Inquiro, the UAB undergraduate science research journal

Volume 2009 | Number 3 Article 27

2009

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Environmental Tobacco Smoke: Role in Progression of Diabetic Nephropathy

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Abstract:

Clinical studies suggest that smoking is a risk factor in the progression of chronic kidney disease, including diabetic nephropathy (DN). The mechanisms involved, however, are not completely understood. We have previously demonstrated that nicotine, one of the compounds present in large amounts in tobacco smoke, promotes mesangial cell proliferation and fibronectin production. In this study, we hypothesized that exposure to environmental tobacco smoke (ETS) promotes the progression of diabetic nephropathy by increasing the expression of cytokines such as TGF-β, resulting in increased mesangial expansion and matrix deposition.

Six-week-old diabetic (db/db) mice were divided into two groups. The experimental group (n=12) was exposed to ETS at a concentration of 30 mg/m3 for 6 hrs/day, 5 days/week for eight weeks. The control group (n=8) was not exposed to smoke. Urine was collected before euthanasia for albumin by ELISA, and creatinine measurements by mass spectrometry. After euthanasia, the kidneys were harvested for morphometric analysis and Western blot analysis. Serum was saved for cotinine measurements using ELISA.

ETS exposure resulted in significant mesangial expansion that was accompanied by concomitant increases in TGF-β and fibronectin expression. There was, however, no difference in albumin urinary excretion between the two groups. Serum levels of cotinine found in the ETS group were similar to those found in smokers.

Introduction

The prevalence of type 2 diabetes mellitus in the United States has doubled since 1990.[1] There are about 24 million people in the United States with diabetes, of which 16.11 million have been diagnosed with type 2 diabetes.[2] A complication of diabetes, diabetic nephropathy, is the most common cause of end stage renal disease in the United States, and is also associated with increased cardiovascular morbidity and mortality.[3] Clinically, it is characterized by increased protein excretion in urine and progressive decrease in glomerular filtration rate (GFR). Pathologically, it is characterized by an initial phase of glomerular hyperfiltration followed by glomerular hypertrophy, mesangial expansion, and increased deposition of extracellular matrix (ECM) proteins such as fibronectin and collagen. Fibrosis and progressive mesangial expansion induce irreversible changes in the structure and function of the glomeruli, effectively reducing the glomerular filtration surface,[4] and eventually resulting in glomerulosclerosis and interstitial fibrosis.[5]
Several clinical and experimental studies have demonstrated the role of transforming growth factor beta (TGF-β) in the pathogenesis of chronic kidney disease, including diabetic nephropathy.[6] This cytokine is largely pro-fibrotic, and plays a significant role in diabetic nephropathy by increasing the production of ECM components in the glomerulus.

The purpose of this study was to investigate the mechanisms by which tobacco smoke causes accelerated progression of existing diabetic nephropathy. While clinical studies have shown that cigarette smoking is an independent risk factor in the progression of chronic kidney disease and diabetic nephropathy,[7] the mechanisms involved are not yet known. Additionally, while studies based on large population samples have in fact found a strong correlation between tobacco smoke exposure and progressive kidney disease in subjects with preexisting nephropathy,[8,9,10,11] some studies have found no association between tobacco smoke exposure and progressive renal failure.[12,13,14,15]

It is important to understand the mechanisms by which tobacco smoke promotes diabetic nephropathy, as this could lead to the development of new therapies or preventative measures in diabetic individuals who are exposed to tobacco smoke, whether through direct inhalation or through secondhand exposure. Secondhand smoke is a major and widespread health concern in the United States, with around 126 million people receiving exposure annually.[16] Of this group, about 49,000 will die prematurely due to secondhand smoke exposure.[17] In these studies we hypothesize that exposure to tobacco smoke worsens the progression of diabetic nephropathy by increasing the severity of extracellular matrix deposition and increasing the expression of the pro-fibrotic cytokine TGF-β.

Methods

Environmental Tobacco Smoke (ETS) Exposure

For these studies we used six-week old db/db (diabetic) mice (Jackson Labs), which were exposed to either room air (n=8) or to ETS (n=12) for eight weeks. ETS exposures were performed at the Center for Health and the Environment at the University of California-Davis. Mice assigned to ETS were exposed to tobacco smoke from Research Cigarettes (University of Kentucky) at a concentration of 30 μg/m^3 for six hours per day, 5 days a week. This concentration was chosen in order to obtain mice smoke exposure levels similar to those of human smokers.[18] After eight weeks of exposure to either room air or ETS, the mice were euthanized, and kidneys harvested for histology and molecular biology. Serum was saved for cotinine measurements.

Measurement of Glomerular Surface Area
Light microscopy of PAS-stained sections (5 µm) was used for morphometric analysis. The surface area and mesangial area (μm^2) of a minimum of 20 glomerular sections from each animal were determined from digital images using the Image-Pro Plus 4.5 software (Media Cybernetics). Glomerular and mesangial surface areas were measured from digital images by tracing around the perimeter of the glomerular tuft (glomerular area) and around the perimeter of intraglomerular PAS positive material (mesangial area). Glomeruli that were incomplete, distorted, tangentially sectioned, or globally sclerosed were excluded from analysis. The analysis software was calibrated to a stage micrometer.

Western blotting
Briefly, kidney cortex homogenates were separated by SDS-PAGE (8% acrylamide gel) under reducing conditions and transferred to a nitrocellulose membrane (Hybond ECL). Blots were incubated for 1 h with rabbit anti-murine polyclonal antibody to fibronectin at a 1:200 dilution (Sigma), TGF-β at a 1:1000 dilution (R&D Systems), or a polyclonal antibody to β-tubulin at 1:200 dilution (Santa Cruz). After washing, the blots were incubated with goat anti-rabbit antibody (Santa Cruz) for one hour, and the signal detected by horseradish polymerase-catalyzed chemiluminescence.

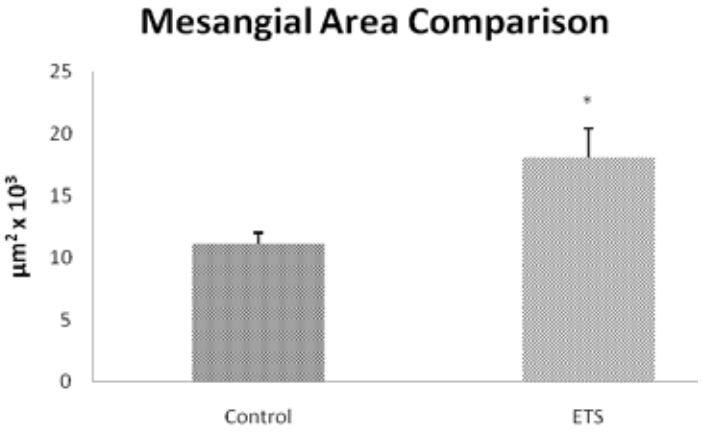
Serum cotinine measurements
Cotinine was measured in serum by ELISA (E-101-25, Bethyl Labs) following the manufacturer's instructions.

Urinary albumin excretion
Urine collections were performed the day prior to sacrifice, and urine saved at –20° C. Urinary albumin concentration was measured by ELISA (E-101-25, Bethyl Labs) following the manufacturer's instructions. Urinary albumin excretion was adjusted for urinary creatinine, which was measured by tandem mass spectrometry (Biochemical Genetics Laboratory, University of Alabama at Birmingham).

Statistical analysis
Data is expressed as mean ± SEM. For statistical comparisons, a student's unpaired *t*-test was used (Microsoft Excel).

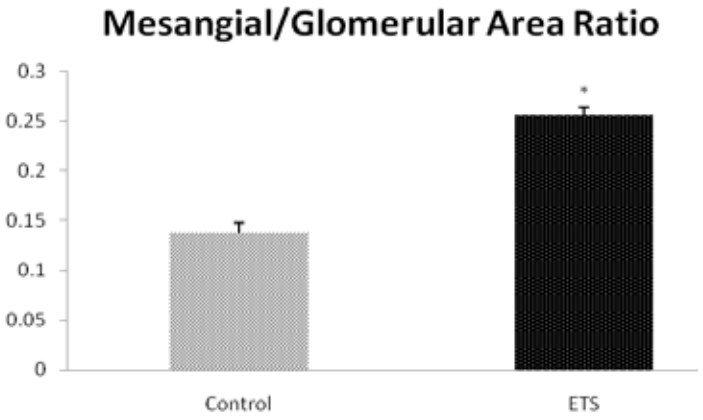
Results
Effects of ETS on mesangial expansion in diabetic mice
Morphometric analysis in PAS-stained sections demonstrated a significant increase in both total mesangial area (Figure 1) and the mesangial/glomerular area ratio (Figure 2) in mice exposed to ETS, suggesting that ETS exposure worsens mesangial expansion and extracellular matrix deposition in a mouse model of diabetic nephropathy. To determine whether these changes were accompanied by changes in the expression of the extracellular matrix protein fibronectin[19] and the pro-fibrotic cytokine TGF-β,[20] we performed Western blot analysis in renal cortex homogenates from air-exposed and ETS-exposed diabetic mice. As shown in Figures 3 and 4, ETS exposure resulted in significant increases in the expression of both fibronectin and TGF-β as assessed by Western blot. In the aggregate, these findings demonstrate that chronic ETS exposure increases mesangial expansion in diabetic mice.

**Note: the following figures include all control group subjects (n=8) and all experimental group subjects (n=12). In choosing the sample size for each group, it was asserted that more subjects should belong in the experimental group due to the variability of data in animal testing. This increased number allowed a more accurate representation of the experimental group data.*



*P < 0.05 versus control group.

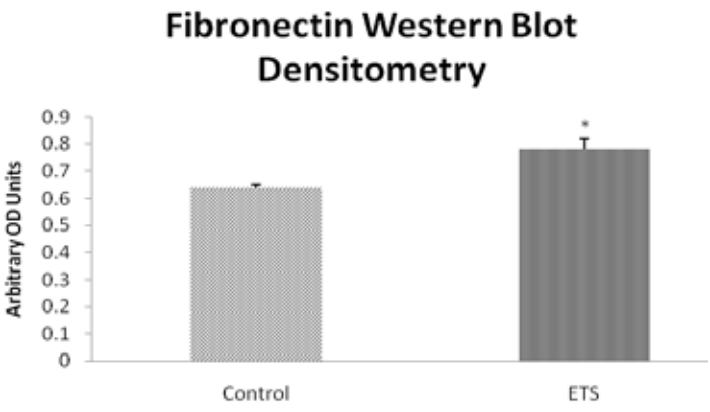
Figure 1: The ETS-exposed group demonstrated a significant increase in mesangial area.



**P < 0.05 versus control group.*

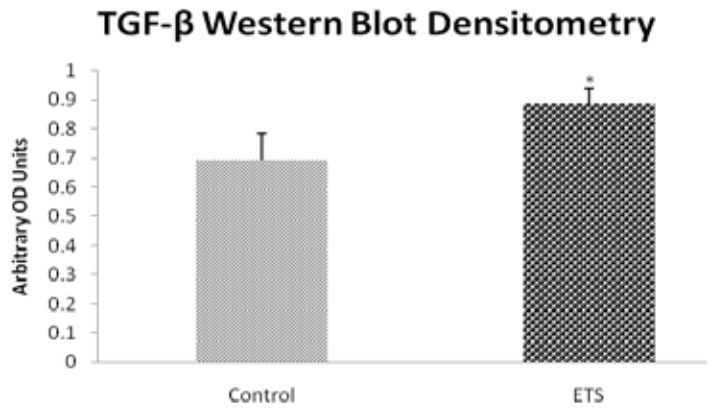
Figure 2: The ETS-exposed group had a significantly larger mesangial-to-glomerular area ratio than control.

Effects of ETS on urinary albumin excretion
To determine whether ETS-induced mesangial cell expansion was associated with changes in urinary protein excretion, we measured albuminuria in air-exposed and ETS-exposed diabetic mice. As shown in Figure 5, air-exposed diabetic mice had urinary albumin excretions in the range reported by others in db/db mice with diabetic nephropathy,[21] however, and in contrast with our morphometric analysis and Western blot analysis, ETS exposure did not significantly modify the urinary excretion of albumin.



*P < 0.05 versus control group.

Figure 3: The ETS-exposed mice showed significantly increased fibronectin expression.



*P < 0.05 versus control group.

Figure 4: TGF-β expression was significantly increased in the ETS group compared to control.

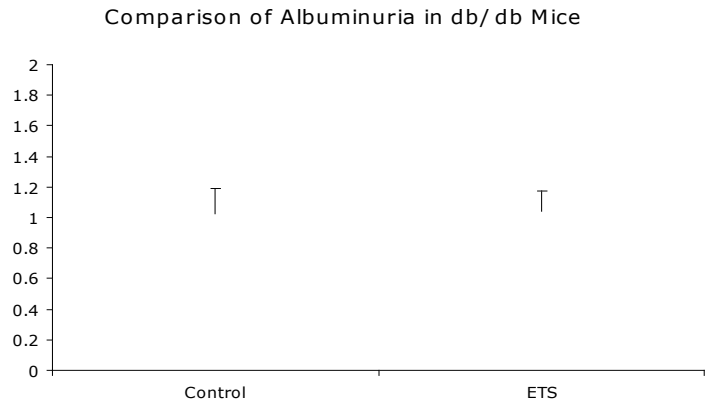


Figure 5: Albuminuria levels were not significantly different between control and ETS groups.

Cotinine levels in ETS-exposed mice

We measured the serum levels of cotinine, a stable metabolite of nicotine, as a marker of ETS exposure. The ETS group had measured plasma cotinine levels of 80.6 ng/ml (± 7.45 SEM), while the control group exhibited virtually undetectable levels. ETS exposure resulted in levels of cotinine similar to those found in the plasma of smokers.[22]

Discussion

These studies demonstrate that long-term ETS exposure worsens the severity of diabetic nephropathy as assessed by mesangial expansion and extracellular matrix deposition in a well- validated mouse model of diabetic nephropathy. ETS exposure, however, did not significantly modify the urinary excretion of albumin, suggesting that at the time of analysis, the urinary excretion of albumin was already at its maximum, and therefore not further increased by ETS exposure.

Several recent epidemiological studies have demonstrated that consumption of tobacco products accelerates the progression of chronic kidney disease, both in diabetic and non-diabetics.[8,9,10,11] The mechanisms involved, however, are not well understood. We have recently demonstrated that human mesangial cells are endowed with nicotine receptors,[19] and that nicotine worsens renal injury in rat model of glomerulonephritis.[4] Our current studies now demonstrate that ETS exposure results in significant mesangial expansion associated with increased expression of the extracellular matrix protein fibronectin and TGF-β, a pro-fibrotic cytokine that plays a major role in the pathogenesis of chronic kidney disease, including diabetic nephropathy. Importantly, our studies were performed with ETS exposures that resulted in levels of the stable nicotine metabolite cotinine in the range observed in the plasma of smokers.[22]

Our studies also suggest that TGF-β plays an important role as a mediator of the deleterious effects of smoking in the progression of diabetic nephropathy. Whether or not the observed increase in TGF-β is the result of a direct effect of compounds present in tobacco on the expression of this cytokine, however, remains to be determined and is the focus of additional studies in our laboratory.

Based on our previous studies,[19] we hypothesize that nicotine, a compound present in large amounts in tobacco smoke, may be mediating in large part the deleterious effects of ETS on the progression of diabetic nephropathy. However, we also recognize that other biologically active compounds present in tobacco smoke may also be playing an important role on these effects. In summary, our studies demonstrate the effects of ETS on the progression of diabetic nephropathy, unveil some of the mechanisms involved, and may result in the development of novel strategies in the treatment and prevention of diabetic nephropathy in smokers

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