitor cocktail and were then centrifuged. Proteins were extracted and were then quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, IL, USA). Aliquots of 80 μ g were loaded onto a SDS-polyacrylamide gel and transferred to nitrocellulose paper after electrophoresis. The membrane was incubated overnight in 5% non-fat milk with the following primary antibodies: anti-collagen IV (sc-11360), anti-actin (sc-1616), anti-NOX 4 (sc-20141), anti-p22phox (sc-20781), anti-SREBP-1 (sc-367), anti-LXR α (sc-1202), anti-SCD-1 (sc-14720), and anti-FAS antibody (sc-1023) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and bands were visualized using an enhanced chemiluminescence substrate (Thermo Fisher Scientific). The densities of the bands were quantified using the GS-700 Imaging Densitometer (Bio-Rad, CA, USA) and their values were normalized to that of actin in the control.

Statistical analysis

The results were calculated as the mean \pm standard deviation. The statistical analyses were performed using SPSS software (version 21.0, IBM, NY, USA). The difference of continuous variables between the groups was analyzed using analysis of variance or Student’s *t*-test, according to the number of groups. *P* values < 0.05 were considered statistically significant.

RESULTS

Physiologic data

Prior to diabetes induction, body weights, food and water intake, and the levels of serum fasting glucose, HbA1c, and creati-

nine, as well as urine volumes and urine albumin to creatinine ratios of SD rats did not significantly differ between the groups (Table 1). Three days after STZ injection, the rats in the DM and Bil groups were confirmed to have induced diabetes, as defined by a fasting glucose level of 300 mg/dL or greater (DM: Bil group = 416 \pm 55:397 \pm 35 mg/dL, *P* = 0.724). The serum creatinine was increased to 0.71 \pm 0.22 mg/dL in the DM group during the 5 weeks following diabetes induction, a higher level compared to that of the Bil group (0.45 \pm 0.02 mg/dL, *P* = 0.025). The levels of fasting glucose and HbA1c, on the other hand, were not different between the DM and Bil groups (Table 2). The amount of 24-hr urine albumin tended to be higher in the DM group than in the Bil group; however, no statistical difference was observed in rats once they were sacrificed. The levels of 24-hr urine albumin and serum creatinine did not differ significantly between the control and Bil groups (ANOVA and post-hoc Tukey test, *P* = 0.613 and *P* = 0.810, respectively).

Renal pathology and ROS

We measured the ratio of mesangial matrix area to glomerular area in each rat to estimate the severity of diabetic change (Fig. 1A). The area ratios were the highest in the DM group, followed

Table 1. Baseline characteristics of rats

Parameters	Control (n = 5)	DM (n = 8)	Bil (n = 5)	<i>P</i> value
Body weight (g)	350.4 \pm 19.1	327.8 \pm 18.4	352.2 \pm 14.3	0.084
Water intake (mL/day)	25.4 \pm 9.8	20.5 \pm 12.2	18.7 \pm 9.6	0.604
Food intake (g/day)	17.9 \pm 6.1	14.6 \pm 5.4	17.5 \pm 3.9	0.462
Fasting glucose (mg/dL)	151 \pm 17	142 \pm 9	150 \pm 13	0.351
HbA1c (%)	3.0 \pm 0.2	3.0 \pm 0.6	3.1 \pm 0.2	0.167
Serum Creatinine (mg/dL)	0.52 \pm 0.08	0.56 \pm 0.22	0.52 \pm 0.02	0.839
Urine volume (mL/day)	14.8 \pm 9.0	10.7 \pm 4.7	13.6 \pm 5.5	0.485
UACR (mg/g cr)*	146 \pm 71	178 \pm 68	201 \pm 53	0.433

*UACR, urine albumin to creatinine ratio sampled from 24-hr urine, presented as mg/g creatinine. The *P* value was estimated using the analysis of variance (ANOVA) test. DM and Bil groups had diabetes induced by intraperitoneal (IP) injection of streptozotocin (STZ). The Bil group was administered bilirubin (60 mg/kg) three times a week for 5 weeks by IP injection 3 days after diabetes induction. No differences were observed between the control and DM groups, DM and Bil groups, or control and Bil groups according to ANOVA and Tukey post-hoc analyses.

Table 2. Characteristics of rats at 5 weeks after induction of diabetes

Parameters	Control (n = 5)	DM (n = 8)	Bil (n = 5)	<i>P</i> value*	<i>P</i> value [†]
Body weight (g)	453.9 \pm 38.4	298.3 \pm 42.4	305.7 \pm 13.3	< 0.001	0.930
Water intake (mL/day)	20.3 \pm 4.1	171.7 \pm 83.2	183.0 \pm 38.7	0.001	0.943
Food intake (g/day)	14.6 \pm 6.2	41.1 \pm 12.6	46.4 \pm 4.6	< 0.001	0.603
Fasting glucose (mg/dL)	141 \pm 4	484 \pm 79	504 \pm 58	< 0.001	0.858
HbA1c (%)	3.6 \pm 0.2	8.7 \pm 0.8	9.1 \pm 0.8	< 0.001	0.460
Serum Creatinine (mg/dL)	0.39 \pm 0.04	0.71 \pm 0.22	0.45 \pm 0.05	0.004	0.025
Urine volume (mL/day)	14.5 \pm 4.5	103.1 \pm 74.1	125.5 \pm 54.2	0.018	0.779
24-hr urine albumin (g/day)	1.31 \pm 0.42	6.19 \pm 4.78	2.84 \pm 0.99	0.036	0.185

**P* values were estimated for the 3 groups by using an ANOVA tests; [†]*P* values for the comparisons of DM and Bil groups by using ANOVA and post-hoc Tukey analysis. The values of all the parameters tested were significantly different between the control and DM groups (*P* value < 0.05). Serum creatinine and 24-hr urine albumin were not different between control and Bil groups (*P* = 0.810 and *P* = 0.613, respectively), as determined using ANOVA and Tukey post-hoc analyses.

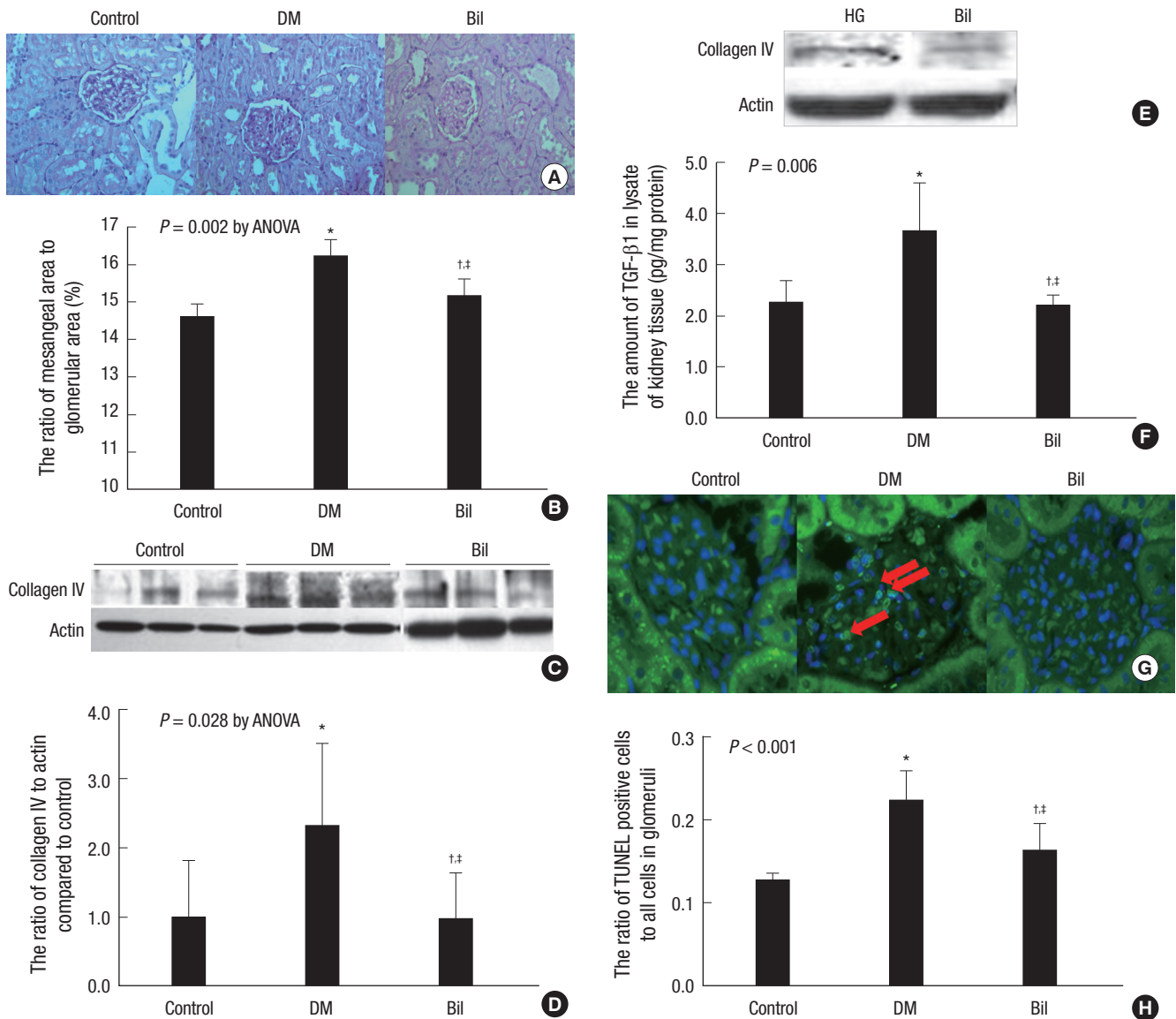


Fig. 1. Changes in kidney tissue. (A) Representative image of PAS staining in kidney tissue ($\times 400$). (B) The ratios of mesangial area to total glomerular area were averaged from 30 glomeruli per rat. Ratios were increased in DM rats compared to controls, an effect that was decreased by bilirubin treatment (Bil group). $*P = 0.002$, $^{\dagger}P = 0.248$ compared to the control group and $^{\ddagger}P = 0.035$ compared to DM group, as determined by analysis of variance (ANOVA and post-hoc Tukey test). (C) Western blot analysis of collagen IV in kidney tissue lysates. (D) The relative density ratios of collagen IV in each group compared to control group, normalized to actin. Collagen IV was increased in the DM group compared to control group and was decreased by bilirubin treatment. $*P = 0.045$, $^{\dagger}P = 0.999$ compared to the control and $^{\ddagger}P = 0.048$ compared to DM group (ANOVA and Tukey post-hoc test). (E) Collagen IV expression in cultured mesangial cells in high glucose media (HG; 450 mg/dL of D-glucose). Bilirubin (0.3 mg/dL) was dissolved in DMSO prior to culturing cells in HG conditions. (F) The concentration of TGF- $\beta 1$, measured in kidney tissue protein extracts by ELISA, was also increased in DM group. This effect was attenuated in the Bil group. $*P = 0.019$, $^{\dagger}P = 0.991$ compared to the control and $^{\ddagger}P = 0.014$ compared to DM group (ANOVA and Tukey post-hoc test). (G) TUNEL staining of glomeruli. The red arrow indicates apoptotic cells with condensed nuclear DNA. (H) The ratio of TUNEL positive to total glomerular cells. $*P < 0.001$, $^{\dagger}P = 0.020$ compared to the control and $^{\ddagger}P = 0.001$ compared to the DM group (ANOVA and Tukey post-hoc test). The bars on the graph indicate the 95% confidence interval of the mean values.

by the Bil and control groups, respectively (16.5 ± 0.78 : 15.2 ± 0.68 : $14.3 \pm 0.98\%$, $P = 0.002$) (Fig. 1B). These data correlated with the protein expression of collagen IV in kidney tissue (Fig. 1C and D). The relative levels of collagen IV protein normalized to actin were increased 2.33 ± 1.12 -fold in the kidneys of the DM group compared to control group. This effect was attenuated by bilirubin treatment (0.98 ± 0.53 folds). Bilirubin treatment (0.3 mg/dL of medium) of mesangial cells under HG conditions also reduced the levels of collagen IV protein (Fig. 1E). The level

of the profibrotic protein TGF $\beta 1$, which was increased in the kidneys of DM rats, were attenuated by bilirubin (control:DM: Bil group = 2.27 ± 0.34 : 3.66 ± 1.12 : 2.20 ± 0.16 pg/ 100 mg protein, $P = 0.006$) (Fig. 1F). The proportion of apoptotic cells present in glomeruli was increased in DM rats, but bilirubin treatment mitigated this increase (control:DM:Bil group = 12.8 ± 0.3 : 22.3 ± 1.4 : $16.3 \pm 1.3\%$, $P < 0.001$) (Fig. 1G and H).

The renoprotective effect of bilirubin was accompanied by a reduction in the level of ROS such as hydrogen peroxide (Fig.

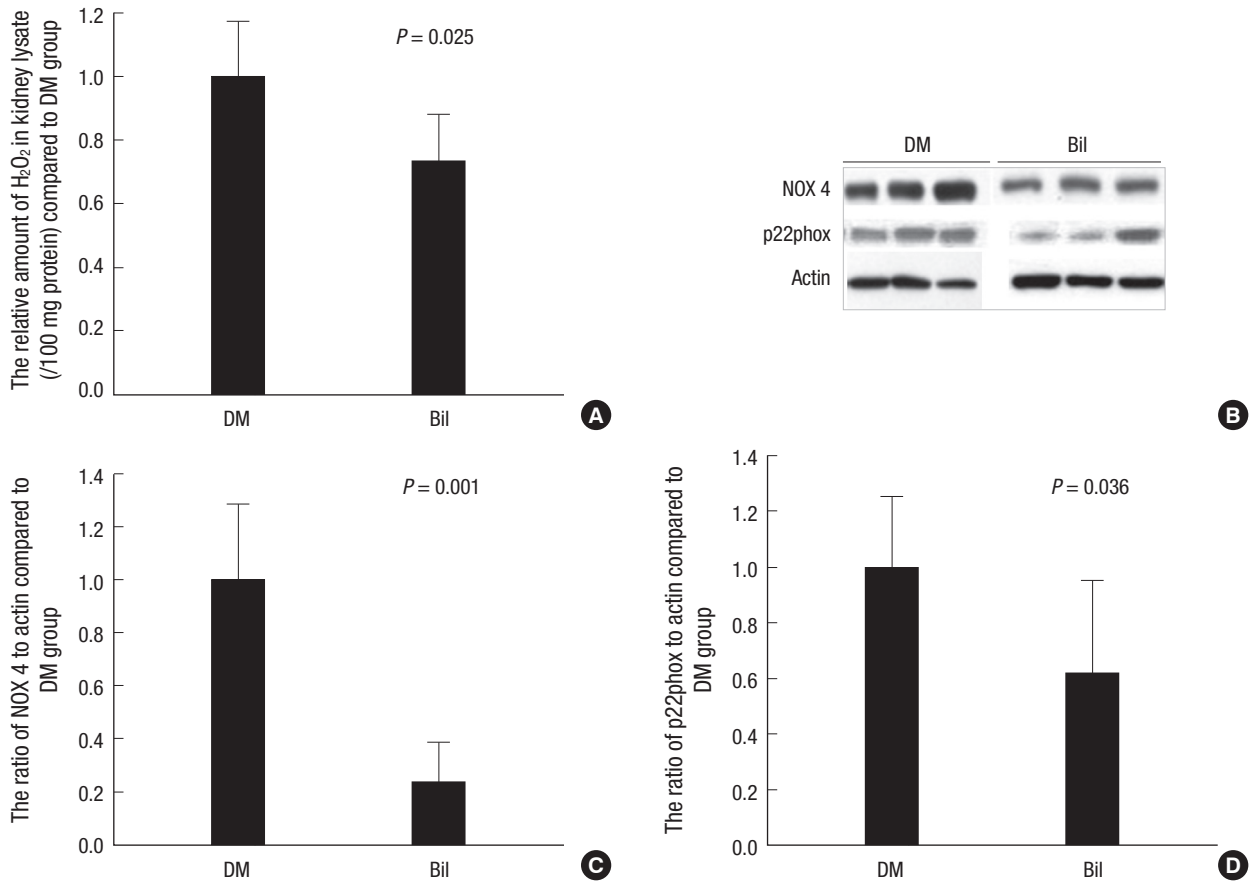


Fig. 2. NADPH oxidase subunits and hydrogen peroxide in kidney tissues. (A) The relative amount of hydrogen peroxide in kidney lysates (100 mg protein). Peroxide levels were lower in the Bil group than in the DM group. (B) Western blot analysis of NOX4 and p22phox levels of kidney tissue extracts. The levels of NOX4 (C) and p22phox (D) were significantly decreased by bilirubin treatment (0.3 mg/dL). The bars on the graph indicate the 95% confidence interval of the mean values. *P* values were estimated using the Student's *t*-test.

Table 3. Lipid profiles in serum and liver tissue

Lipids	Control (n = 5)	DM (n = 8)	Bil (n = 5)	<i>P</i> value*	<i>P</i> value [†]
Serum cholesterol (mg/dL)	83 ± 9	116 ± 31	73 ± 10	0.026	0.023
Serum HDL-C (mg/dL)	29 ± 3	41 ± 6	30 ± 8	0.019	0.042
Serum TG (mg/dL)	54 ± 15	791 ± 269	162 ± 70	< 0.001	< 0.001
Serum FFA (mEq/mL)	710 ± 35	2,048 ± 1,062	975 ± 324	0.015	0.012
TG in liver (mg/g tissue)	0.85 ± 0.02	1.01 ± 0.01	0.87 ± 0.06	0.005	0.049

**P* values estimated for the 3 groups using ANOVA tests; [†]*P* values compared between DM and Bil groups by using ANOVA and Tukey post-hoc analyses. FFA, free fatty acid.

2A). The protein levels of NADPH oxidase subunits NOX4 and p22phox were also reduced in renal tissues (Fig. 2B). Bilirubin decreased the expression of NOX4 and p22phox to 23.9% ± 11.9% (Fig. 2C) and 61.9% ± 26.9% (Fig. 2D) in diabetic rats, respectively.

The effect of bilirubin on dyslipidemia in diabetic rats.

Serum lipid levels were elevated in DM rats (Table 3). Total cholesterol, HDL-C, free fatty acid, and TG levels were increased in the DM group. However, bilirubin treatment effectively attenuated the severity of dyslipidemia. Of the lipids evaluated in sera, TGs were increased to the greatest extent in the DM group, 14.7 ± 5.0-fold compared to the control group (*P* < 0.001, ANOVA and

post-hoc Tukey test). TG levels in the Bil group were not markedly different from that of the control group (*P* = 0.410, ANOVA and post-hoc Tukey test). TGs, which are synthesized in liver, were increased by 1.19 ± 0.02-fold compared to control group (*P* = 0.005, ANOVA and post-hoc Tukey test) in liver tissues. Levels of the mature form of SREBP-1 (mSREBP-1, 68 kDa, detected by anti-SREBP1 antibody sc-367), which is cleaved from precursor SREBP-1 (125 kDa, detected by anti-SREBP1 antibody sc-367), and its downstream proteins, SCD-1 and FAS, were enhanced under diabetic conditions. The upstream factors mSREBP-1 and LXRα were also increased in the kidneys (Fig. 3A) and livers of DM rats (Fig. 3B). The protein levels of

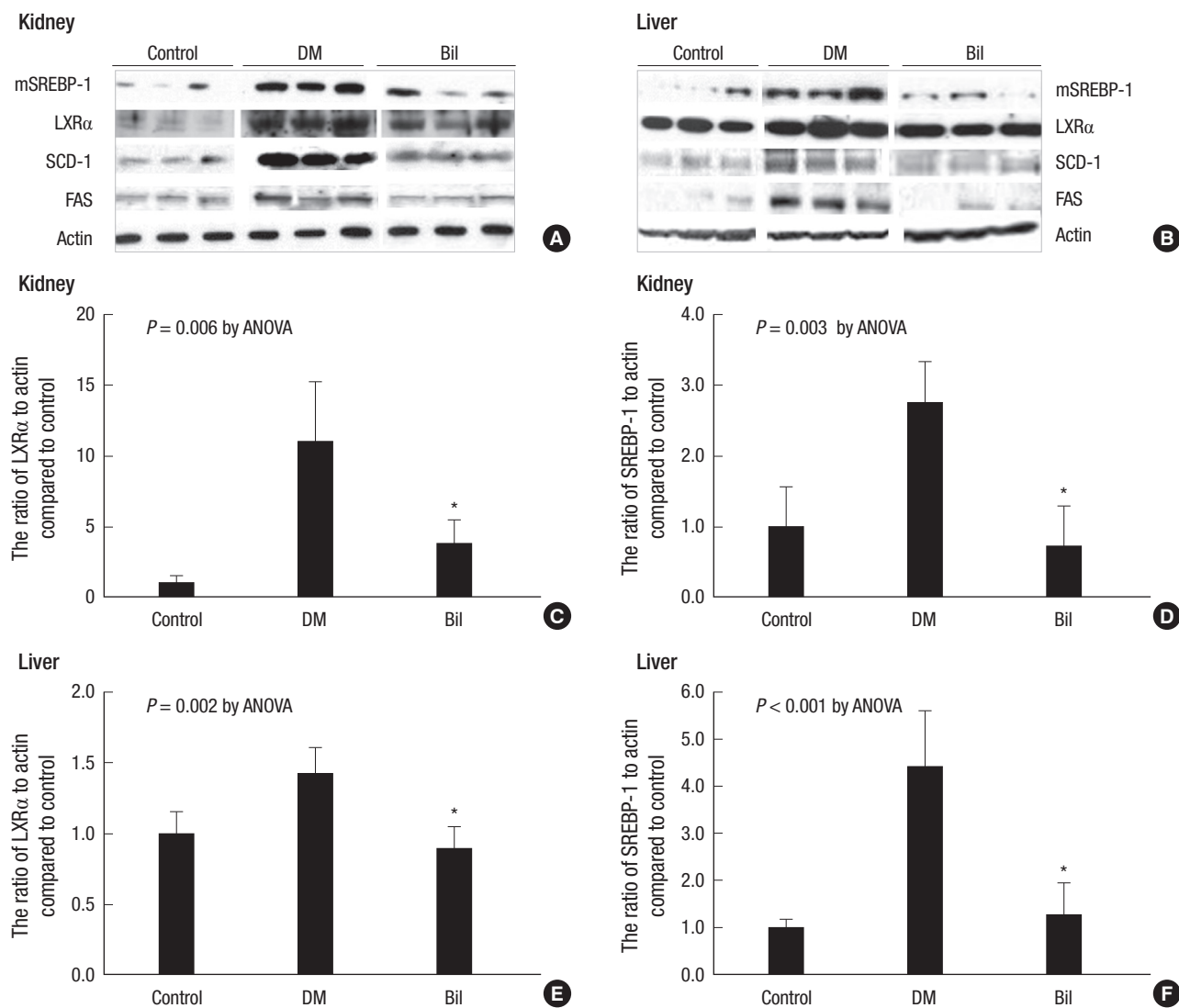


Fig. 3. Expression of factors that regulate TG synthesis. Western blot analysis of factors related to synthesis of TG in the kidney (A) and liver (B). Diabetic conditions increased the levels of LXR α , SREBP-1, FAS, and SCD-1 in kidney and liver tissues. These effects were attenuated by bilirubin treatment. The relative expression of LXR α and SREBP-1 in the kidney (C, D) and liver (E, F), normalized to actin are shown. * $P < 0.05$ in F compared to the DM group (ANOVA and Tukey post-hoc test). The bars on the graph indicate standard errors of the mean values.

LXR α and mSREBP-1 in the DM group were increased by 11.0 ± 4.23 -fold (Fig. 3C) and 2.75 ± 0.58 -fold (Fig. 3D), respectively, in kidney tissues and by 1.42 ± 0.26 -fold (Fig. 3E) and 4.41 ± 1.68 -fold (Fig. 3F) in liver tissues compared to control group. These increases in mSREBP-1 and LXR α protein levels were significantly attenuated by bilirubin injection. The levels of SCD-1 and FAS showed a similar trend as mSREBP-1 in DM rats before and after bilirubin injection (Fig. 3A and B).

In mesangial cells, bilirubin treatment did not change the protein levels of mSREBP-1 and LXR α (data not shown). However, in hepatoma cell lines, the protein levels of mSREBP-1 and LXR α were increased when they were cultured in HG medium, but this effect was attenuated by bilirubin treatment (Fig. 4A). The fold-changes of mSREBP-1 and LXR α expression are shown in Fig. 4C. Transfection of LXR α siRNA suppressed the expres-

sion of mSREBP-1; however, the LXR α siRNA was not as effective as bilirubin in suppressing mSREBP-1 expression (Fig. 4B and D). These data suggest that the suppressive effect of bilirubin on mSREBP-1 protein levels depended not only on the suppression of LXR α , but also on another pathway, such as direct suppression of mSREBP-1 expression.

DISCUSSION

In this study, bilirubin attenuated renal dysfunction, decreased mesangial matrix, TGF- β 1 production, and apoptosis in the glomeruli of diabetic kidneys, and reduced TGF- β 1 production in mesangial cells cultured in HG medium. The renoprotective effect of bilirubin was attributed to not only the suppression of NADPH oxidase levels and ROS production in the kidneys, but

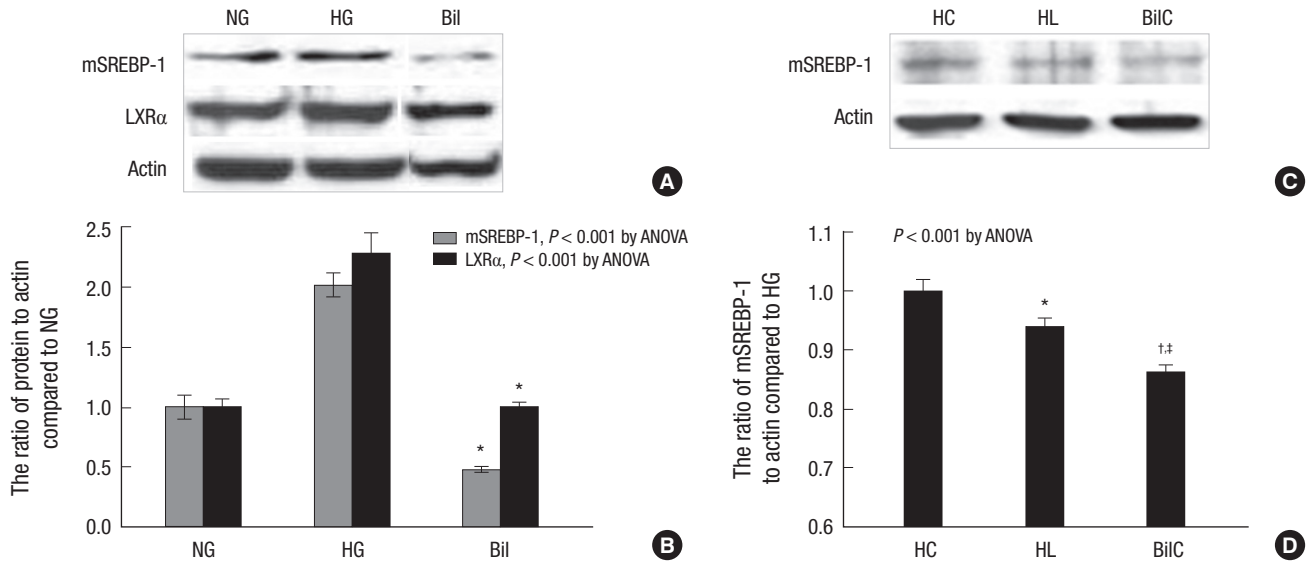


Fig. 4. Expression of factors that regulate TG synthesis in hepatoma cells. **(A)** Western blot analysis of factors related to synthesis of TG in hepatoma cell lines. The relative levels of SREBP-1 and LXRα normalized to actin were increased in cells cultured in HG medium compared to those cultured in normal glucose medium. These effects were attenuated by bilirubin treatment **(B)**. * $P < 0.001$ compared to the HG group (ANOVA and Tukey post-hoc test). **(C)** Western blot analysis of SREBP-1 in untransfected cells and those transfected with LXRα siRNA, cultured in high glucose media. HC: untreated control cells (no bilirubin or siRNA); HL: cells transfected with siRNA; BilC cells treated with bilirubin but not transfected. **(D)** The relative level of SREBP-1 normalized to actin. * $P < 0.001$, † $P = 0.003$ compared to the HG group and † $P = 0.010$ compared to the HL group (ANOVA and Tukey post-hoc test). The bars on the graph indicate the 95% confidence interval of the mean values.

also to attenuation of dyslipidemic genes in the sera, kidneys, and livers of diabetic rats. Bilirubin suppressed lipogenic genes, LXRα, mSREBP-1, FAS, and SCD-1 in kidney and liver tissues.

In this experiment, the serum creatinine level in DM rat increased at 5 weeks after streptozotocin injection, which was manifested earlier compared to the other report (16). Streptozotocin induces DNA damage in the kidney lasted for 20 days after treatment (17) and provoked acute nephrotoxic effect with a single of 65 mg/kg injection into rats (18). Nevertheless, the toxic effect could not explain all the changes in the kidney because the kidneys are known to recover from acute mild nephrotoxic effects within 3 weeks after STZ injection (17) and we sacrificed the rats at 5 weeks after injection. Also, the pathologic change in the kidney of DM rat showed typical diabetic changes, such as increase of mesangial extracellular matrix. In other reports, serum creatinine was increased at 42-56 days after injection streptozotocin into rats with typical diabetic changes in the kidney (19-21). Therefore, the changes of renal function and albuminuria in this study would be mainly from diabetic nephropathy and, from toxic effect by streptozotocin in minor aspect.

The serum TG level was decreased to a greater extent by bilirubin treatment than were the levels of cholesterol, HDL-C, and free fatty acids in this study. Recently, Blumer et al. (22) evaluated the lipid status in Gilbert's syndrome and the association between serum bilirubin and lipids in clinical studies. Lower concentrations of total cholesterol and TG and a higher concentration of HDL-C were found in individuals with higher bilirubin concentration (22). Decreasing HDL-C concentration with

increasing bilirubin has been reported in only one study (6). In studies using the hyperbilirubinemic Gunn rat, total cholesterol and TG were also decreased (3, 23, 24). In contrast to the results in humans and similar to the results of our study, HDL-C in Gunn rats is lower than in genetic control (23). Notably, in every study examining the relationship between bilirubin and lipid status in healthy subjects, reduced TG and elevated bilirubin levels were reported (22). That relationship was confirmed in this animal-based study. In mammals, biosynthesis of cholesterol, fatty acids, and TGs is regulated by SREBPs (25). Two SREBP encoding genes, *SREBP-1* and *SREBP-2*, exist. *SREBP-1* generates two proteins, SREBP-1a and SREBP-1c. SREBP-1 regulates free fatty acid and TG metabolism and promotes acetyl-CoA carboxylase, FAS (26), SCD (27), and LDL receptor (28). The transcription of *SREBP-1c* is primarily induced by insulin and mediated by a phosphatidylinositol 3'-kinase (PI3K) dependent pathway (29) and by the activation of LXRα (30). LXRα induces the expression of genes involved in cholesterol efflux and clearance (31) and induction of *SREBP-1c* by binding retinoid X receptor/LXR DNA-binding sites in the *SREBP-1c* promoter (32).

Increased renal expression of SREBP-1c has been implicated in diabetic and hyperlipidemia-associated nephropathies through activation of ROS, NADPH oxidase, and TGF-β (33). The absence of SREBP-1c completely abolishes the increase of albuminuria induced by diabetes in STZ-treated mice (34). In diabetic *db/db* mice, n-3 polyunsaturated fatty acid-mediated *SREBP-1* attenuation in the kidney improved albuminuria and renal dysfunction (35). Even in the absence of hyperglycemia or hyperlipi-

demia, increased renal expression of *SREBP-1a* resulted in renal accumulation of lipid and increased TGF- β , plasminogen activator inhibitor 1, and vascular endothelial growth factor, leading to renal hypertrophy, accumulation of extracellular matrix proteins, and mesangial expansion (36). SREBP-1 directly binds to the TGF- β promoter in cultured mesangial cells in HG medium (37).

Consistent with the findings of previous studies, we confirmed that bilirubin injection into STZ-induced type I diabetic animals improved renal functional derangement and morphologic changes, as well as decreased ROS production by suppressing the levels of NADPH subunits. Fujii et al. (4) used Gunn rats and biliverdin-treated *db/db* mice to verify the renoprotective effects of bilirubin and biliverdin on diabetic nephropathy (4). Both rodent models had less albuminuria and they were protected against progression of mesangial expansion. Moreover, levels of TGF- β , fibronectin, ROS, and NADPH oxidase subunits were normalized in the kidney (4). Our study showed bilirubin decreased serum cholesterol and both serum and hepatic TG. This effect was accompanied by suppression of SREBP-1, its upstream regulator LXR α , and downstream factors FAS and SCD-1 in the kidney and liver. Knockdown of LXR α by siRNA decreased SREBP-1 expression, but did so to a lesser degree than bilirubin treatment in hepatoma cells, implying that bilirubin directly suppressed SREBP-1 expression or other LXR α -independent pathways. Therefore, the renoprotective effect of bilirubin in diabetic rats resulted not only from scavenging ROS and suppressing NADPH oxidase subunits, but also by attenuating diabetes-induced renal dyslipidemia. Notably, HDL-C levels were altered by bilirubin in this study. As previously mentioned, Gunn rats have a lower level of HDL-C than genetic controls, which is similar to rats injected with bilirubin. One mechanism by which HDL-C is decreased by bilirubin might involve decreased expression of ATP-binding cassette transporter A1 (ABCA1). HDL-C is assembled from apolipoprotein A1, cholesterol, and phospholipids by a reaction mediated by ABCA1 (38). Overexpression of hepatic ABCA1 raises HDL-C levels (39). Exon 1 of the *ABCA1* gene contains an LXR response element, through which LXR regulates ABCA1 in peripheral tissue (38). However, the liver-specific *ABCA1* gene lacks exon 1 and possesses a functional SREBP response site in the upstream region of exon 2 (38). Therefore, suppressed expression of LXR and SREBP-1 by bilirubin would decrease expression of *ABCA1*, decreasing HDL assembly in the livers and in peripheral tissues of rats, such as fatty tissues or kidneys.

In conclusion, bilirubin attenuated renal dysfunction and dyslipidemia in diabetes by suppressing levels of LXR α and SREBP-1 and oxidative stress.

DISCLOSURE

There is no conflict of interest for all authors.

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REFERENCES

1. Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A, et al. *Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature* 2000; 403: 776-81.
2. Dennery PA, McDonagh AF, Spitz DR, Rodgers PA, Stevenson DK. *Hyperbilirubinemia results in reduced oxidative injury in neonatal Gunn rats exposed to hyperoxia. Free Radic Biol Med* 1995; 19: 395-404.
3. Fu YY, Kang KJ, Ahn JM, Kim HR, Na KY, Chae DW, Kim S, Chin HJ. *Hyperbilirubinemia reduces the streptozotocin-induced pancreatic damage through attenuating the oxidative stress in the Gunn rat. Tohoku J Exp Med* 2010; 222: 265-73.
4. Fujii M, Inoguchi T, Sasaki S, Maeda Y, Zheng J, Kobayashi K, Takayanagi R. *Bilirubin and biliverdin protect rodents against diabetic nephropathy by downregulating NAD(P)H oxidase. Kidney Int* 2010; 78: 905-19.
5. Jenko- Pražnikar Z, Petelin A, Jurdana M, Žiberna L. *Serum bilirubin levels are lower in overweight asymptomatic middle-aged adults: an early indicator of metabolic syndrome? Metabolism* 2013; 62: 976-85.
6. Hwang HJ, Kim SH. *Inverse relationship between fasting direct bilirubin and metabolic syndrome in Korean adults. Clin Chim Acta* 2010; 411: 1496-501.
7. Kwon KM, Kam JH, Kim MY, Kim MY, Chung CH, Kim JK, Linton JA, Eom A, Koh SB, Kang HT. *Inverse association between total bilirubin and metabolic syndrome in rural Korean women. J Womens Health (Larchmt)* 2011; 20: 963-9.
8. Jung CH, Lee MJ, Kang YM, Hwang JY, Jang JE, Leem J, Park JY, Kim HK, Lee WJ. *Higher serum bilirubin level as a protective factor for the development of diabetes in healthy Korean men: a 4 year retrospective longitudinal study. Metabolism* 2014; 63: 87-93.
9. Han SS, Na KY, Chae DW, Kim YS, Kim S, Chin HJ. *High serum bilirubin is associated with the reduced risk of diabetes mellitus and diabetic nephropathy. Tohoku J Exp Med* 2010; 221: 133-40.
10. Mashitani T, Hayashino Y, Okamura S, Tsujii S, Ishii H. *Correlations between serum bilirubin levels and diabetic nephropathy progression among Japanese type 2 diabetic patients: a prospective cohort study (Diabetes Distress and Care Registry at Tenri [DDCRT 5]). Diabetes Care* 2014; 37: 252-8.
11. Chin HJ, Song YR, Kim HS, Park M, Yoon HJ, Na KY, Kim Y, Chae DW, Kim S. *The bilirubin level is negatively correlated with the incidence of hypertension in normotensive Korean population. J Korean Med Sci* 2009; 24: S50-6.
12. Chin HJ, Cho HJ, Lee TW, Na KY, Oh KH, Joo KW, Yoon HJ, Kim YS, Ahn

- C, Han JS, et al. *The mildly elevated serum bilirubin level is negatively associated with the incidence of end stage renal disease in patients with IgA nephropathy. J Korean Med Sci* 2009; 24: S22-9.
13. Schwertner HA, Jackson WG, Tolani G. *Association of low serum concentration of bilirubin with increased risk of coronary artery disease. Clin Chem* 1994; 40: 18-23.
14. Kamisako T, Masuda S, Tanaka Y. *Relationship between serum bilirubin and remnant lipoprotein cholesterol level. Clin Lab* 2013; 59: 435-8.
15. Shepherd RE, Moreno FJ, Cashore WJ, Fain JN. *Effects of bilirubin on fat cell metabolism and lipolysis. Am J Physiol* 1979; 237: E504-8.
16. Degenhardt TP, Alderson NL, Arrington DD, Beattie RJ, Basgen JM, Steffes MW, Thorpe SR, Baynes JW. *Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. Kidney Int* 2002; 61: 939-50.
17. Kraynak AR, Storer RD, Jensen RD, Kloss MW, Soper KA, Clair JH, DeLuca JG, Nichols WW, Eydeloth RS. *Extent and persistence of streptozotocin-induced DNA damage and cell proliferation in rat kidney as determined by in vivo alkaline elution and BrdUrd labeling assays. Toxicol Appl Pharmacol* 1995; 135: 279-86.
18. Junod A, Lambert AE, Orci L, Pictet R, Gonet AE, Renold AE. *Studies of the diabetogenic action of streptozotocin. Proc Soc Exp Biol Med* 1967; 126: 201-5.
19. Zhang S, Yang J, Li H, Li Y, Liu Y, Zhang D, Zhang F, Zhou W, Chen X. *Skimmin, a coumarin, suppresses the streptozotocin-induced diabetic nephropathy in wistar rats. Eur J Pharmacol* 2012; 692: 78-83.
20. Roy S, Metya SK, Sannigrahi S, Rahaman N, Ahmed F. *Treatment with ferulic acid to rats with streptozotocin-induced diabetes: effects on oxidative stress, pro-inflammatory cytokines, and apoptosis in the pancreatic β cell. Endocrine* 2013; 44: 369-79.
21. Fang J, Wei H, Sun Y, Zhang X, Liu W, Chang Q, Wang R, Gong Y. *Regulation of podocalyxin expression in the kidney of streptozotocin-induced diabetic rats with Chinese herbs (Yishen capsule). BMC Complement Altern Med* 2013; 13: 76.
22. Bulmer AC, Verkade HJ, Wagner KH. *Bilirubin and beyond: a review of lipid status in Gilbert's syndrome and its relevance to cardiovascular disease protection. Prog Lipid Res* 2013; 52: 193-205.
23. Boon AC, Hawkins CL, Bisht K, Coombes JS, Bakrania B, Wagner KH, Bulmer AC. *Reduced circulating oxidized LDL is associated with hypocholesterolemia and enhanced thiol status in Gilbert syndrome. Free Radic Biol Med* 2012; 52: 2120-7.
24. Sakamoto S, Kusuhara H, Miyata K, Shimaoka H, Kanazu T, Matsuo Y, Nomura K, Okamura N, Hara S, Horie K, et al. *Glucuronidation converting methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921) to a potent apical sodium-dependent bile acid transporter inhibitor, resulting in a hypocholesterolemic action. J Pharmacol Exp Ther* 2007; 322: 610-8.
25. Goldstein JL, DeBose-Boyd RA, Brown MS. *Protein sensors for membrane sterols. Cell* 2006; 124: 35-46.
26. Magaña MM, Osborne TF. *Two tandem binding sites for sterol regulatory element binding proteins are required for sterol regulation of fatty acid synthase promoter. J Biol Chem* 1996; 271: 32689-94.
27. Le Lay S, Lefrère I, Trautwein C, Dugail I, Krief S. *Insulin and sterol-regulatory element-binding protein-1c (SREBP-1C) regulation of gene expression in 3T3-L1 adipocytes. Identification of CCAAT/enhancer-binding protein beta as an SREBP-1C target. J Biol Chem* 2002; 277: 35625-34.
28. Yokoyama C, Wang X, Briggs MR, Admon A, Wu J, Hua X, Goldstein JL, Brown MS. *SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. Cell* 1993; 75: 187-97.
29. Fleischmann M, Iynedjian PB. *Regulation of sterol regulatory-element binding protein 1 gene expression in liver: role of insulin and protein kinase B/cAkt. Biochem J* 2000; 349: 13-7.
30. Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuzaka T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, et al. *Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter. Mol Cell Biol* 2001; 21: 2991-3000.
31. Steffensen KR, Gustafsson JA. *Putative metabolic effects of the liver X receptor (LXR). Diabetes* 2004; 53: S36-42.
32. Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, et al. *Role of LXRs in control of lipogenesis. Genes Dev* 2000; 14: 2831-8.
33. Shimano H. *SREBPs: physiology and pathophysiology of the SREBP family. FEBS J* 2009; 276: 616-21.
34. Ishigaki N, Yamamoto T, Shimizu Y, Kobayashi K, Yatoh S, Sone H, Takahashi A, Suzuki H, Yamagata K, Yamada N, et al. *Involvement of glomerular SREBP-1c in diabetic nephropathy. Biochem Biophys Res Commun* 2007; 364: 502-8.
35. Chin HJ, Fu YY, Ahn JM, Na KY, Kim YS, Kim S, Chae DW. *Omacor, n-3 polyunsaturated fatty acid, attenuated albuminuria and renal dysfunction with decrease of SREBP-1 expression and triglyceride amount in the kidney of type II diabetic animals. Nephrol Dial Transplant* 2010; 25: 1450-7.
36. Sun L, Halaihel N, Zhang W, Rogers T, Levi M. *Role of sterol regulatory element-binding protein 1 in regulation of renal lipid metabolism and glomerulosclerosis in diabetes mellitus. J Biol Chem* 2002; 277: 18919-27.
37. Uttarwar L, Gao B, Ingram AJ, Krepinsky JC. *SREBP-1 activation by glucose mediates TGF- β upregulation in mesangial cells. Am J Physiol Renal Physiol* 2012; 302: F329-41.
38. Sato R. *Sterol metabolism and SREBP activation. Arch Biochem Biophys* 2010; 501: 177-81.
39. Wellington CL, Brunham LR, Zhou S, Singaraja RR, Visscher H, Gelfer A, Ross C, James E, Liu G, Huber MT, et al. *Alterations of plasma lipids in mice via adenoviral-mediated hepatic overexpression of human ABCA1. J Lipid Res* 2003; 44: 1470-80.