Diabetes and hyperlipidemia induce dysfunction of VSMCs: contribution of the metabolic inflammation/miRNA pathway

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Li T, Yang G, Zhu Y, Wu Y, Chen X, Lan D, Tian K, Liu L. Diabetes and hyperlipidemia induce dysfunction of VSMCs: contribution of the metabolic inflammation/miRNA pathway. Am J Physiol Endocrinol Metab 308: E257–E269, 2015. First published November 25, 2014; doi:10.1152/ajpendo.00348.2014.—Vascular endothelial cell injury is considered to be the major factor inducing vascular complications in metabolic diseases and plays an important role in other organ damage. With diabetic and hyperlipidemic rats and cultured VSMCs, the present study was aimed at investigating whether the early damage of VSMCs during metabolic diseases plays a critical role in vascular dysfunction and the underlying mechanisms and would be a promising treatment target. With diabetic and hyperlipidemic rats and cultured VSMCs, the changes and relationships of vascular relaxation and contractile function to the vital organ damage and the underlying mechanisms were investigated; meanwhile, the protective and preventive effects of lowering blood lipid and glucose and inhibition of diabetes and hyperlipidemia-induced vascular hyperreactivity were observed. Diabetic and hyperlipidemic rats presented hyperreactivity in vascular contractile response in the early stages. Hyperglycemia and hyperlipidemia directly affected the contractile function of VSMCs. Early application of fasudil, a specific antagonist of Rho kinase, significantly alleviated diabetes and hyperlipidemia-induced organ damage by inhibiting vascular hyperreactivity. Diabetes and hyperlipidemia-induced inflammatory response could upregulate the expression of connexins and Rho kinase by selective downregulation of the expression of miR-10a, miR-139b, miR-206, and miR-222. These findings suggest that hyperglycose and lipid may directly impair VSMCs and induce vascular hyperreactivity in the early stages. Metabolic inflammation-induced changes in the miRNA-connexin/Rho kinase regulatory pathway are the main mechanism for vascular hyperreactivity and organ damage. Measures inhibiting vascular hyperreactivity are promising for the prevention of organ damage induced by metabolic diseases.

vascular smooth muscle cells; vascular contraction; metabolic disease; Rho kinase; microRNA

METABOLIC DISEASES SUCH AS DIABETES AND HYPERLIPIDEMIA threaten human health with an increasing morbidity year by year. There were 190 million diabetic patients globally as of 2012. In the UK, diabetic patients had increased from 1.4 million in 1996 to 2.9 million in 2011 (20). In China, the morbidity of diabetes is about 9.6%, and there are 18 million diabetic patients. In addition, the number of patients with hyperlipidemia is also increasing rapidly, and its morbidity in China is greater than 20%, with more than 180 million patients. Diabetes and hyperlipidemia-induced vascular complications are the critical reasons for vital organ failure, including heart and renal failure. Gilbert reported that nearly 50% of diabetes patients would develop cardiovascular complications (7). Among patients with documented cardiovascular disease, diabetes accounts for 50% in mortality and hospitalization because of heart failure compared with nondiabetic subjects. The approaches for preventing metabolic disease-induced cardiovascular complications include lowering blood glucose or lipid levels, antihypertensive medications, panretinal photocoagulation, agents targeting vascular endothelial growth factor, and statin therapy (14). Despite these advances in treatment, metabolic disease-induced vascular complications remain an enormous problem.

Previous studies have shown that injury of the vascular endothelial cell (VEC) plays a critical role in the development of vascular complications induced by diabetes and hyperlipidemia (15). Based on lowering lipids and glucose, some VEC repairment measures have shown some beneficial effects in preventing vascular complications (1, 8). However, we know that, except for VEC, as an important component of the vasculature, vascular smooth muscle cells (VSMCs) play an important role in vascular dilation and constriction, the key factors in determining tissue perfusion. Recent studies have shown that VSMCs in diabetes and hyperlipidemia may be functionally impaired and thus contribute to the increased incidence of vascular complications (16). However, its importance in organ damage and the underlying mechanisms are currently unknown. Previous studies have focused mainly on late-stage diabetes and hyperlipidemia (10). Whether VSMCs have a functional abnormality in the early stages of hyperlipidemia and diabetes and the functional abnormality of VSMCs plays a critical role in organ damage, as well as the underlying mechanisms, is not known.

To elucidate these problems with diabetes and hyperlipidemia rat renal artery and cultured VSMCs, we investigated 1) the relationship of vascular relaxation and constriction of renal artery in diabetes and hyperlipidemia with regard to renal function, 2) the underlying mechanisms responsible for the early damage of VSMC in diabetes and hyperlipidemia, and 3) the protective effects on vascular and organ function based on inhibition of diabetes and hyperlipidemia-induced vascular hyperreactivity.
RESEARCH DESIGN AND METHODS

Ethical Approval

The present study and protocol were approved by the Research Council and Animal Care and Use Committee of the Research Institute of Surgery, Daping Hospital, Third Military Medical University (Chongqing, China). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The approval no. for the study is YYLL (2012) 018.

Animal Management

Male and female Sprague-Dawley (SD) rats were purchased from the Animal Center of Research Institute of Surgery, Third Military Medical University, housed under controlled conditions (22°C, 55–65% humidity, and 12:12-h light-dark cycle), and fed a standard rat pellet diet.

Preparation of diabetic rat model. Six-week-old SD rats were fed a diabetic diet, including 67.5% basic forage, 2.5% yolk, 20% sucrose, and 10% lard oil. After 7 wk of the diabetic diet, rats were administered streptozotocin (35 mg/kg), and blood glucose was measured after 1 wk. If the blood glucose level was >14 mmol/l, the model was considered successful. The success rate of the model was ~80% in the present study (Fig. 1).

Preparation of the hyperlipidemia rat model. Six-week-old SD rats were fed with a hyperlipemia diet, including 77.6% basic forage, 10% yolk, 10% lard oil, 0.2% holo-cholic acid, and 0.2% propylsulphyrymidine. After 8 wk, blood lipids were measured. If the total cholesterol was >6.2 mmol/l, triglycerides were >6 mmol/L, and low density lipoprotein (LDL) >6 mmol/l, the model was considered successful. The success rate of this model was >95% in the present study (Fig. 1).

Experimental Protocol and Design

Animal experiments. At 4, 8, 16, and 24 wk after the diabetes or hyperlipidemia model was established, the contractile response of renal artery, the renal function and blood flow, the expression of contraction-related proteins, the miRNAs, and some cytokine production were detected. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and 2 ml of blood was sampled to test the kidney damage biomarker, including cystatin C (CYS), β-glycerolphosphate dehydrogenase (β-G), blood urea nitrogen (BUN), and serum creatinine (Scr). The rats were then euthanized with an overdose of pentobarbital sodium, and blood was tested for the kidney damage biomarker. In the left renal artery (LRA) was taken for the measurement of vasoconstriction and vasodilation. Meanwhile, the expression of connexins (Cx37, Cx40, Cx43, and Cx45), PKCα, PKCε, Rho kinase, miRNAs, and the concentration of TNFα, IL-1β, and IL-6 was measured to determine the metabolic inflammation in the LRA were determined (12, 27). The detailed measurement methods are discussed in Parameter Measurement.

VSMC Experiments

Effects of high concentrations of lipid and glucose and cytokines on the contractile response of VSMCs and the expression of miRNAs, Cx43, and Rho kinase. The third passage of VSMCs from normal rats was incubated with high concentrations of glucose (25 mmol/l) or lipid (palmitic acid 50 μmol/l) for 12, 24, 48, and 72 h or 50, 100, and 200 ng/ml TNFα, IL-1β, and IL-6 for 24 h, respectively. The contractile response of VSMC to norepinephrine (NE), the expressions of miR10a, miR139b, miR206, and miR222, and Cx43 and Rho kinase in VSMCs were observed.

Effect of Cx43-overexpressed adenovirus and RNAi on the expression of Rho kinase. The third passage of VSMCs from normal rats was transfected by Cx43-overexpressed and Cx43 RNAi adenovirus according to the manufacturer’s instructions and cultured for 24 h. The expression of Rho kinase was determined by Western blotting.

Effects of miRNAs on the contractile response of VSMCs and relationship to Cx43 and Rho kinase. Experiments included two parts. First, there were the effects of miRNAs on the contractile response of normal VSMCs. The third passage of VSMCs from normal rats was transfected by the antisense of miR10a, miR139b, miR206, and miR222 according to the manufacturer’s instructions; 24 h later, the contractile responses of VSMC were determined. Second, the effects of miRNAs on the contractile response in hyperglucose or hyperlipid medium cultured VSMCs and the relationship to Cx43 and Rho kinase. Hyperglucose, or hyperlipid medium cultured VSMCs (48 h) were transfected with highly expressed miR-10a, miR-139b, miR-206, and miR-222 adenovirus and the antisense oligodeoxynucleotide of Cx43 (Cx43 AODN, 100 μmol/l); 24 h later, the contractile responses of VSMCs to NE were determined. For the role of Y-27632 (Rho kinase antagonist), highly expressed miRNA-transfected VSMCs were incubated with Y-27632 (10−5 mol/l) for 30 min, and the contractile response of VSMCs to NE was determined. The antisense sequences of miR-10a, miR-139b, miR-206, and miR-222 are ATA CAC ACT CCT TTA CAT TTC A, CAG CTG GTT GAA GGG GAC CAA A, CCA CAC ACT TTA CAT TCC A, and ACC CAG TAG CCA GAT GTA GCC, respectively. The sequence numbers are RmiRQP0884, RmiRQP1191, RmiRQP0308, and RmiRQP0339, respectively.

Regulatory effects of miRNAs on Rho kinase and Cx43 in VSMCs. The third passage of normal VSMCs was transfected by dual-fluorescence reporters (firefly and renilla) with promoter of Rho kinase and Cx43 and highly expressed miR10a, miR139b, miR206, and miR222 adenovirus. Twenty-four hours later, the ratio of firefly and renilla was determined according to assay kit instructions. Meanwhile, VSMCs were transfected by highly expressed miR10a, miR139b, miR206, and miR222 adenovirus and cultured for 24 h, and the expression of Rho kinase and Cx43 was determined.

Beneficial Effects of Vascular Contractile Response Modulation on Organ Function

Metformin hydrochloride (DMBG; 200 mg·kg−1·day−1, oral administration) (TianFang Medical, Henan, China) was used to lower the blood glucose by diminishing the production of glycogen and increasing sensitivity to insulin. DMBG was administered at the time of the diabetes model completion. The blood glucose level was decreased to normal after 1 wk of DMBG administration in diabetic rats (Table 1). Simvastatin (20 mg·kg−1·day−1, oral administration; Merck Sharp & Dohme, Hoddesdon, UK) was used to decrease the blood lipids by inhibiting the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase to decrease the production of cholesterol, increase the receptor activity of LDL, and accelerate decomposition of LDL. Simvastatin was administered at hyperlipidemia model completion (Fig. 1). The total blood cholesterol, triglyceride, and LDL were decreased to normal levels after 1 wk of simvastatin administration in hyperlipidemia rats. Fasudil, a Rho kinase antagonist, was administered at 5 mg·kg−1·day−1 (oral administration) in the early stages of diabetes.
phase (at model completion) or later phase (16 wk after model finished) until the end of the experiment. Twenty-four weeks from the beginning of the early treatment, the renal blood flow, the kidney function and mitochondrial function, and vascular relaxation and contraction responses of the LRA were measured.

**Parameter Measurement**

**Vascular contractile and relaxation function.** The vascular contractile and relaxation functions were expressed as the responsiveness of the arteries to vasoconstrictors (NE) and vasodilators (acetylcholine (Ach)). According to experimental design, after the diabetic or hyperlipidemia were supplemented into the lower compartment after medium without or with fasudil for hyperlipemia at 16 wk after the finished model. *P < 0.05; **P < 0.10 vs. healthy animals at the same time point group with the same disease.

**Table 1. Demographic parameters of rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight, g</th>
<th>MAP, mmHg</th>
<th>Glucose, mmol/l</th>
<th>CHOL, mmol/l</th>
<th>TG, mmol/l</th>
<th>HDL, mmol/l</th>
<th>LDL, mmol/l</th>
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<tr>
<td><strong>Healthy</strong></td>
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<td>4 wk</td>
<td>212.6 ± 12.4</td>
<td>112.3 ± 12.5</td>
<td>5.27 ± 0.76</td>
<td>0.92 ± 0.13</td>
<td>0.24 ± 0.06</td>
<td>0.66 ± 0.10</td>
<td>0.40 ± 0.07</td>
</tr>
<tr>
<td>8 wk</td>
<td>223.6 ± 13.0</td>
<td>110.5 ± 12.3</td>
<td>5.47 ± 0.78</td>
<td>0.92 ± 0.13</td>
<td>0.29 ± 0.06</td>
<td>0.64 ± 0.10</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td>16 wk</td>
<td>278.5 ± 15.9</td>
<td>114.2 ± 12.7</td>
<td>5.90 ± 0.82</td>
<td>1.04 ± 0.14</td>
<td>0.31 ± 0.07</td>
<td>0.74 ± 0.11</td>
<td>0.40 ± 0.08</td>
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<tr>
<td>24 wk</td>
<td>302.6 ± 17.2</td>
<td>120.6 ± 13.3</td>
<td>5.01 ± 0.74</td>
<td>0.99 ± 0.13</td>
<td>0.26 ± 0.06</td>
<td>0.79 ± 0.11</td>
<td>0.42 ± 0.08</td>
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<td><strong>Hyperlipidemia</strong></td>
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<td>4 wk</td>
<td>256.8 ± 14.8*</td>
<td>110.2 ± 12.3</td>
<td>5.99 ± 0.83</td>
<td>7.66 ± 0.20*</td>
<td>6.90 ± 0.13*</td>
<td>0.52 ± 0.15*</td>
<td>7.90 ± 0.13*</td>
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<tr>
<td>8 wk</td>
<td>388.6 ± 21.7*</td>
<td>117.8 ± 13.0</td>
<td>6.17 ± 0.85</td>
<td>7.71 ± 0.21*</td>
<td>6.95 ± 0.13*</td>
<td>0.47 ± 0.17*</td>
<td>7.92 ± 0.13*</td>
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<tr>
<td>16 wk</td>
<td>405.6 ± 22.6*</td>
<td>120.5 ± 13.3</td>
<td>6.46 ± 0.88</td>
<td>8.06 ± 0.24*</td>
<td>7.36 ± 0.17*</td>
<td>0.48 ± 0.23*</td>
<td>8.46 ± 0.18*</td>
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<tr>
<td>24 wk</td>
<td>445.5 ± 24.7*</td>
<td>126.9 ± 13.9</td>
<td>6.92 ± 0.84</td>
<td>8.14 ± 0.25*</td>
<td>7.46 ± 0.18*</td>
<td>0.43 ± 0.24*</td>
<td>8.56 ± 0.19*</td>
</tr>
<tr>
<td>Simva</td>
<td>365.5 ± 20.5#</td>
<td>128.5 ± 13.1</td>
<td>6.25 ± 0.86</td>
<td>2.03 ± 0.14##</td>
<td>1.32 ± 0.07##</td>
<td>0.78 ± 0.12#</td>
<td>2.56 ± 0.09##</td>
</tr>
<tr>
<td>Simva-Fasu-Ear</td>
<td>306.2 ± 17.4</td>
<td>112.7 ± 12.5</td>
<td>6.23 ± 0.86</td>
<td>2.05 ± 0.14##</td>
<td>1.30 ± 0.07##</td>
<td>0.82 ± 0.12#</td>
<td>2.54 ± 0.09##</td>
</tr>
<tr>
<td>Simva-Fasu-Late</td>
<td>395.6 ± 22.1</td>
<td>123.5 ± 13.6</td>
<td>6.35 ± 0.87</td>
<td>2.99 ± 0.13##</td>
<td>1.43 ± 0.08##</td>
<td>0.81 ± 0.13#</td>
<td>2.63 ± 0.10##</td>
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</table>

Values are means ± SD. *P < 0.05; **P < 0.01 vs. healthy animals at the same time point group with the same disease. ##P < 0.05; ###P < 0.01 vs. 24-wk group with the same disease.

was determined by a mitochondrion function analyzer (MT 200; Strathkelvin). Mitochondrion function was reflected by respiratory control rate (consumed oxygen rate with and without adenosine diphosphate) (13).

**Expressions of miRNA, Rho kinase, and Cx and the concentrations of TNFα, IL-1β, and IL-6.** The renal artery tissues from 24 wk of diabetic and hyperlipidemic rats were used. TNFα, IL-1β, and IL-6 levels in renal artery tissues were determined by ELISA kits. After total protein and RNA were extracted, the change profiles of miRNAs in the renal artery were measured with the 352 miRNAs chip. Based on the chip results, the expressions of miR-10a, miR-139b, miR-206, and miR-222 were further determined with quantitative RT-PCR (miR10a: ACA GAU UCG AUU CUA GGA GGA; miR139b: UGG AGA CCG GCC CUCU GUU GGA G; miR206: TGG AAT GTA AGG AAG TGT GTG G; miR222: AGC TAC ATC TGG CTA CTG GGT). The expressions of Cx37, Cx40, Cx43, Cx45, PKCa, PKCe, and Rho kinase were tested with Western blotting.

**Contractile response of VSMCs.** VSMCs were seeded in the insert wells of both FITC-labeled bovine serum albumin (BSA) was measured to reflect the basal contractile function of VSMCs. VSMCs were seeded in the insert wells of the transwell, and the infiltration rate of isothiocyanate fluorescein-labeled bovine serum albumin (BSA) was measured to reflect the contraction strength of VSMC. Briefly, 5 μl of both FITC-labeled BSA (4 mg/ml) and NE at a final concentration of 10−5 mol/l was added into the upper compartment of transwells with cultured VSMCs. One-hundred microliters of medium was collected from the lower compartment, fluorescence was measured at 5, 10, 15, 25, 35, 45, 60, and 75 min after NE was added, and 100 μl of fresh medium was supplemented into the lower compartment after medium withdrawal each time (24).

**Statistical Analyses**

Data were presented as means ± SD of n observations. Statistical differences were analyzed by repeated-measures three-way ANOVA analyses followed by the post hoc Tukey test (SPSS version 15.0; SPSS, Chicago, IL). A P value of <0.05 (2-tailed) was considered statistically significant.

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RESULTS

Changes of Vascular Contractile and Relaxation Function Following Hyperlipidemia and Diabetes in Rats and the Relationship to Blood Flow and Organ Dysfunction

To observe whether VSMCs are functionally impaired at the early stage after diabetes and hyperlipidemia and whether it depends on VECs, we observed the change of vascular contraction and relaxation response of LRA with intact and denuded endothelium. The results showed that vascular contractile responses of LRA in healthy rats demonstrated no significant change at different ages during the observation period (38 wk). In hyperlipidemic or diabetic rats, the vascular contractile responses of LRA with intact endothelium (Fig. 2A) or denuded endothelium (Fig. 2B) all presented significant increases in the early stages and appeared to be hyperreactive ($P < 0.05$ or $0.01$). The increased rates in diabetic rats were higher than in hyperlipidemic rats. This vascular hyperreactivity in endothelium-denuded and endothelium-intact LRA was similar, suggesting that diabetes and hyperlipidemia-induced vascular hyperreactivity was not endothelium dependent.

Also, in healthy normal rats, the relaxation responses of LRA had no significant changes at different time points, whereas they were decreased significantly after diabetes and hyperlipidemia (Fig. 2C). This decrease also began in the early stages of diabetes and hyperlipidemia, and as the illness prolonged, the decrease extent was more serious. This finding was more obvious in diabetes than in hyperlipidemia.

Renal blood flow was significantly decreased at 4 wk after hyperlipidemia or diabetes ($P < 0.05$ or $0.01$). As the illness prolonged, the renal blood flow was decreased further (Fig. 2D). The decreased rate was negatively correlated with increased vascular contractile response (vascular hyperreactiveness), and the coefficients were $-0.927$ and $-0.947$ in hyperlipidemia or diabetes, respectively. Mitochondrial function (reflected by respiratory control rate) of the kidney was...
significantly decreased after hyperlipidemia or diabetes \((P < 0.05\) or 0.01; Fig. 2E). The decreased rate was positively correlated with the decreased renal blood flow. The coefficients were 0.9988 and 0.9306 in hyperlipidemia and diabetes, respectively.

Cyc and \(\beta_2\)-MG, reflecting glomerulus function, were increased significantly at 4 wk after hyperlipidemia or diabetes and BUN, and Scr began to increase at 8 wk after hyperlipidemia or diabetes \((P < 0.05\) or 0.01; Fig. 2, F–I). The increase of these variables in diabetes was more obvious than in hyperlipidemia, which was negatively correlated with a decrease in renal blood flow and mitochondrial respiratory control rate of the kidney.

**Relationship of Vascular Hyperreactivity Following Hyperlipidemia and Diabetes in Rats to Metabolic Inflammation, miRNAs, Connexins, and Rho Kinase**

To understand the mechanisms of diabetes and hyperlipidemia-induced vascular hyperreactivity, we preliminarily observed the relationship of diabetes and hyperlipidemia-induced vascular hyperreactivity to metabolic inflammation, miRNAs, and some vascular contraction regulatory molecules such as connexins, Rho kinase, and PKC in LRA.

Compared with healthy rats, the concentrations of TNF\(\alpha\), IL-1\(\beta\), and IL-6 in blood were significantly increased after hyperlipidemia or diabetes \((P < 0.01)\). Their increased rates in diabetes were higher than that in hyperlipidemia (Fig. 3, A–C).

The miRNA chip study showed that 36 miRNAs in hyperlipidemia and 47 in diabetes appeared significantly changed compared with healthy rats (data not shown) in renal artery tissues. Based on these results and bioinformatic analysis, we further quantified the expressions of miR-10a, miR-139b, miR-206, and miR-222 distributed and involved in the regulation of cardiovascular function. The results showed that they were significantly decreased in the renal artery in hyperlipidemia or diabetes \((P < 0.01)\). The changes in miR-10a, miR-139b, miR-206, and miR-222 were negatively correlated with vascular contractile response.

The protein expressions of Cx43 and Rho kinase in renal artery were significantly increased after hyperlipidemia or diabetes \((P < 0.01)\), whereas Cx37, Cx40, Cx45, PKC\(\alpha\), and PKC\(\xi\) had no significant changes. At 24 wk after hyperlipidemia or diabetes, the expressions of Cx43 and Rho kinase were increased 3.75- and 3.02-fold in diabetes rats and 3.65- and 2.23-fold in hyperlipidemic rats, respectively, compared with the normal healthy rats (Fig. 3, H–J).

**Effects of High Concentrations of Glucose, Lipid, and Cytokines on the Contractile Response of VSMC and the expressions of miRNA, Cx43, and Rho Kinase**

To further confirm whether there was direct and early damage from high concentrations of lipids and glucose or metabolic inflammation on VSMC and the mechanisms, we observed the effects of high concentrations of glucose and lipid incubation and TNF\(\alpha\), IL-1\(\beta\), and IL-6 on the contractile response of cultured VSMCs and the relationship to miRNA, Cx43, and Rho kinase.

The results showed high concentrations of glucose (25 mmol/l) or lipid (palmitic acid 50 \(\mu\)mol/l) incubated with VSMCs for 12–72 h, and the contractile response of VSMC was significantly increased \((P < 0.01)\). The accumulative infiltration rate of FITC-BSA after administration of NE was gradually increased. The infiltration rates were increased by 29.3–44.7% and 26.1–40.2% in the hyperglucose and hyperlipid groups, respectively, after 12–72 h of incubation (Fig. 4, A and B). Further study found that high concentrations of glucose (25 mmol/l) or lipid (50 \(\mu\)mol/l palmitic acid) incubated with VSMCs for 12–72 h significantly decreased the expressions of miR-10a, miR-139b, miR-206, and miR-222 and increased the expressions of Cx43 and Rho kinase \((P < 0.01); Fig. 4, C–I\).

Overexpressed Cx43 adenovirus could increase the expression of Rho kinase in VSMC, whereas Cx43 RNAi could decrease the expression of Rho kinase in VSMC (Fig. 4, J and K).

TNF\(\alpha\), IL-1\(\beta\), and IL-6 at concentrations of 50, 100, and 200 ng/ml, respectively, incubated with VSMCs for 24 h significantly decreased the expressions of miR-10a, miR-139b, miR-206, and miR-222 expression \((P < 0.01)\). TNF\(\alpha\) could downregulate the expression of miR-10a, miR-139b, and miR-222 but did not affect the expression of miR-206. IL-1\(\beta\) could downregulate miR-10a and miR-206 expression but had no effects on miR139b and miR222. IL-6 could downregulate the expression of all of these miRNAs (miR10a, miR 139b, miR206, and miR222; \(P < 0.01\)). Further study found that TNF\(\alpha\), IL-1\(\beta\), and IL-6 at the concentrations of 50, 100, and 200 ng/ml, respectively, increased the Cx43 and Rho kinase expressions and the contractile response in VSMCs (Fig. 5).

**Regulatory Effects of miR10a, miR139b, miR206, and miR222 on the Contractile Response of VSMCs and the Relationship to Rho Kinase and Cx43**

Under normal culture conditions, downregulation of miR-10a, miR-139b, miR-206, and miR-222 with the antisense of these miRNAs significantly increased the contractile response of VSMCs \((P < 0.01); Fig. 6A\). Whereas overexpressed miR-10a, miR-139b, miR-206, and miR-222 adenovirus significantly decreased hyperglucose- and hyperlipid-induced hyperresponsiveness of VSMC \((P < 0.01); Fig. 6, B and C\), inhibition of Rho kinase with Y-27632, antagonist of Rho kinase, and Cx43 with Cx43 AODN could antagonize hyperglucose- and hyperlipid-induced hyperresponsiveness of cultured VSMCs. The role of Y-27632 was stronger than Cx43 AODN \((Fig. 6, D and E)\).

To know whether and which miRNAs (miR10a, miR139b, miR206, and miR222) directly regulate Rho kinase and Cx43, we performed the psiCHECK assay and effects of highly expressed miR10a, miR139b, miR206 and miR222 adenovirus on the expression of Cx43 and Rho kinase. The results were that dual-fluorescence reporter experiments showed that miR-10a and miR-139b can directly act with 3' untranslated region (UTR) of Rho kinase but have no direct effect on Cx43 3'-UTR, whereas miR-206 and miR-222 can act directly with 3'-UTR of Cx43 but have no direct effect on Rho kinase 3'-UTR \((Fig. 6, F and G)\). Further studies found that overexpressed miR-10a and miR-139b adenovirus significantly decreased the protein expression of Rho kinase, whereas overexpressed miR-206 and miR-222 decreased the expression of Cx43 \((P < 0.01); Fig. 6, H–K\).
Fig. 3. The changes in cytokines, miRNAs, connexins (Cx), and contraction-related proteins following hyperlipidemia or diabetes in LRA. A–C: changes in TNFα, IL-1β, and IL-6 (n = 10). D–G: relative level of miR10a, miR139b, miR206, and miR222 (in healthy rats, the value is defined as 1; n = 6). H–J: changes in Cx37, Cx40, Cx43, Cx45, PKCα, PKCε, and Rho kinase (n = 3). *P < 0.05; **P < 0.01 vs. healthy rats at the same time point. Ctl, normal control group.
Beneficial Effects of Vascular Contractile Response Modulation on Organ Function for Diabetes and Hyperlipidemia in Rats

Blood glucose and lipids. Body weight was gradually increased in healthy rats as age increased, whereas their blood pressure, blood glucose and blood lipids, and HDL and LDL did not change significantly during the whole observation period. In hyperlipidemia rats, the body weight, blood lipids, and LDL were significantly increased after being fed a high-lipid diet, and they were significantly higher than that in healthy rats of the same age. HDL was significantly decreased after the feeding of a high-lipid diet. Simvastatin in combina-
Fig. 5. Effects of TNF-α, IL-1β, and IL-6 on the expression of miR10a, miR139b, miR206, miR222, Cx43, and Rho kinase in VSMCs and contractile response of VSMCs. A–D: effects of TNF-α, IL-1β, and IL-6 on expression of miR10a (n = 6; A), miR139b (n = 6; B), miR206 (n = 6; C), and miR222 (n = 6; D); control group is defined as 1. E–J: effects of TNF-α, IL-1β, and IL-6 on expression Cx43 and Rho kinase (n = 3). K: effects of TNF-α, IL-1β, and IL-6 on the contractile response of VSMCs (n = 8). *P < 0.05; **P < 0.01 compared with control group.
tion with or without fasudil significantly decreased serum cholesterol, triglyceride, and LDL and increased HDL levels compared with model rats (24 wk). In diabetic rats, the body weight appeared to decrease and the serum glucose was increased compared with healthy rats after being fed a diabetic diet. DBMG in combination with or without fasudil significantly decreased blood glucose and slightly decreased the blood lipids, including cholesterol and triglyceride levels, compared with the model rats (24 wk; Table 1). Blood LDL and HDL had no obvious changes before and after treatment.

Vascular contraction and relaxation function. Compared with diabetic or hyperglycemic rats, simple administration of
simvastatin in hyperlipidemia or DMBG in diabetes and lowering of the blood lipid or blood glucose level (Table 1) only slightly decreased diabetes and hyperlipidemia-induced hyperresponsiveness of vascular reactivity. Based on this treatment, the application of fasudil (24 wk), the inhibitor of Rho kinase, could significantly decrease diabetes and hyperlipidemia-induced hyperresponsiveness of vascular reactivity ($P < 0.01$). Early application of fasudil was superior to late application of fasudil. In addition, fasudil’s effect on decreasing diabetes and hyperlipidemia-induced vascular hyperresponsiveness was endothelium independent. The contractile responses of LRA with fasudil did not significantly improve the blood flow and mitochondrial function of the kidney, with no significant differences compared with controls. In combination with fasudil in the early stages of diabetes or hyperlipidemia, it significantly improved the renal blood flow and mitochondrial function of the kidney ($P < 0.05$). However, late application of fasudil did not significantly improve the blood flow and mitochondrial function of the kidney (Fig. 7, G and H).

Organ function. Also, simply decreasing the blood lipids or blood glucose level with simvastatin and DMBG did not significantly alleviate the damage of renal function. In combination with fasudil in the early stages of diabetes and hyperlipidemia, we could significantly alleviate the damage in renal function ($P < 0.05$; Fig. 7, I–L). It appeared that the blood CYC, β2-MG, BUN, and Scr were significantly decreased after application of fasudil at the early stage in combination with lowering blood glucose and lipids. Meanwhile, late application of fasudil did not alleviate kidney damage.

DISCUSSION

Vascular complication, the main complication in metabolic diseases, plays an important role in organ damage, in which vascular endothelium damage is most critical. However, whether the direct damage of VSMCs plays an important role in organ damage in diabetes and hyperlipidemia has not been determined. Our present study found that hyperglucose and hyperlipid at the early stages of diabetes and hyperlipidemia could directly induce the functional impairment of VSMCs. This functional impaired VSMC could induce the vascular hyperreactivity and hypersensitivity and then reduce the tissue perfusion and organ damage.

Previous studies have demonstrated that high concentrations of lipid and glucose could induce the change of biological features in VSMCs, including increasing the proliferation and migration activity of VSMCs (3, 10, 18). Except for high glucose, oxidized low-density lipoproteins can also promote VSMC proliferation, migration, and apoptosis (2, 11, 22). Previous studies have considered that the functional impairment of VSMCs in diabetes and hyperlipidemia results mainly from the phenotype transformation of VSMCs (21), which is induced by the long-term metabolic inflammation, oxidative stress, and so on. Our present study found that high concentrations of lipid and glucose at the early stages of diabetes and hyperlipidemia can directly interfere with the contractile response of VSMCs, inducing vascular hyperreactivity.

The mechanism responsible for hyperglucose and hyperlipids in diabetes and hyperlipidemia inducing the contractile function change of VSMCs is not clear. Our present study found that the concentrations of TNFα, IL-1β, and IL-6 were significantly increased and that the expressions of miR-10a, miR-139b, miR-206, and miR-222 were significantly decreased in renal artery tissue in diabetic and hyperlipidemic rats. Hyperglucose and hyperlipid, TNFα, IL-1β, and IL-6 could downregulate the expression of miR-10a, miR-139b, miR-206, and miR-222 and upregulate the expression of Cx43 and Rho kinase in VSMCs. Overexpressed miR-10a, miR-139b, miR-206, and miR-222 could downregulate the expression of Cx43 and Rho kinase, and overexpressed Cx43 could upregulate, whereas Cx43 RNAi could downregulate the expression of Rho kinase in VSMC. These results suggested that hyperglucose and hyperlipids could induce metabolic inflammation. Metabolic inflammation could downregulate the expression of miRNAs and then upregulate the expression of connexin and Rho kinase and finally induce the hyperreactivity of VSMCs (Fig. 8).

In short, single-stranded RNAs and miRNAs participate in the occurrence, development, and outcome of many diseases via inhibiting the target gene expression. Studies have shown that the cardiovascular system has various miRNAs, and their changes in expression are part of the pathogenesis of cardiovascular diseases (4, 5). For example, miR-1 is found to be high in expression, whereas miR-21 is found to be expressed lower in the pathogenesis of myocardial hypertrophy (26). miR146a has been found to play a critical role in pathological changes of atherosclerosis (28). miRNAs have also been found to be associated with the pathogenesis of diabetes (29). Although several studies have implicated the roles of miRNAs in the pathogenesis of diabetes, their roles in diabetic vascular complications are not widely studied and determined. With the miRNA chip, we found 36 miRNAs in hyperlipidemia and 47 in diabetes that appeared to be significantly changed compared with healthy rats in renal artery tissues. Based on the results and bioinformatic analysis, we further quantified the expressions of miR-10a, miR-139b, miR-206, and miR-222 and their role in hyperglucose- and hyperlipid-induced hyperreactivity in VSMC. The results showed that hyperglucose and hyperlipid could decrease the expression of the four miRNAs in VSMCs and found that they play important roles in hyperglucose- and hyperlipid-induced hyperreactivity in VSMCs.

Among all changed miRNAs, we selected only four miRNAs (miR-10a, miR-139b, miR-206, and miR-222) for further investigation. The main rationale is that the four miRNAs had the most obvious change in diabetic rats and rats with hyperlipidemia, and they are distributed and involved in the regulation of cardiovascular function based on the bioinformatic analysis. Of course, whether other miRNAs also participate in the regulation of vascular contractile function in diabetes and hyperlipidemia needs further investigation. In addition, renal artery tissue used to observe the alterations of miRNAs in the present study was endothelium intact, and we did not use an endothelium-denuded artery to measure. The change profile of miRNAs in endothelium-intact and -denuded arteries may have some differences. Since the in vivo experiment in the present study showed that endothelium denuded or intact did not signifi-
Fig. 7. Effects of lowering lipid and glucose in combination with fasudil on vascular contractile response, blood flow, organ, and mitochondrial function in DM or HLP rats (n = 10). A–C: contractile response of LRA from hyperlipidemia rats with or without endothelium to NE and relaxation. D–F: contractile response of LRA from diabetic rats with or without endothelium to NE and relaxation. G and H: tissue blood flow and mitochondrial function (mitochondrial RCR). I–L: kidney function damage parameters. Fasudil-Ear, early treatment with fasudil; Fasudil-Lat, late treatment with fasudil; Simva, simvastatin; DMBG, melamine hydrochloride. *P < 0.05 vs. no treatment group; #P < 0.05 vs. Simva or DMBG treatment group; @P < 0.05; @@P < 0.01 vs. late simva or DMBG + fasudil treatment group.
cantly affect the contractile response of renal artery, and cultured VSMC experiments showed that miR-10a, miR-139b, miR-206, and miR-222 had the same change pattern after incubation with hyperglucose and hyperlipids, TNFα, IL-1β, and IL-6 in renal artery tissue in diabetic rats and rats with hyperlipidemia, we did not use endothelium-denuded renal artery tissue to detect the changes in miRNAs in the animal part of the experiment. The possible differences in the miRNA change between endothelium-intact and -denuded arteries need to be studied further.

miRNAs are regulated by many factors, but cytokines are the important ones. Previous studies have found that cytokines participate in many physiological and pathophysiological processes via regulation of miRNAs (30). Meerson et al. (16) found that TNFα could downregulate miR-221 in cultured human preadipocytes. Ruan et al. (23) found that TNFα could upregulate miR126, miR23a, and miR125b and downregulate miR1975, miR222, and miR1974 in endothelial cell injury. Our present study found that TNFα, IL-1β, and IL-6 could selectively decrease the expressions of miR-10a, miR-139b, miR-206, and miR-222 in VSMCs. TNFα could downregulate the expression of miR-10a, miR-139b, and miR-222, but it could not affect the expression of miR-206. IL-1β could downregulate the expressions of miR-10a and miR-206, but had no effects on miR139b and miR222. IL-6 could downregulate the expression of all of these miRNAs. Nevertheless, how TNFα, IL-1β, and IL-6 regulate miR-10a, miR-139b, miR-206, and miR-222 needs further study.

Connexin is a kind of protein that forms a membrane channel called myoendothelial gap junction (MEGJ), connecting endothelial and smooth muscle cells. MEGJ is constructed by two connexons, which are formed by oligomerization of six connexins (25). Basic research has demonstrated that connexins between VSMCs VECs express mainly Cx37, Cx40, Cx43, Cx45, and Cx46. Our previous studies found that MEGJ took part in the regulation of the vascular contractile response after shock, and the types involved include Cx37, Cx40, and Cx43. The mechanism may be related to the regulation of intracellular Ca^{2+} and calcium sensitivity of VSMCs (17). Our present study found that diabetes and hyperlipidemia can induce the expression of Cx43 in renal artery via miRNAs. Increased expression of Cx43 contributed to the hyperreactivity of the vascular contractile response of renal artery in diabetes and hyperlipidemia. Further study found Cx43 could directly (regulation of the intracellular Ca^{2+} and calcium sensitivity of VSMCs) or via Rho kinase regulate vascular reactivity. miRNAs could directly or via Cx43 regulate Rho kinase to regulate the vascular reactivity. Because our results found that miRNAs had selective regulation on Cx43 and Rho kinase, miR206 and miR222 regulated mainly Cx43, whereas miR10a and miR139b regulate mainly Rho kinase (Fig. 8).

Rho kinase, a small G protein family member, has been shown to have an important role in vascular contraction regulation (9). Our previous study showed that Rho kinase played a very important role in vascular reactivity regulation following shock (13). Inhibition of Rho kinase activity can significantly decrease vascular reactivity and blood pressure (6). Fasudil, an inhibitor of Rho kinase, can inhibit vascular spasm in patients with subarachnoid hemorrhage (19). Our present study found that early application of fasudil could significantly suppress the diabetes- and hyperlipidemia-induced vascular hyperreactivity, increase the tissue perfusion, and prevent organ damage. However, late application of fasudil had no significant effect. The main reason for this may be that, at the early stages of diabetes and hyperlipidemia, only functional impairment exists in the vasculature; some functional measurement can improve function, whereas at the late stages of diabetes and hyperlipidemia, when the organ structure gets damaged, either it is difficult for a simple functional measurement to have an effect or it has only a limited effect. These results suggest that the prevention of VSMC dysfunction that started in the early stages of diabetes or hyperlipidemia may be a promising approach to prevent metabolic disease-induced organ damage.

Some limitations need further investigation in the present study. 1) Only fasudil, based on Rho kinase, was used to observe the beneficial effects on diabetes- and hyperlipidemia-induced vascular hyperreactivity and organ damage; whether the same good effects target connexins or miRNAs needs further investigation. 2) The present study was conducted in animals, and the observed organ is only in kidney; whether the same results can be obtained in human beings and other organs needs further confirmation. 3) Although we found that TNFα, IL-1β, and IL-6 can selectively regulate miRNAs, miRNAs can selectively regulate connexin and Rho kinase, and the precise mechanisms need further investigation.

CONCLUSION

Diabetes or hyperlipidemia can cause hyperreactivity in vascular contraction beginning in the early stages and is not endothelium dependent. Vascular contractile dysfunction plays...
a critical role in diabetes- and hyperlipidemia-induced vital organ damage. The main mechanisms are that diabetes and hyperlipidemia may induce metabolic inflammation and production of cytokines, which further regulate the expression of miRNAs and then the vascular contractile regulation pathway, to damage vascular contractile function. Measures aimed at vascular hyperreactivity are promising approaches to prevent metabolic disease-induced organ damage.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

T.L. and L.-m.L. conception and design of research; T.L., G.-m.Y., Y.Z., Y.W., X.-y.C., D.L., and K.-l.T. interpreted results of experiments; T.L., Y.Z., and L.-m.L. performed experiments; T.L., G.-m.Y., Y.Z., Y.W., and X.-y.C. prepared figures; T.L. drafted manuscript; T.L. and L.-m.L. revised and edited manuscript; T.L., G.-m.Y., Y.Z., Y.W., X.-y.C., D.L., K.-l.T., and L.-m.L. approved final version of manuscript; G.-m.Y., Y.W., X.-y.C., D.L., K.-l.T., and L.-m.L. analyzed data.

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