Reversibility of renal injury with cholesterol lowering in hyperlipidemic diabetic mice

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Abstract Hyperlipidemia is a risk factor for development and progression of diabetic nephropathy. However, it is not known if reduction of hyperlipidemia is protective against progression of disease. The goal of this study was to determine if reduction of hypercholesterolemia could limit progression of diabetic nephropathy. Diabetic and nondiabetic LDL receptor deficient (LDLR−/−) mice were fed diets containing either no cholesterol (0%) or high cholesterol (0.12%) for 36 weeks. One group each of diabetic and nondiabetic mice were fed the high-cholesterol diet for 26 weeks then changed to the 0% cholesterol diet for the last 10 weeks. Consumption of the high-cholesterol diet exacerbated the development of diabetic nephropathy with elevations in urine albumin excretion, glomerular and renal hypertrophy, and mesangial matrix expansion. Increased glomerular lipid and apolipoprotein B accumulation was found in diabetic mice that consumed the 0.12% cholesterol diet compared with other groups. However, diabetic mice that changed from the high-cholesterol diet to the 0% cholesterol diet for the last 10 weeks had lower urine albumin excretion and mesangial matrix expansion compared with mice that consumed the 0.12% cholesterol diet throughout. This suggests that hyperlipidemia causes continuous renal injury, and that lowering cholesterol levels by dietary means can improve renal function in diabetic LDLR−/− mice.—Taneja, D., J. Thompson, P. Wilson, K. Brandewie, L. Schaefer, B. Mitchell, and L. R. Tannock. Reversibility of renal injury with cholesterol lowering in hyperlipidemic diabetic mice. J. Lipid Res. 2010. 51: 1464–1470.

Supplementary key words diabetes • mesangial matrix • apolipoprotein B

Diabetes is the leading cause of end stage renal disease in the US. In addition to hyperglycemia, other risk factors for the development or progression of diabetic nephropathy have been identified, including hypertension and hyperlipidemia. Several studies have demonstrated mechanisms by which renal lipid accumulation can be damaging to the kidney, including induction of mesangial cell synthesis of cytokines monocyte chemotactic protein-1 (MCP-1) and monocyte colony-stimulating factor (M-CSF) (1, 2), increased mesangial cell synthesis of extracellular matrix components (3–5), and mesangial cell proliferation (6). Although hyperlipidemia is not thought to be a primary cause of renal injury in humans, several studies suggest that hyperlipidemia is a risk factor for the progression of established renal disease (7, 8).

This association between hyperlipidemia and renal disease has led to an interest in the potential use of lipid-lowering therapies to preserve renal function. Inhibitors of HMG-CoA reductase (statins) have been studied in a variety of populations for their ability to reduce cardiovascular disease events, where they have been shown to have profound benefits. However, most studies excluded subjects with renal disease or did not collect information on markers of renal injury. Thus, the data on statin use in nephropathy is inconclusive; although several studies suggest a benefit, others report no benefits [for review see (9)]. Statins robustly lower plasma cholesterol levels in humans, but also have a number of other, pleiotropic effects, including anti-inflammatory effects. Thus, reported benefits of statins in human diabetic nephropathy could be due to either their lipid-lowering or pleiotropic effects (10).

Abbreviations: apo, apolipoprotein; LDLR, LDL receptor deficient; MCP-1, monocyte chemotactic protein-1; M-CSF, monocyte colony-stimulating factor; STZ, streptozotocin; TGF, transforming growth factor.

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The goal of this study was to test the hypothesis that lipid lowering would limit the progression of diabetic nephropathy, using a murine model. Although previous studies have provided evidence that statins are protective against the development of diabetic nephropathy in mice (11–13), we elected to use dietary means to address the topic, as mice do not uniformly respond with lowered cholesterol levels to statin treatment (14). The diets selected are free of cholate or antioxidants, are very similar in terms of calories per gram, but differ in cholesterol content (15). We now provide data demonstrating that lowering cholesterol levels limits progression of diabetic nephropathy in this murine model.

METHODS

Chemicals and reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified.

Murine studies

Female hyperlipidemic LDL receptor deficient mice (LDLR\(^{-/-}\)) were generously provided by Dr. Alan Daugherty, University of Kentucky. Unlike many mouse models, LDLR\(^{-/-}\) mice carry their cholesterol in LDL particles, develop further elevations in cholesterol when fed high-cholesterol diets, and are susceptible to renal injury. Mice were housed in a specific pathogen free facility with 12 h light/dark cycles, with ad libitum access to food and water. These studies were approved by the Institutional Animal Care and Use Committee, in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Streptozotocin (STZ) was used to induce diabetes. Female LDLR\(^{+/+}\) mice received STZ 40 mg/kg/d intraperitoneally daily for 5 days at age 8 weeks and age 10 weeks. Control mice received an identical schedule of injections of the citrate buffer. All mice that received STZ had hyperglycemia (glucose > 250 mg/dl) by age 11 weeks (data not shown) and were started on diets containing either 0% cholesterol (g/kg, with 11% calories from fat) or 0.12% cholesterol (g/kg, 40% calories from fat; Harlan Teklad, Madison WI) at age 12 weeks, as previously described (15). To determine if reduction of hypercholesterolemia via dietary means could attenuate progression of nephropathy, one group each of control and diabetic mice were changed back to 0% cholesterol diets for the last 10 weeks after 26 weeks of consumption of the 0.12% cholesterol diet. Mice were weighed weekly and blood glucose was measured from the tail vein (Freestyle Flash\textsuperscript{®} Complete Blood Glucose Monitoring System, Abbott Laboratories, Abbott Park, IL) every other week or when mice lost weight. The majority of diabetic mice received insulin in the form of slow-release subcutaneous pellets (Linshin Canada Inc., ON, Canada) to avoid weight loss, but insulin dose was not titrated to achieve euglycemia. Insulin administration was repeated every 2 to 5 weeks as needed. Blood pressure was measured in conscious mice via tail cuff apparatus (Visitech Systems Inc., Apex, NC) during weeks 8, 16, 24, and 34 following one week of acclimation.

Metabolic characterization

Blood samples were obtained prior to study initiation, and in weeks 14, 26, and 36. Cholesterol, triglyceride, and glycated hemoglobin levels were measured as described previously (15). Plasma transforming growth factor (TGF-B)\textsubscript{1} Emax\textsuperscript{®} ImmunoAssay system (Promega, Madison, WI) according to manufacturer’s directions. Urine albumin excretion was measured during weeks 9, 17, 25, and 35 from urine samples obtained by housing mice individually for 24 h in metabolic cages (urine albumin excretion was not measured at baseline, and data is extrapolated to 0 at this time). Urinary albumin and creatinine were measured using commercially available kits (Exocell, Inc., Philadelphia, PA, and R and D Systems, Minneapolis, MN, for albumin and creatinine, respectively), and data is expressed as mg albumin per g creatinine.

Renal analyses

Mice were euthanized by lethal anesthesia after 36 weeks on the diets. Mice were perfused through the left ventricle at constant, near-physiological pressure with 10 ml sterile phosphate buffered saline. The kidneys were removed, decapsulated, and weighed. The right kidney was divided transversely, with half embedded in OCT and the other half snap-frozen in liquid nitrogen. The left kidney was divided transversely and fixed in 4% paraformaldehyde then embedded in paraffin. Histological analyses were performed on 4 μm thick tissue sections stained with periodic acid Schiff reagent. Matrix accumulation was scored using a semi-quantitative scale by two blinded observers (D.T. and L.R.T.) as previously described (16). Glomerular cross-sectional area was measured using computer-assisted morphometry (Image Pro, Media Cybernetics Inc., Bethesda, MD) in at least 30 glomeruli/mouse in glomeruli located in the outer cortex sectioned through the glomerular tuft. Renal pathology was evaluated by our expert renal pathologist (B.M.) blinded to group.

Renal lipid accumulation

Frozen renal sections embedded in OCT were sectioned 5 μm thick and stained with Oil Red O. Paraffin embedded sections (4 μm thick) were immunostained for apolipoprotein (apo)B (K23300R, Meridian Life Sciences Inc., Saco, ME). Negative controls were obtained with isotype-matched irrelevant antibodies, no primary antibody or no secondary antibody. Total renal protein was extracted and apoB content was also analyzed using Western blot analyses. Actin was used as the loading control (A2066, Sigma). Blots were scanned and densitometry performed using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analyses

Data is presented as mean ± SEM unless otherwise described. All data was analyzed by two-way ANOVA with multiple pairwise comparisons using Holm-Sidak method (SigmaStat Software Inc., San Jose, CA). \(P\) values < 0.05 were considered statistically significant.

RESULTS

Effect of diabetes and diets on survival

One nondiabetic mouse died during the study (unknown cause; mouse in the 0.12% diet group) and eight of 39 diabetic mice died during the study. Two diabetic mice died of hypoglycemia following insulin administration (both in the 0% diet group at week 14). Two diabetic mice were euthanized early per study protocol due to poor body condition and hyperglycemia despite insulin administration (one each from the 0% diet and diet change groups in weeks 14 and 21, respectively). One mouse (diabetic, diet change group) died of intestinal obstruction and three diabetic mice died from hyperglycemia and dehydration despite insulin administration during the study (one from the 0% diet group, two from the 0.12% diet group; weeks of death ranged from week 8 to week 31).
Effect of diabetes and diets on metabolic parameters

Diabetes was induced by STZ injections, and all STZ mice had elevations in blood sugar starting 2 weeks after injections, which persisted throughout the 36 week study (Fig. 1A). There was no effect of the high-cholesterol diet on blood glucose levels. Although 90% of diabetic mice required insulin periodically, the glucose levels remained elevated throughout the study. Glycated hemoglobin levels were significantly higher for diabetic mice than control mice (P < 0.001), but were not affected by diet (Table 1, showing 36 week measurements). Diabetic mice had less weight gain than control mice, but consumption of the 0.12% cholesterol diet led to increased weight gain compared with the 0% cholesterol diet within both control and diabetic mice. The mice that changed from the 0.12% diet to the 0% diet for the last 10 weeks of the study had minor weight loss, whereas the mice that continued on the 0.12% diet for the last 10 weeks continued to gain weight (Fig. 1B). Consumption of the high-cholesterol diet led to significant elevations of plasma cholesterol in both control and diabetic mice, but there was no effect of diabetes on plasma cholesterol levels. Interestingly the cholesterol levels increased between 26 and 36 weeks for all groups (Fig. 1C). Diabetic, but surprisingly not control mice, had a decrease in plasma cholesterol level when switched from the 0.12% cholesterol diet to the 0% cholesterol diet. There was no effect of either diet or diabetes on triglyceride levels (Table 1, showing 36 week values). Blood pressure was measured daily for 5 consecutive days every 8 weeks. There were no differences in blood pressure between any groups at any time (data not shown). As expected, TGF-β concentrations were increased in the diabetic mice compared with control mice overall (P < 0.001; Table 1), but were also affected by diet (P = 0.028). Pairwise comparisons revealed that diabetic mice fed the 0.12% cholesterol diet had higher TGF-β concentrations than diabetic mice fed the 0% cholesterol diet, but there was no effect of the diet change on plasma TGF-β concentrations in either diabetic or control mice.

Effect of diabetes and diets on renal parameters

Urinary albumin excretion was significantly elevated in diabetic mice as early as 9 weeks following induction of diabetes (P < 0.001). By 17 weeks of diet and diabetes, there was an apparent effect of both diabetes (P < 0.001) and diet (P = 0.008), with higher urinary albumin excretion levels in diabetic mice on the 0.12% cholesterol diets compared with the 0% cholesterol diet (P = 0.001). Both control and diabetic mice that changed diets from the 0.12% cholesterol diet to the 0% cholesterol diet for the last 10 weeks had no further elevations in albumin excretion, whereas all other groups had continued rise in albuminuria (Fig. 2A). There was no effect of diet or diabetes on kidney weight (not shown), or kidney weight corrected for body weight (Table 1). Mesangial matrix expansion was measured after 36 weeks of diet and/or diabetes (Fig. 2B, C). The diabetic group that changed diets had a matrix score less than the diabetic mice fed either the 0% or 0.12% diets throughout, suggesting that they either had less matrix expansion during the 0.12% diet period or possibly had regression of matrix expansion. However, the matrix expansion in the diet change groups was not significantly different to the other diet groups.

Effect of diabetes and diet on renal lipid accumulation

Renal sections were stained for neutral lipid with Oil Red O or immunostained for apoB to evaluate renal lipid accumulation. Oil Red O staining was detectable in sections from both control and diabetic mice fed the 0.12% cholesterol diet, with greater staining in diabetic mice than controls. No Oil Red O staining was seen in mice fed the 0% cholesterol diet throughout, or in mice fed the 0% cholesterol diet for the last 10 weeks of study (Fig. 3A). Using immunohistochemistry, apoB was detectable in all groups, but the greatest apoB staining was seen in diabetic mice and control mice fed the 0.12% cholesterol diets throughout.
There was a reduction in apoB staining in both control and diabetic mice that received the 0% cholesterol diets for 10 weeks following 26 weeks consumption of the 0.12% cholesterol diet (Fig. 3B). To evaluate this further, total renal protein was analyzed by Western blot for apoB content. Minimal apoB was present in control mice fed the 0% cholesterol diet, with increased amounts in all other groups. There was a significant effect of both diabetes (P = 0.038) and diet (P = 0.047) on renal apoB; however, by pairwise analyses there were no significant changes in renal apoB content in the groups that changed diet (Fig. 3C, D).

**DISCUSSION**

In this study we confirm previous reports that hypercholesterolemia exacerbates diabetic nephropathy (17–19). However, our data expands on previous reports by including mice that had dietary manipulations of cholesterol consumption to determine the effect of changing dietary cholesterol on progression of diabetic nephropathy. Two major markers of diabetic nephropathy, namely urinary albumin excretion and mesangial matrix accumulation, had attenuated progression in the mice that changed diets, compared with the mice that remained on the high-cholesterol diet throughout. In fact, both the urine albumin excretion and the mesangial matrix scores in diabetic mice that changed diets were similar to the scores in diabetic mice that consumed the 0% cholesterol diet throughout. These improvements were seen in the control mice that changed diets, despite the lack of reduction in plasma cholesterol levels with the diet change in these mice. Because we used LDLR−/− mice, even those mice fed the 0% cholesterol diet are hyperlipidemic (plasma cholesterol ~400 mg/dl on 0% cholesterol diet). Furthermore, the mice fed the 0% cholesterol diet had an increase in plasma cholesterol over time (levels increased to ~700 mg/dl by 36 weeks on the diet). This hypercholesterolemia could account for the renal injury that did develop in control mice on the 0% cholesterol diet. These data suggest that hypercholesterolemia causes renal injury in a continuous insult manner, and that lowering cholesterol levels may lead to improved renal function.

In support of this, we demonstrate that renal lipid accumulation was decreased in diabetic mice that changed diets, compared with diabetic mice that consumed the 0.12% cholesterol diet throughout, suggesting clearance of the renal lipid deposition. Immunohistochemistry for apoB similarly suggested clearance of renal lipoprotein deposition, although this was not confirmed by Western blot analyses. This discrepancy may be due to different sensitivities of the techniques and/or increased antigen accessibility in the protein extracts used for Western blots. It is conceivable that with prolonged period of study there would have been a reduction in renal apoB content in the groups that changed diets.

The pathogenic role of TGF-β is well established in diabetic nephropathy, and appears to be a major cause of extracellular matrix accumulation. We observed the expected elevations in TGF-β concentrations in diabetic mice, but also observed an increase in TGF-β levels in mice on the high cholesterol diet. Previous studies have suggested that hypercholesterolemia leads to increased activity of the renin-angiotensin system (20) and elevated angiotensin II leads to increased TGF-β (21). Thus, the modest mesangial matrix accumulation observed even in control mice fed the 0% cholesterol diet could be attributed to the modest elevations in TGF-β concentrations caused by hyperlipidemia, and the more extensive matrix expansion observed in diabetic mice could reflect the elevated TGF-β concentrations in these groups. However, there was no change in TGF-β concentrations in the diabetic mice that changed diets, suggesting that the improvement in mesangial matrix accumulation was not due to altered TGF-β signaling, but perhaps due to the changes in renal lipid accumulation.

There are some limitations with our study. First, the control mice that changed diets did not have the expected drop in plasma cholesterol concentrations, whereas the diabetic mice did. The reasons for this are unclear, as both groups consumed the same diet and there were no other abnormalities between groups. Despite the lack of change in plasma cholesterol levels in the control mice that changed diets, the mice did have a modest weight loss and an improvement in urine albumin excretion, similar to

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**TABLE 1. Effect of diabetes and diets on metabolic parameters**

|                      | 0% Cholesterol Diet | 0.12% Cholesterol Diet | Diet Change |
|----------------------|----------------------|-------------------------|-------------|
|                      | Control              | Diabetes                | Control     | Diabetes               |
| Glycated hemoglobin (%) | 5.9 ± 0.3            | 9.4 ± 0.7               | 7.4 ± 0.4   | 9.7 ± 1.4              | 7.0 ± 0.6 | 12.3 ± 1.6 |
| Triglycerides (mg/dl) | 42 ± 8               | 48 ± 11                 | 82 ± 15     | 77 ± 20                | 65 ± 17 | 57 ± 9    |
| Plasma TGF-β (pg/ml) | 1073 ± 162           | 3035 ± 294              | 2250 ± 526  | 5582 ± 1046            | 1456 ± 116 | 5320 ± 1044 |
| Renal weight/body weight (mg/g) | 4.8 ± 0.2 | 6.0 ± 0.5 | 4.4 ± 0.2 | 5.2 ± 0.3 | 5.1 ± 0.3 | 5.8 ± 0.7 |

Data shown is mean ± SEM for N = 7–12 per group as indicated, measured after 36 weeks of 0% or 0.12% cholesterol diets and/or diabetes. Diet change groups were fed the 0.12% cholesterol diet for 26 weeks and then changed to the 0% cholesterol diet for the last 10 weeks. All analyses were done by two-way ANOVA with pairwise comparisons by Holm-Sidak method.

a represents P < 0.05 compared with control group on 0% diet.
b represents P < 0.05 compared with control group on 0.12% diet.
c represents P < 0.05 compared with diabetic group on 0% diet.
d represents P < 0.05 compared with control group that changed diets.

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**Reversibility of renal injury**
the diabetic group that changed diets. This suggests that despite their lack of change in plasma cholesterol levels, the mice did have some metabolic response to the diet change. Second, the C57BL6 mouse model has been reported to be relatively resistant to the development of diabetic nephropathy (22, 23). However, the major focus of this study was to evaluate the effect of lowering lipid levels on the progression of diabetic nephropathy. Thus, the diet-responsive hyperlipidemic LDLR<sup>−/−</sup> model (C57BL6 genetic background) was selected. The LDLR<sup>−/−</sup> mouse does not develop atherosclerosis while fed normal rodent chow, but does develop atherosclerosis when challenged with high-fat/ high-cholesterol diets. We observed similar effects on development of diabetic nephropathy: despite their hypercholesterolemia while fed the 0% cholesterol diet, the nondiabetic LDLR<sup>−/−</sup>/H<sub>11002</sub>/H<sub>11002</sub> mice had minimal development of nephropathy; when challenged with the 0.12% cholesterol diet, increased development of diabetic nephropathy was observed. Thus, this model developed sufficient renal injury for us to test the hypothesis that lipid lowering after establishment of diabetic nephropathy would limit progression of renal disease. Third, the duration of study following the diet change was short. It is possible that with a longer study the changes in metabolic and renal parameters following the diet change would have become more apparent. However, despite this relatively brief
period of study following the diet change, we did observe improvements in renal function (urine albumin excretion) and pathology (mesangial matrix expansion).

In humans, dyslipidemias appear to accelerate the decline of renal function in established nephropathy (8, 24, 25), and have a modest effect to predict initiation of renal dysfunction when studied in cohorts with other known risk factors for renal insufficiency, namely hypertension and diabetes (26, 27). Several studies demonstrate intra-glomerular lipid deposition on human renal biopsies (28–30). Increased apoB and E staining on renal biopsies occurred in the presence of more advanced renal disease compared with biopsies with minimal or no apoB and E staining (31). However, patients with hyperlipidemia do not usually develop renal insufficiency in the absence of another renal insult. Thus, in humans, dyslipidemia does not initiate renal injury, but contributes to progression of renal dysfunction, raising the question of whether lipid lowering could be a therapeutic option in subjects with early renal disease. However, to date, most clinical studies using lipid-lowering drugs have either excluded subjects with impaired renal function, or have studied patients with very advanced renal failure, in whom no effect of lipid lowering on renal function would be expected. Nevertheless, some statin trials have documented modest improvements in markers of renal function, suggesting a possible benefit of lowering LDL levels in humans with early renal disease (32). The Study of Heart and Renal Protection, currently ongoing, is anticipated to provide definitive evidence of the role of lipid-lowering medications in the progression of renal disease. Murine studies using statins have indicated a protective role for statins on the development of renal disease (11–13), but whether this is a direct result of lipid lowering or due to pleiotropic effects of statins is not clear, as mice do not consistently respond to the lipid-lowering effects of statins (14). Our data suggests that hyperlipidemia provides continuous renal insult, and that reduction of hyperlipidemia by dietary means can limit progression of renal disease.14

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