The antioxidant silybin prevents high glucose-induced oxidative stress and podocyte injury in vitro and in vivo

Khaled Khazim, Yves Gorin, Rita Cassia Cavaglieri, Hanna E. Abboud, and Paolo Fanti

South Texas Veterans Health Care System, Audie L. Murphy Memorial Hospital Division, San Antonio, Texas; Department of Medicine, University of Texas Health Science Center, San Antonio, Texas; and Nephrology and Hypertension Unit, Western Galilee Hospital, Nahariya, Israel

Corresponding author.
Address for reprint requests and other correspondence: P. Fanti, Univ. of Texas Health Science Center, Dept. of Medicine, Division of Nephrology MC 7882, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900 (e-mail: fanti@uthscsa.edu).

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Abstract

Podocyte injury, a major contributor to the pathogenesis of diabetic nephropathy, is caused at least in part by the excessive generation of reactive oxygen species (ROS). Overproduction of superoxide by the NADPH oxidase isoform Nox4 plays an important role in podocyte injury. The plant extract silymarin is attributed antioxidant and antiproteinuric effects in humans and in animal models of diabetic nephropathy. We investigated the effect of silybin, the active constituent of silymarin, in cultures of mouse podocytes and in the OVE26 mouse, a model of type 1 diabetes mellitus and diabetic nephropathy.

Exposure of podocytes to high glucose (HG) increased 60% the intracellular superoxide production, 90% the NADPH oxidase activity, 100% the Nox4 expression, and 150% the number of apoptotic cells, effects that were completely blocked by 10 µM silybin. These in vitro observations were confirmed by similar in vivo findings. The kidney cortex of vehicle-treated control OVE26 mice displayed greater Nox4 expression and twice as much superoxide production than cortex of silybin-treated mice. The glomeruli of control OVE26 mice displayed 35% podocyte drop out that was not present in the silybin-treated mice. Finally, the OVE26 mice experienced 54% more pronounced albuminuria than the silybin-treated animals. In conclusion, this study demonstrates a protective effect of silybin against HG-induced podocyte injury and extends this finding to an animal model of diabetic nephropathy.

Keywords: albuminuria, apoptosis, diabetic nephropathy, NADPH oxidase, phytochemicals

The podocytes are terminally differentiated and highly specialized glomerular visceral epithelial cells that, along with the endothelial cell layer and the glomerular basement membrane (GBM), participate in the formation of the glomerular filtration barrier and in the prevention of urinary protein loss. Podocyte injury leads to proteinuria and is considered a major contributor to the initiation and progression of both diabetic and nondiabetic glomerular disease. Patients with early type 1 and type 2 diabetes mellitus experience loss of podocytes and show correlation between the rate of albumin excretion and the drop in podocyte number. In addition, mouse models of both type 1 and 2 diabetes demonstrate a sharp increase in podocyte apoptosis and reduction in podocyte/slit diaphragm protein expression shortly after the onset of hyperglycemia and proteinuria.

Oxidative damage from both free radical and nonradical oxygen species contributes to the pathogenesis of diabetic complications including onset and progression of diabetic kidney disease. Both experimental and clinical studies have documented a link among hyperglycemia, oxidative stress, and diabetic nephropathy. More recently, reactive oxygen species (ROS)-mediated effects of HG have been implicated in podocyte injury and apoptosis. In this context, the NADPH oxidase isoform Nox4 has emerged as one of the most important sources of ROS in the kidney.

The flavonolignan silybin is known as silibinin, the most abundant (50–70%) and the most active component of silymarin, an extract from the plant milk thistle (silybum marianum, Asteraceae family). Silymarin exerts hepatoprotective effects and has been used for centuries as an herbal remedy for liver disease. In vitro studies showed...
that silymarin has both antioxidant and anti-inflammatory effects (9), including inhibition of superoxide production in Kupffer cells (13). In addition, silybin prevents hydrogen peroxide-induced apoptosis of endothelial cells (56). Silymarin also appears to reduce proteinuria in a rat model of streptozotocin-induced diabetes and in patients with type 2 diabetes (17, 55). The mechanisms by which silymarin exerts these effects are unknown.

These observations led us to formulate the hypothesis that silybin through its antioxidant effects reduces proteinuria by direct effects on the podocyte. The studies described in this study explore the effect of silybin on podocyte injury in vitro in cultured podocytes and in vivo in a mouse model of type 1 diabetes.

**MATERIALS AND METHODS**

**Materials**

Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Silybin stock solution (20 mM final concentration) was prepared in dimethyl sulfoxide (DMSO); for the in vitro experiments, the stock was directly dissolved in the culture media. For the in vivo experiments, silybin was first diluted 1:10 with DMSO and then 4/46/46 (vol/vol/vol) with propylene glycol and normal saline to a final concentration of 80 µM.

**Cell Culture**

Conditionally immortalized mouse podocytes were grown to near confluence and reseded three times under growth-permissive conditions (33°C) in flasks coated with type I collagen in a humidified chamber with 5% CO₂. Growth media consisted of RPMI 1640 with 10% fetal bovine serum, 100 U/ml of penicillin/streptomycin, and 5 mM D-glucose (NG). The medium was supplemented with 50 U/ml of mouse interferon-γ (INF-γ) during the first passage and then 10 U/ml INF-γ in the second and third passage. Cell differentiation was then induced by subculturing the cells under nonpermissive conditions (37°C) in serum-containing medium without INF-γ for 10–14 days (46). Cells were serum starved for 24 h before the experiment with RPMI 1640 containing NG and 0.2% BSA. The experiment consisted of overnight pretreatment of the cells with silybin at a final concentration of 10 µM or with vehicle. Cells were then exposed to NG or 25 mM D-glucose (HG) with or without silybin for 24 h.

**Animal Models**

OVE26 mice (FVB background; The Jackson Laboratory, Bar Harbor, ME) were used as a model of type 1 diabetes mellitus and FVB mice as nondiabetic control animals. At 6 wk of age, mice were started on an animal protein-based diet (Teklad irradiated global soy protein-free extruded rodent diet-2920X) and animals were provided food and water ad libitum; at 10 wk they were started on 100 mg/kg silybin or vehicle given intraperitoneally for 6 wk (6 animals in each group). Urine collections were done in metabolic cages, and the urine albumin-to-creatinine ratio was measured using commercial kits for mouse albumin (Bethyl Laboratories, Montgomery, TX) and creatinine (Enzo Life Science, Farmingdale, NY). Blood glucose was measured using a glucose meter with detection range 10–600 mg/dl (Contour; Bayer Healthcare, Tarrytown, NY). The animals were killed by exsanguination under anesthesia. The cortex of one kidney was flash-frozen in liquid nitrogen for microscopy and image analysis, while the contralateral organ was fixed in 10% formalin for 24 h and then embedded in paraffin for immunohistochemistry. The Institutional Animal Care and Use Committee of the University of Texas Health Science Center San Antonio approved the protocol.

**NADPH Oxidase Activity**

NADPH oxidase activity was measured in cultured podocytes using lucigenin-enhanced chemiluminescence as described previously (23). Cells were washed with ice-cold phosphate-buffered saline and scraped from the plate in lysis buffer (20 mM KH₂PO₄ pH 7.0, containing protease inhibitor cocktail; Roche Diagnostics, Indianapolis, IN). The material was homogenized (100 strokes) using an ice-cold Dounce homogenizer. To start the assay, 25 µg of homogenates were added to 50 mM phosphate buffer (pH 7.0) containing 150 mM sucrose, 5 µM lucigenin, and 100 µM NADPH. Photon emission expressed as relative light units was measured every 30 s for 5 min by luminometry. Superoxide production was expressed as relative light units per minutes per milligrams of protein. Protein content was measured using Bio-Rad Protein Assay Reagent (Bio-Rad laboratories, Hercules, CA).

**Detection of Intracellular Superoxide**

Intracellular superoxide anion production was measured by analyzing the conversion of the fluorescent probe dihydroethidium (DHE) into dihydroxyethidium (2-OH-E+ ) adopting an established high-performance liquid
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For analysis of Wilm's tumor 1 (WT-1) expression, frozen kidney sections were fixed in acetone for 10 min. Formalin, embedded in paraffin, and subjected to microwave irradiation at 2,400 W for 6 min in citrate buffer to enhance antigen retrieval. For analysis of Wilm's tumor 1 (WT-1) expression, frozen kidney sections were fixed in acetone for 10 min.

Immunohistochemistry

For analysis of Nox4 and Wilm's tumor 1. For analysis of Nox4 expression, kidney sections or cell monolayers were fixed in 10% formalin, embedded in paraffin, and subjected to microwave irradiation at 2,400 W for 6 min in citrate buffer to enhance antigen retrieval. For analysis of Wilm's tumor 1 (WT-1) expression, frozen kidney sections were fixed in acetone for 10 min.
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Studied on a type 1 diabetic mouse model. Podocyte apoptosis was examined in vitro by two different methods, Hoechst staining and DNA fragmentation. As shown by Hoechst staining, exposure of the podocytes to HG for 24 h resulted in significant podocyte apoptosis compared with NG, while silybin protected against HG-induced podocyte apoptosis. Likewise, silybin completely prevented DNA fragmentation, a measure of podocyte apoptosis induced by HG. 

Studies on a type 1 diabetic mouse model. To validate the findings in cultured immortalized mouse podocyte, we studied the effect of silybin on an established mouse model of type 1 diabetes, the OVE26 mouse. These mice develop morphological and structural changes characteristics of human diabetic nephropathy. Three groups of mice were studied: J) FVB...
control animals, 2) control diabetic OVE26 animals treated with vehicle, and 3) OVE26 diabetic mice treated with silibin for 6 wk. Blood glucose levels were 130.4 ± 12.4 mg/dl in the FVB control animals and above the detection limit (600 mg/dl) in the OVE26 diabetic groups, irrespective of treatment with vehicle or silibin.

**Effects of silybin on superoxide production in the renal cortex.** Superoxide production was measured in kidney cortex using DHE and HPLC. 2-OH-E\(^+\) production was increased in the renal cortex from diabetic mice and silibin treatment significantly reduced the increase of 2-OH-E\(^+\) in OVE26 mice (Fig. 4A). In addition, immunohistochemistry of kidney cortex showed increased expression of Nox4 in the OVE26 mice compared with the FVB control mice (Fig. 4B). Silibin treatment prevented the increased expression of Nox4 in the OVE26 mice (Fig. 4B).

**Effects of silybin on the number of resident podocytes.** Loss of podocytes in diabetic mice was detected by counting the number of synaptopodin-positive cells in glomeruli (Fig. 5A and B). Dual-label immunohistochemistry/immunofluorescence was used to identify and count podocytes relative to the GBM stained with collagen IV (Fig. 5B). As shown in Fig. 5A and B, synaptopodin staining is significantly reduced in type 1 diabetic OVE26 mice compared with FVB mice. Treatment with silibin for 6 wk prevented diabetes-induced podocytes loss (Fig. 5A and B). The expression of WT-1 is restricted to podocytes in mature glomeruli (37, 49). Therefore, we also evaluated the number of podocytes in glomeruli using WT-1 staining. The number of the WT-1-positive cells in glomerular sections (20–25 glomeruli per animal, 3 animals per group) was reduced in the diabetic mice, and this effect of diabetes was prevented by treatment with silibin (Fig. 5C).

**Effect of silybin on albuminuria.** The diabetic mice developed severe albuminuria when compared with the nondiabetic mice. Silibin treatment attenuated the albuminuria in the diabetic mice by 54% (P = 0.06; Fig. 5D).

**DISCUSSION**

The present study demonstrates a protective effect of the flavonolignan silybin on renal injury and albuminuria in the OVE26 mouse, an animal model of type 1 diabetes. In addition, the study provides in vivo and in vitro evidence demonstrating that the mechanisms by which silybin exerts its renoprotective effect involve inhibition of NADPH oxidase activity and prevention of podocyte apoptosis (Fig. 6).

Consistent with the original description of the OVE26 model, our mice were hyperglycemic shortly after birth and they displayed severe proteinuria and significant loss of podocytes at the age of 16 wk. As a novel observation, we report that treatment with silibin for 6 wk prevented podocyte loss and reduced albuminuria by 54% without affecting blood glucose levels. Albuminuria is the strongest independent clinical predictor of progression to end stage renal disease in diabetic patients (1, 3, 11, 28), and reduction of albuminuria and proteinuria is associated with slower decline in glomerular filtration rate and reduced risk for end stage renal disease (7, 44). Furthermore, glomerular podocyte loss is an early event in the pathogenesis of diabetic nephropathy (48, 60, 61) and murine models of diabetic nephropathy have shown that podocyte apoptosis precedes the onset of albuminuria and mesangial matrix expansion (35). In this context, the ability of silybin to correct both podocyte loss and albuminuria in diabetic nephropathy represents a new and/or additional therapeutic option to the conventional established antiproteinuric treatment strategies. In this study, we did not monitor blood pressure because prior research showed that 16-wk-old OVE26 mice have similar or slightly lower blood pressure than control FVB mice and that OVE26 mice experience an increase in the systolic blood pressure only at 8 mo of age (10, 63).

Of interest is that milk thistle and silymarin, a silybin-enriched milk thistle extract, have recently been reported to increase the renal activity of certain antioxidant enzymes (catalase and glutathione peroxidase) and to protect the kidneys from diabetic damage in streptozotocin-treated rats (55). Treatment with silymarin also reduced albuminuria in type 2 diabetic patients (17). Furthermore, the effect of silybin seems not to be limited to diabetic nephropathy since this flavonoid was shown to prevent glomerular and tubular cell injury and apoptosis in cisplatin- and arsenic-treated rats (5, 19, 42). The present study adds to the existing evidence not only by demonstrating a renoprotective effect of silybin in a new animal model of type 1 diabetes but also by providing strong evidence that prevention of podocyte injury is a major underlying mechanism of this protective effect.

Podocyte injury in diabetes results from oxidative stress mediated primarily by NADPH oxidases of the Nox family (50). Indeed, Nox-dependent ROS generation and specifically the ROS produced by the isoform Nox4 are now recognized as a key effector of renal cell damage, including podocyte injury and depletion that characterize the early stages of diabetic kidney disease. In the present study, we confirm previous observations indicating that Nox4 expression is increased in podocytes exposed to glucose (16, 22, 26, 45). We have previously shown that impairment of Nox4 function with an adenovirus encoding a dominant-negative form of Nox4 significantly inhibits glucose-induced NADPH oxidase activity and
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Our in vitro data demonstrate that silybin decreases superoxide generation in cultured podocyte and the in vivo study showed a similar effect in the kidney cortex. These findings, together with previous reports of silybin-induced reduction of fibronectin accumulation by human mesangial cells exposed to HG (57) and of protection of the kidney from arsenic toxicity via a decrease in ROS generation and apoptosis of tubular cells (40), clearly support the concept that silybin targets several cell types involved in the pathogenesis of diabetic nephropathy. This is not surprising considering that oxidative stress and enhanced generation of ROS is the underlying mechanism of injury to multiple cell types. It is tempting to speculate that inhibition of Nox4 function may be the common protective mechanism of silybin in all these cell types, since it is also known that Nox4-derived ROS play a key role in glucose-mediated mesangial cell injury.

Contrary to what is observed in diabetic renal tissue where Nox4 promotes apoptosis, Nox-derived ROS are described as antiapoptotic in cancer cells (36, 53). Interestingly, in cancer cells, silybin exerts proapoptotic rather than antiapoptotic effects as seen in diabetes (12, 27, 51). Therefore, it appears that Nox-derived ROS and silybin effects on cell survival differ in normal vs. malignant cells. Given that Nox4 has been implicated in numerous cancers (4), it is possible that Nox4 constitutes a prominent target of silybin in cells. These findings emphasize the complexity of the interaction between therapeutic or toxic xenobiotics and the biological conditions under which oxidative stress occurs.

Besides the herein described effect of silybin on NADPH oxidase and superoxide production, several other antioxidant effects targeting a variety of cells and tissues have been attributed to this compound and/or analogs including direct scavenging of free radicals, inhibition of the formation of other unstable compounds besides superoxide (14, 54, 62), maintenance of glutathione and other endogenous antioxidant redox balance (31, 33), as well as enhanced expression of the antioxidant enzymes glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase (8, 38). Furthermore, silybin suppresses the production of inflammatory cytokines incriminated in the pathogenesis of diabetic nephropathy including tumor necrosis factor-α (2, 32, 52) and transforming growth factor-β1 (25, 39, 47). The effects of silybin therefore may not be limited to inhibition of NADPH oxidase activity and to prevention of podocyte injury and loss, and they may be extended to protection against other renal and nonrenal complications of diabetes mellitus.

In conclusion, this study demonstrates a protective effect of the antioxidant silybin against HG-induced podocyte injury and extends this finding to an animal model of type 1 diabetes and diabetic nephropathy. Our data support the concept that silybin may represent a novel therapeutic intervention for the treatment of diabetic nephropathy. Clinical trial aiming to determine the efficacy of silybin-containing flavonoids (milk thistle) in diabetic nephropathy is currently underway (ClinicalTrials.gov No. NCT01265563).

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: K.K., Y.C.G., and R.C.C. performed experiments; K.K., Y.C.G., R.C.C., and P.F. analyzed data; K.K., Y.C.G., H.E.A., and P.F. interpreted results of experiments; K.K. and P.F. prepared figures; K.K. drafted manuscript; Y.C.G.,
REFERENCES


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Fig. 1.

Silybin inhibits high glucose (HG)-induced superoxide generation. Exposure of mouse podocyte cultures to normal glucose (NG; 5 mM glucose) or to HG (25 mM glucose) with or without 10 µM silybin. Assessment of superoxide generation with the dihydroethidium (DHE) stain using 2 different methods: A, left: representative fluorescence microscopic images of podocytes after incubation with 10 µM DHE for 15 min. A, right: bar graph of semiquantitative analysis of the DHE fluorescence (means ± SE of 3 different experiments). ***P < 0.0001 HG vs. NG; ###P < 0.0001 HG vs. HG + silybin. B, left: representative HPLC elution profiles of the dihydroxyethidium (2-OH-E·) peak, a DHE byproduct specific for superoxide generation, and the nonspecific ethidium (E·) peak in cells extracts from cultured podocytes. B, right: bar graph of quantitative analysis of 2-OH-E· generation (means ± SE of 4 different experiments each experiment in triplicate). ***P < 0.001 HG vs. NG; ##P < = 0.01 HG vs. HG + 10 µM silybin. Results are expressed as nmol 2-OH-E·/µM consumed DHE. C: dose-dependent response of cultured podocytes to silybin. Cells exposed to HG in the presence of 0.1, 1, or 10 µM silybin or vehicle. 2-OH-E· generation assessed by HPLC, as in B (***P < 0.001 HG vs. NG; #P < 0.05 HG vs. HG + 0.1 µM silybin; ##P < 0.01 HG vs. HG + 1 µM silybin; ###P < 0.01 HG vs. HG + 10 µM silybin).
Effect of silybin on NADPH oxidase activity and Nox4 expression. Exposure of mouse podocytes to NG or HG without and with 10 µM silybin. A, left: representation of a representative individual experiment of NADPH-dependent superoxide generation in cultured cells, analyzed by lucigenin-enhanced chemiluminescence. Superoxide anion generation was determined by photoemission every 30 s for 4–5 min and was expressed as relative light units (RLU)/mg protein. A, right: bar graph of quantitative analysis of photoemission intensity expressed as RLU·min⁻¹·mg protein⁻¹ (means ± SE). **P < 0.01 NG vs. HG; #P < 0.05 HG vs. HG + silybin.

B, left: Western blotting analysis of Nox4 expression in homogenized podocytes. Actin was included as control for loading and for specificity of change in protein expression. B, right: bar graph of quantitative analysis of Nox4 densitometry corrected for actin band density (means ± SE; *P < 0.05 NG vs. HG; ##P < 0.01 HG vs. HG + silybin).

C, left: representative Western blotting analysis of Nox1 expression in homogenized podocytes. Actin was included as control for loading and for specificity of change in protein expression. C, right: bar graph of quantitative analysis of Nox1 densitometry corrected for actin band density (means ± SE of 3 experiments).
Effect of silybin on HG-induced podocyte apoptosis. Exposure of serum-deprived mouse podocytes to NG or HG for 24 h without or with 10 µM silybin. A, left: detection of apoptotic nuclei using Hoechst 33258. Chromatin condensation (white arrows) examined by fluorescent microscopy. A, right: bar graph of apoptotic cells as percent count of total cells (3 different experiments). **$P < 0.01$ NG vs. HG; ##$P < 0.01$ HG vs. HG + silybin. B: ELISA for cellular DNA fragmentation. Bar graph expresses the fold increase (means ± SE of 3 experiments in triplicate). *$P < 0.05$ NG vs. HG; #*$P < 0.05$ HG vs. HG + silybin.
Silybin inhibits superoxide generation in diabetic mice kidney cortex and decreases the Nox4 protein expression. Measurement of superoxide generation in kidney cortex of 16-wk-old FVB mice, OVE26 mice, and OVE26 mice treated with silybin 100 mg/kg ip for 6 wk. A: bar graph of the percent increase in 2-OH-E\(^2\)† generation in kidney cortex (means ± SE; \(n = 4\) per group). **\(P < 0.01\) FVB vs. OVE26; #\(P < 0.05\) OVE26 vs. OVE26 + silybin. B: representative immunoperoxidase staining images of Nox4 protein in kidney sections from FVB (nondiabetic control), OVE26 (diabetic control), and OVE26 + silybin mice (diabetic treated).
Silybin decrease podocyte injury in diabetic mice. A, left: representative immunofluorescence images of glomeruli stained for synaptopodin. A, right: bar graph of fluorescence intensity (means ± SE; n = 3 per group). ***P < 0.001 FVB vs. OVE26; ###P < 0.001 OVE26 vs. OVE26 + silybin. B, left: representative dual-label immunohistochemistry/fluorescence staining of glomeruli for podocyte count; basement membranes stained with collagen IV. B, right: bar graph of podocyte count per glomerulus (means ± SE; n = 3 per group). ***P < 0.001 FVB vs. OVE26; ###P < 0.001 OVE26 vs. OVE26 + silybin. C, left: representative immunoperoxidase staining of glomeruli for WT-1 protein expression. Right panel, bar graph of podocyte count per glomerulus (mean ± SE; n = 3 per group). ***P < 0.001 FVB vs. OVE26; ###P < 0.001 OVE26 vs. OVE26 + silybin. D: bar graph of albumin/creatinine ratio in 24-h urine samples from FVB, OVE26, and silybin-treated OVE26 mice. Urine collected over 24 h in metabolic cages after acclimatization. Albumin measured by ELISA and creatinine by colorimetry. Results expressed as µg protein/mg creatinine (means ± SE). ***P < 0.001 FVB vs. OVE26; P = 0.06 OVE26 vs. OVE26 + silybin.
Proposed mechanism of the protective effect of silybin on HG/diabetes-induced podocyte injury/apoptosis.

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