Advanced glycation end products facilitate bacterial adherence in urinary tract infection in diabetic mice

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One-sentence summary: Advanced glycation end products (AGEs) accumulate on urothelium in Diabetes Mellitus, and aid bacteria in developing urinary tract infections (UTI).

ABSTRACT

Diabetic individuals have increased susceptibility to urinary tract infection (UTI), a common, painful condition. During diabetes mellitus, non-enzymatic reactions between reducing sugars and protein amine groups result in excessive production of advanced glycation end products (AGEs) that accumulate in tissues. Since bacteria adhere to cell surfaces by binding to carbohydrates, we hypothesized that adherence of bacteria to the bladder in diabetics may be enhanced by accumulation of AGEs on urothelial surface proteins. Using a murine model of UTI, we observed increased adherence of type 1 fimbriated uropathogenic Escherichia coli (UPEC) to the bladder in streptozotocin-induced diabetic female mice compared with age-matched controls, along with increased concentrations of two common AGEs in superficial urothelial cells from diabetic bladders. Several lectins with different specificities exhibited increased binding to urothelial homogenates from diabetic mice compared with controls, and two of those lectins also bound to AGEs. Furthermore, mannose-binding type 1 fimbriae isolated from UPEC bound to different AGEs, and UPEC adherence to the bladder in diabetic mice, were inhibited by pretreatment of mice with the AGE inhibitor pyridoxamine. These results strongly suggest a role for urothelial AGE accumulation in increased bacterial adherence during UTI in diabetes.

Key words: lectin; glycoprotein; Nε-(carboxyethyl)lysine; Nε-(carboxymethyl)lysine; enzyme-linked lectin assay
INTRODUCTION

Urinary tract infection (UTI) has been reported to be the second most common type of infection among outpatients (Schappert and Rechtsteiner 2008) and the most common nosocomial infection in the USA, affecting more than 8 million people per year (Klevens et al. 2007). UTI causes substantial morbidity and accounts for annual treatment-related costs of over $5.1 billion (Schappert and Rechtsteiner 2008; Litwin and Saigal 2012, NIH Publication No. 12–7865). Infection of the bladder, referred to as lower UTI (LUTI), is the most prevalent presentation, accounting for 95% of all UTIs (Mulvey 2002). Women are about four times as susceptible as men to this painful disease and are at greatest risk during their reproductive years (Grieben 2007). It has been estimated that roughly 50% of women will experience a UTI in their lifetime, with a recurrence rate reaching 30–40% (Foxman 2002).

Several studies have shown that both the prevalence and severity of UTI increase with diabetes mellitus, the most common endocrine disease worldwide (Joshi et al. 1999; Geerlings 2008; Funfstuck et al. 2012). The clinical and social ramifications of LUTI in this population are significant, including frequent hospitalizations and long-term antibiotic therapy with no long-term benefit, but with increased antibiotic resistance (Gupta et al. 2001; Foxman 2002). Diabetic individuals are more prone to UTI-related complications such as pyelonephritis (Funfstuck et al. 2012). Delineation of the mechanisms of increased LUTI susceptibility with diabetes may lead to new therapeutic targets and more effective preventive strategies.

Uropathogenic Escherichia coli (UPEC) are the most frequently isolated uropathogens, responsible for 80% of community-acquired LUTI (Ronald 2002). Although the detailed pathogenesis of UPEC-induced UTI is not fully understood, many studies conducted to date have demonstrated that it is a highly complex multistep process with host-pathogen interactions at every stage (Hannan et al. 2012). The pathogenesis of LUTI in diabetic patients appears to be even more complex and multifactorial than in non-diabetic individuals, owing to diverse factors such as conformational and molecular changes in the bladder epithelium (urothelium) and alterations of the host immune response. UPEC are thought to ascend the urethra from the peritoneum to colonize the bladder. After entering the bladder, bacterial adherence to luminal urothelial surfaces is the very first and crucial step for ascending UPEC to establish infection (de Man et al. 1988). The type 1 fimbria is the major adhesion appendage on many UPEC strains that enables them to attach to and invade urothelial cells, and it plays a critical role in the development of cystitis (Hultgren et al. 1985; Connell et al. 1996; Bahrami-Mougeot et al. 2002). The luminal lining of the bladder consists of stratified urothelium, with large umbrella cells comprising the superficial layer. A strong impermeable barrier is established on the apical surface of the umbrella cells, consisting of plaques composed of unique urothelial membrane glycoproteins, the uroplakins (Wu et al. 2009). Each type 1 fimbria contains a single lectin-like adhesion protein (adhesin) on its tip called FimH, which can recognize and bind to terminal oligomannose moieties on cell surface glycoproteins, thereby mediating bacterial adherence to and enabling invasion of target host cells (Krogfelt et al. 1990; Mulvey 2002). During a UPEC infection, uroplakins Ia and IIa on the luminal urothelial surface serve as the main receptors for FimH (Zhou et al. 2001; Thumbikat et al. 2009). FimH-mediated adherence is the first and the most critical step of UPEC pathogenesis in LUTI before the downstream events of invasion, intracellular replication and efflux of bacteria from dying urothelial cells (Langemmann et al. 1997; Mulvey et al. 1998).

The ability of bacteria to adhere to bladder mucosa might be enhanced in diabetic individuals due to changes in the carbohydrate content of the luminal urothelial surface. In a small in vitro study, urothelial cells harvested from diabetic patients were found to have increased binding capacity for type 1 fimbriated UPEC strains compared with cells from non-diabetic individuals, but the mechanism behind the enhanced binding remains unclear (Geerlings et al. 2002). Since binding of UPEC to host urothelial cells relies on FimH adherence to mannose and possibly other carbohydrates on glycoproteins, changes in the carbohydrate moieties on the urothelial surface in diabetes may alter the binding ability of UPEC during LUTI.

Over the course of progression of diabetes, there is increased accumulation of small carbohydrate moieties called advanced glycation end products (AGEs) due to hyperglycemia and exacerbated in cases of poor glucose control. AGEs are implicated as mediators of several recognized diabetic complications (Monnier 1990; Brownlee 2001; Goldin et al. 2006; Puddu and Viiani 2011). AGEs are generated in vivo and in vitro by a series of non-enzymatic chemical reactions between aldose sugars, including glucose and mannose, and macromolecules including proteins, nucleic acids and lipids (Abraham et al. 1985). The initial product is a Schiff base, which is spontaneously converted to a more stable Amadori product, of which hemoglobin-A1c, a clinical parameter used to monitor mid-range glucose control in diabetic patients, is a representative. AGE formation and accumulation may perturb various cell functions and structures [reviewed in Brownlee (2001)]. Despite well-studied functional perturbations of molecular signaling pathways by AGEs within cells, little information is available about a possible mechanistic role in host–pathogen interactions. Given that microbes colonize cell surfaces predominantly by binding to carbohydrate moieties on glycoproteins and glycolipids, the possibility that AGE accumulation could have an impact on susceptibility to microbial infection merits investigation.

The aim of this study was to investigate the possible role of AGEs that have accumulated on bladder urothelium in diabetes as alternative binding receptors for type 1 fimbriated UPEC, which could be a mechanism for increased susceptibility to LUTI in diabetes. We confirmed increased adherence of UPEC to the urothelium in diabetic mice relative to non-diabetic mice and showed that it corresponds to increased accumulation of carbohydrates in the urothelium and AGEs Nε-(carboxyethyl)lysine (CEL) and Nε-(carboxymethyl) lysine (CML) in superficial urothelial cells of diabetic mice. Furthermore, we determined that AGEs bind directly to type 1 fimbriae in vitro and inhibit adherence of type 1 fimbriated UPEC to the bladder in diabetic mice, and that the AGE inhibitor pyridoxamine similarly inhibits UPEC adherence in diabetic mice, potentially impacting bacterial colonization of the urothelium.

MATERIALS AND METHODS

Propagation and characterization of type 1 fimbriated UPEC

Growth of type 1 fimbriated UPEC and characterization of the fimbriae were conducted as described with minor modifications (Martinez et al. 2000). For each experiment involving bacterial inoculation, UPEC strain 53498 (American Type Culture Collection, Manassas, VA) was freshly streaked from a frozen glycerol stock onto a Luria agar plate and grown at 37°C overnight. To
stimulate expression of abundant type 1 fimbriae, a single UPEC colony was inoculated into Luria-Bertani (LB) broth and incubated at 37 °C overnight without shaking (static conditions), and then the bacterial suspension was subcultured at 1:1000 into 2 ml of fresh LB broth and incubated again at 37 °C overnight without shaking. Bacteria were washed and concentrated to 1.6 × 10^9 cells/ml in 1x PBS, and type 1 fimbria expression was confirmed by mannose-sensitive agglutination of a 2% solution of guinea pig erythrocytes. Bacteria were maintained on ice for a maximum of 2 h before installation.

**Type 1 diabetes induction and pyridoxamine treatment in mice**

Female C57BL/6J mice eight-weeks-old (Jackson Laboratory, Bar Harbor, ME) received one or two intraperitoneal injections of high-dose streptozotocin (STZ, 150 mg/kg) in 0.1 M sodium citrate, pH 4.5 to induce diabetes; controls received sodium citrate vehicle alone. Mice with blood glucose levels > 300 mg/dl three days after STZ injection were considered diabetic and were monitored weekly until experiments were performed six weeks after STZ injection. Only diabetic mice that maintained glucose levels > 300 mg/dl were used in experiments. For measuring the effects of pyridoxamine on bacterial adherence in diabetic and age-matched control mice, the drinking water of treated mice was replaced with water containing 1 g/l pyridoxine hydrochloride (Sigma-Aldrich, St Louis, MO) for seven weeks, beginning one week before STZ injection and continuing until completion of the experiment. All protocols involving mice were pre-approved by the Institutional Animal Care and Use Committee of Case Western Reserve University in compliance with the Public Health Service policy on humane care and use of laboratory animals.

**Experimental UTI in mice and bacterial adherence assays**

We followed established inoculation and adherence assay protocols (Ruggieri et al. 1985; Mulvey et al. 1998) with some modifications. Six weeks after injection of STZ or vehicle, mice were anesthetized with isoflurane and sterilized in the pelvic area, the bladder was emptied by manual massaging and washing, and 50 µl of type 1 fimbriated UPEC (8.0 × 10^6 CFU) suspended in 1x PBS was inoculated via transurethral catheterization, using a 24 GA 0.75 IN 0.7 × 19 mm shielded IV catheter (BD Angiocath™ Autoguard™). After 10 seconds of catheterization and injection, catheters were removed and mice were returned to their cages. At 15 min after UPEC inoculation, mice were euthanized by cervical dislocation under anesthesia and their bladders were removed aseptically, weighed and incised vertically from fundus to urethral trigone. The bladder parts were rinsed five times in five different 200 ml sterile 1xPBS flasks and homogenized in 1 ml of 0.025% Triton X-100 in 1xPBS. To test for any pre-existing infection, urine was collected aseptically from mice before inoculation of bacteria. Bacterial titers were determined by plating serial dilutions of homogenates or urine onto LB agar plates and incubating at 37 °C overnight.

**Lectin binding activity of urothelium**

Urothelial contents of mannose-containing and other carbohydrate structures were assessed using enzyme-linked lectin assays (ELLA) as described (McCoy et al. 1984; Garcia et al. 2005; Wu et al. 2008). Mouse urothelium was detached from the underlying basement membrane and suspended in 0.6 ml of ice-cold 1x PBS containing 0.1% Triton X-100 and 1 tablet/10 ml of Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). The tissue was homogenized on ice for 2 to 3 min with a Power Gen 125 Homogenizer at maximum speed, and then centrifuged at 12 000 g for 10 min at 4 °C to pellet insoluble material. The protein concentration in the supernatant was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) and adjusted to 10 µg/ml. Aliquots of 100 µl of homogenate were added to wells of a 96-well plate and incubated for 2 h at 37 °C to allow non-specific adsorption of glycoproteins. The wells were then washed twice with 0.1% Tween in 1x PBS, and non-specific lectin binding was blocked by adding 1% BSA (Sigma-Aldrich) to the wells and incubating for 1 h at 37 °C, followed by two washes with 0.1% Tween in 1x PBS.

Biotin-conjugated lectins (concanavalin A, Galanthus nivalis agglutinin, Lens culinaris lectin, Phaseolus vulgaris leucoagglutinin, Ricinus communis agglutinin I, Helix pomatia agglutinin, Maackia amurensis agglutinin, Sambucus canadensis agglutinin, Datura stramonium lectin and Triticum vulgare agglutinin) were purchased from EY Laboratories, San Mateo, CA. The biotinylated lectins (5 µg/ml in 1 × PBS) were preincubated at 37 °C for 30 min with a competitive sugar (5% w/v), a non-competitive sugar (5% w/v), or alone, and then 100 µl of lectin/sugar mixture was added per well of adsorbed proteins and incubated at 37 °C for 1 h. Wells without homogenate were blocked with 1% BSA and incubated with the lectin/sugar mixtures as negative controls. Following incubation, the wells were washed five times with 0.05% Tween 20 in 1x PBS. For detection, 100 µl of horseradish peroxidase (HRP)-conjugated streptavidin solution (Sigma-Aldrich) was added to each well and incubated for 30 min. The wells were washed five times with 0.05% Tween 20, incubated with 2,2'-azinoibis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) and hydrogen peroxide for 10 to 30 min, and then the reaction was stopped with N, N-dimethylformamide solution. Absorbance at 405 nm was measured using a Versamax ELISA microplate reader (Molecular Devices, Sunnyvale, CA), and the adherence of biotinylated lectins to urothelium was determined by subtracting the values of lectin/sugar mixtures incubated in BSA-coated wells from the values of the same lectin/sugar mixtures incubated in wells coated with urothelium and BSA.

**Trypsin-assisted protein harvesting and AGE ELISA**

Proteins of the superficial urothelium surface were harvested using trypsin treatment of ‘inverted bladder balls’, a method originally developed for collection of urothelial cells for primary cell cultures (Kurzrock et al. 2005), and collecting proteins in the cell-free supernatant instead of intact cells. Briefly, bladders harvested from six-week STZ-diabetic and control mice were inverted by pushing the dome downward through the bladder neck with a blunt 18-gauge needle. A suture was placed around the bladder neck and tightened with a half knot. The inverted bladder was inflated with 1x PBS through the needle and the suture was tightened while recruiting the catheter, producing a distended ball with only the urothelial surface exposed. The bladders were then placed in Eppendorf tubes containing 1 ml of trypsin (0.05%/EDTA (0.53 mM) without phenol red (Sigma-Aldrich) and incubated at 37 °C on an oscillating platform for 5 min, removing the tube every minute for brief vigorous shaking. Next, the bladders were removed and paraffin-embedded for H&E staining to ensure that the digest effectively removed only one or two cell layers of urothelium.
The remaining trypsin digest mixture was heat inactivated and centrifuged to pellet intact cells, and the supernatant was used for ELISAs after measurement of total protein concentration and dilution to 10 μg/ml in 1×PBS. ELISAs of CML and CEL were performed using HRP-conjugated anti-CML and anti-CEL monoclonal antibodies (Abnova Corp., Jongli, Taiwan), and detection with ABTS and hydrogen peroxide was performed as in Section Materials and Methods, 'Lectin binding activity of urothelium'. The average absorbance of controls in which the trypsinization procedure was carried out without bladder tissue was subtracted from the sample absorbances. ELISA of CML was also performed on urine samples collected aseptically from mice six weeks after diabetes induction and diluted 20 times with 1×PBS before use.

Production of glucose-AGE-collagen
Collagen type 1 for production of AGE-modified collagen was generously provided by Dr VM Monnier (Department of Pathology, Case Western Reserve University). We followed the AGE-collagen preparation method described by Makita et al. (1992). Briefly, 5, 10, 50 and 100 μl aliquots of collagen stock (5 mg/ml) were added to different wells in a 96-well plate, along with 135, 130, 90 and 40 μl respectively, of 100 mM NaPO4 to neutralize the collagen and allow gel formation. The plate was shaken for 10 min and then wrapped tightly with parafilm and incubated at 4 °C O/N. After O/N incubation, each well was washed twice with 0.1 M sodium phosphate buffer, pH 7.4 to remove acetic acid, and then 200 μl of filter-sterilized 50 mM glucose in 100 mM NaPO4 buffer, pH 7.4 was added under sterile conditions. The samples were wrapped tightly with parafilm and incubated at 4 °C for 21 days to allow formation of AGE-modified collagen (glucose-AGE-collagen). Following incubation, unincorporated sugars or dicarbonyl compounds were removed, the products were aliquoted by incubating BSA without sugar at 37 °C for 7 days. Mannose inhibition was exposed to 24% ammonium sulfate at 4 °C for 1 h to precipitate the type 1 fimbriae and then centrifuged at 15 000 g for 30 min, followed by centrifugation of the supernatant at 27 000 g for 20 min. The 27 000 g pellet was resuspended in 20 ml of 10 mM Tris-HCl, pH 7.8, containing 0.02% sodium azide, applied to an Amicon Ultra 30 kDa centrifugal filter unit (EMD Millipore, Billerica, MA), and washed serially with sterile water. The purity of the concentrated type 1 fimbriae suspension was assessed by electrophoresis on a 0.1% SDS–12% polyacrylamide gel and staining with Coomassie Blue (Laemmli, 1970), and was estimated to be at least 95%. The protein concentration of the type 1 fimbriae suspension was measured using a Bio-Rad Protein Assay Kit. Biotinylation of the purified type 1 fimbriae was performed using a BiotinTag™ Micro Biotinylation Kit (Sigma-Aldrich), following the provided protocol. The biotinylated protein mixture was applied to a Centricon 10 kDa centrifugal filter unit (EMD Millipore) and washed serially with sterile water to remove unreacted BAC-SulfoNHS reagent. The protein concentration of the final concentrated, biotinylated type 1 fimbriae suspension in sterile water was measured using a Bio-Rad Protein Assay Kit.

Production of glucose- and glyceraldehyde-AGE-BSA
Glucose-AGE-BSA and glyceraldehyde-AGE-BSA were produced as described with minor modifications (Valencia et al., 2004; Zhao et al., 2009). Briefly, to prepare glucose-AGE-BSA, low lipopolysaccharide and fatty acid-free BSA (50 mg/ml, Sigma-Aldrich) was incubated with 0.5 M glucose in 0.2 M sodium phosphate buffer (pH 7.4) at 37 °C for eight weeks under sterile conditions. For glyceraldehyde-AGE-BSA, the BSA was incubated at 10 mg/ml with 0.1 M D-glyceraldehyde in 0.2 M sodium phosphate buffer (pH 7.4) at 37 °C for seven days. Sham-modified BSA was prepared by incubating BSA without sugar at 37 °C for eight weeks. Unincorporated sugars were removed, the products were aliquoted and stored at −20 °C, and the levels of AGEs were tested by ELISA as described in Section Materials and Methods, 'Production of glucose-AGE collagen'.

Lectin–AGE binding assays
Lectin binding abilities of AGEs were assessed by ELLA, using glyceraldehyde-AGE-BSA and glucose-AGE-collagen generated as described above and diluted to 10 μg/ml in 1×PBS. Glucose-AGE-collagen was homogenized on ice for 10 min with a Power Gen 125 Homogenizer at top speed, and then 100 μl aliquots of the two AGE-modified protein solutions were added to wells of a 96-well plate and incubated for 2 h at 37 °C for non-specific adsorption. The wells were then washed with 1×PBS and blocked by adding 1% BSA (Sigma-Aldrich) and incubating at 37 °C for 1 h to prevent non-specific lectin binding. Following additional washes with 1×PBS, incubation with biotinylated lectins and detection with HRP-streptavidin and ABTS proceeded as described in Section 2.4 above for ELLA of urothelium.

Isolation and biotinylation of type 1 fimbriae
Purification of type 1 fimbriae was conducted as reported by Horst et al. with some modifications (Horst et al., 2001). Type 1 fimbriated UPEC were prepared as described above and centrifuged for 20 min at 6000 g, and then the pellet was resuspended in 20 ml of 10 mM Tris-HCl, pH 7.2. The fimbriae were detached from the bacteria by homogenization on ice for 10 min with a Power Gen 125 Homogenizer. The bacterial debris was removed by centrifugation at 8000 g for 20 min and repeating centrifugation of the supernatant two more times. The supernatant was exposed to 24% ammonium sulfate at 4 °C for 1 h to precipitate the type 1 fimbriae and then centrifuged at 15 000 g for 30 min, followed by centrifugation of the supernatant at 27 000 g for 20 min. The 27 000 g pellet was resuspended in 20 ml of 10 mM Tris-HCl, pH 7.8, containing 0.02% sodium azide, applied to an Amicon Ultra 30 kDa centrifugal filter unit (EMD Millipore, Billerica, MA), and washed serially with sterile water. The purity of the concentrated type 1 fimbriae suspension was assessed by electrophoresis on a 0.1% SDS–12% polyacrylamide gel and staining with Coomassie Blue (Laemmli, 1970), and was estimated to be at least 95%. The protein concentration of the type 1 fimbriae suspension was measured using a Bio-Rad Protein Assay Kit. Biotinylation of the purified type 1 fimbriae was performed using a BiotinTag™ Micro Biotinylation Kit (Sigma-Aldrich), following the provided protocol. The biotinylated protein mixture was applied to a Centricon 10 kDa centrifugal filter unit (EMD Millipore) and washed serially with sterile water to remove unreacted BAC-SulfoNHS reagent. The protein concentration of the final concentrated, biotinylated type 1 fimbriae suspension in sterile water was measured using a Bio-Rad Protein Assay Kit.

In vitro type 1 fimbriae–AGE binding assays
Binding of biotinylated type 1 fimbriae to AGE products was performed using a direct ELISA procedure with some modifications. AGE-modified BSA and sham-modified BSA were diluted in 1×PBS to a concentration of 10 μg/ml and adsorbed to wells of 96-well plates as described above for lectin–AGE binding assays. Glucose-AGE-BSA and glyceraldehyde-AGE-BSA were produced as described in Section Materials and Methods, 'Production of glucose- and glyceraldehyde-AGE-BSA'; CML-BSA and CEL-BSA were obtained from MBL International, Woburn, MA. Biotinylated type 1 fimbriae (10 μg/ml) were preincubated with 5% D-mannose in 1×PBS, with an AGE- or sham-modified BSA solution in 1×PBS, or with 1×PBS alone at room temperature for 20 min, with the final concentrations of total fimbrial protein and BSA each 10 μg/ml. Then, 100 μl of each preincubated, biotinylated type 1 fimbriae solution or 1×PBS alone was added to quadruplicate wells for each AGE-modified BSA and sham-modified BSA and incubated at 37 °C for 1 h. Mannose inhibition
Table 1. General characteristics of diabetes and age-matched control mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dl)</th>
<th>Body weight (g)</th>
<th>Bladder weight (mg)</th>
<th>Bladder weight/body weight (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>111 ± 8.50</td>
<td>22.4 ± 1.13</td>
<td>27.4 ± 1.43</td>
<td>1.25 ± 0.12</td>
</tr>
<tr>
<td>Diabetes</td>
<td>562 ± 32.1†</td>
<td>18.5 ± 1.07†</td>
<td>36.4 ± 1.11†</td>
<td>1.99 ± 0.13†</td>
</tr>
</tbody>
</table>

Values are means ± SEM; n = 5 mice/group. Symbols in the diabetes row indicate significant differences between diabetic and control mice by unpaired, two-tailed Student’s t-tests: †p < 0.0001 using Welch’s correction for significantly different variances, §p = 0.04, ‡p < 0.004.

of type 1 fimbriae binding is well known, since mannosylated glycoproteins are established receptors for the FimH adhesin (Krogfelt et al. 1990).

Statistical analysis

Data are expressed as means with SEM. The methods of statistical analysis, performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA), are described in the figure legends. Values of p < 0.05 were considered significant.

RESULTS

Type 1 diabetes induction in female C57BL/6j mice

At the time of euthanasia, six weeks after injection of STZ or vehicle, the mean blood glucose level in diabetic mice was about five times higher than in controls (p < 0.0001, Table 1). The mean body weight was significantly lower, and the mean bladder weight and bladder weight-to-body weight ratio were significantly higher in the diabetic mice compared with the control mice (Table 1), indicative of bladder hypertrophy, as we have also observed in STZ-diabetic male mice (Daneshgari et al. 2006). Although bladder volumes were not measured in this study, comparison with our previous study in which bladder weight and capacity were measured (Daneshgari et al. 2006) suggested that the 1.3-fold greater mean bladder weight observed in the diabetic mice in this study corresponds to an approximately 2-fold higher luminal urothelial surface area.

Increased bacterial adherence and carbohydrate content in urothelium of diabetic mice

Fifteen minutes after transurethral inoculation of type 1 fimbriated UPEC strain 53498 into the bladder in six-week STZ diabetic mice, the mean number of bacteria adhering to the urothelium per mg of bladder weight was 2.7-fold greater than in control mice (p = 0.016, Fig. 1A). This experiment was repeated within the independent experiment shown in Fig. 5 with similar results. Since the carbohydrate composition of the urothelial surface is critical for host-pathogen interactions, and mannosylated glycoproteins in particular are established receptors for type 1 fimbriated UPEC (Sharon 1987), the increased adherence we observed may be due in part to increased and/or altered carbohydrate levels on the urothelium of diabetic mice. We compared the levels of...
carbohydrate structures on the urothelium of diabetic and healthy mice using lectins, small carbohydrate binding proteins with different sugar moeity specificities, using an ELISA-based technique (ELLA) (McCoy et al. 1984). Three mannose-selective lectins (ConA, GNA and LCH), along with a lectin (PHA-L) selective for a complex oligosaccharide including mannose exhibited significantly greater mannose-competitive binding to urothelium homogenates containing 1 µg of protein from diabetic mice compared with control mice, suggesting increased numbers of mannose-containing binding sites for type 1 fimbriae on diabetic urothelium (Fig. 1B). We also observed increased binding of lectin RCA-I, selective for β-D-galactopyranoside, to urothelium from diabetic mice relative to control mice (Fig. 1B). RCA-I binding was inhibited by preincubation with galactose, but not by preincubation with mannose, indicating increased levels of galactose-containing or other non-mannose carbohydrate structures in diabetic mice.

**Increased AGE content on the urothelial surface of diabetic mice**

To determine if the increased levels of carbohydrate structures on the urothelium of diabetic mice include AGEs, we measured the superficial urothelial levels of CEL and CML, two common AGEs that have been shown to be increased in skin collagen of diabetic humans and rodents (Dyer et al. 1993; Degenhardt et al. 2002). For this purpose, we used urothelial surface proteins released by trypsin treatment of inverted bladders as described in the section ‘Materials and Methods’. Direct ELISAs of samples containing 1 µg of protein showed significantly increased levels of CEL and CML, by about 7.5- and 5-fold, respectively, on the urothelial surface of diabetic mice compared with control mice (Fig. 2A and B). CML levels were also significantly higher in the urine of diabetic mice compared with controls (Fig. 2C).

**Lectins bind to AGEs**

We next assessed the abilities of mannose-selective and other lectins to bind to AGE-modified proteins, which could shed light on the nature of the increased carbohydrate levels in diabetic urothelium and also provide suggestive evidence about the AGEl binding ability of the mannose-selective lectin-like structure FimH. Using an ELLA to detect lectin–AGE product interactions, we observed that several different lectins bound to the AGE products glyceraldehyde-AGE-BSA and/or glucose-AGE-collagen significantly more than to sham-modified BSA and collagen, respectively (Fig. 3A and B). Interestingly, the mannose-selective lectin GNA bound preferentially to both glyceraldehyde-AGE-BSA and glucose-AGE-collagen, as did RCA-1 (selective for β-D-galactopyranoside). Those results, together with the findings that both of those lectins exhibited increased binding to urothelium from diabetic mice vs. controls (Fig. 1B), suggest that AGEs account for a portion of the increased carbohydrate levels detected by lectins in diabetic urothelium. Other lectins with different specificities that bound to both glyceraldehyde-AGE-BSA and glucose-AGE-collagen significantly more than to the corresponding sham-modified proteins included HPA (selective for α-N-acetylgalactosamine), MAA (selective for sialic acid α(2,3) galactose) and DSA (selective for β(1,4)-N-acetylglucosamine oligomers (HPA).

**Type 1 fimbriae bind to AGEs**

The type 1 fimbria tip adhesin FimH is a lectin-like structure possessing an N-terminal carbohydrate-binding pocket capable of binding to mannose-containing moieties on cell surface glycoproteins such as uropakins (Mulvey 2002). Based on our finding that multiple lectins with selectivities for mannose also bound AGEs (Fig. 3), we next determined if UPEC type 1 fimbriae can bind to AGEs. We found that purified, biotinylated type 1 fimbriae bound to CML-BSA, CEL-BSA, glyceraldehyde-AGE-BSA and glucose-AGE-BSA significantly more than to sham-modified BSA (Fig. 4A and B). Furthermore, preincubation of type 1 fimbriae with either the FimH competitor mannose or with the corresponding AGE-modified BSA inhibited binding of the fimbriae to the immobilized AGE-modified BSA significantly and to similar extents, but did not affect binding of the fimbriae to immobilized sham-modified BSA (Fig. 4A and B). Those results indicate that UPEC type 1 fimbriae bound specifically to AGEs in a mannose- and AGE-competitive manner.

**Figure 2. Elevated AGEs on urothelium and in urine from diabetic mice.** Urothelial surface proteins were released by trypsin, and urine samples were collected from diabetic and control mice as described in the section ‘Materials and Methods’. One hundred µl aliquots of samples (urothelial lysates at a concentration of 10 µg total protein/ml or 20-fold diluted urine) were assayed by ELISA to detect AGE-modified proteins. (a) Urothelial proteins with anti-CEL antibody. (b) Urothelial proteins with anti-CML antibody. (c) Urine with anti-CML antibody. The average absorbance of controls and experimental samples was assayed in triplicate and the mean absorbance value for each mouse was plotted. The wide horizontal lines bisected by error bars indicate the overall mean ± SEM for each mouse group (one experiment with n = 10 mice per group). The mean AGE levels in each pair of diabetic and control groups were compared by the Student’s t-test with Welch’s correction for significantly different variances; the levels of statistical significance are indicated on the graphs.
Inhibition of UPEC adherence by pyridoxamine treatment

To further evaluate a role for increased AGE levels in adherence of UPEC to urothelium in diabetes, the effects of the AGE inhibitor pyridoxamine on UPEC adherence in diabetic and control mice were measured. Pyridoxamine, a naturally occurring form of vitamin B₆, scavenges reactive carbonyl species formed during oxidation of glucose and lipids, and inhibits the formation of AGEs from Amadori compounds (Chetyrkin et al. 2008). The concentration of pyridoxamine used in this experiment (1 g/l in the drinking water) was shown previously to ameliorate nephropathy in a mouse model of type 2 diabetes (Tanimoto et al. 2007). Approximately 4.7-fold more UPEC per mg bladder weight were recovered from the bladders of diabetic mice compared with control mice 15 min after inoculation of $8.0 \times 10^7$ CFU, a slightly higher difference than that observed in the earlier experiment of Fig. 1A. Pyridoxamine markedly inhibited UPEC adherence to the urothelium in diabetic mice ($p = 0.018$), while it had no effect on UPEC adherence in the control mice (Fig. 5). These
results further support the hypothesis that AGEs that accumulate on the urothelium in diabetes can facilitate adherence, and possibly pathogenesis, of type 1 fimbriated UPEC.

DISCUSSION

In the experimental model of UPEC-mediated LUTI, type 1 fimbriate mediate adherence of bacteria to the urothelium and initiate downstream events including invasion of urothelial cells, replication within the invaded cells and emergence and dispersion of bacteria from the dying urothelial cells (Hultgren et al. 1985; Mulvey et al. 1998). In this study, we have shown that, in addition to the well-known mannose residue receptors of UPEC type 1 fimbriae, AGEs play an important role in bacterial adherence during UTI in diabetic mice and may enhance bacterial pathogenesis.

We observed increased UPEC adherence to the bladder in diabetic mice, by 2.7-fold (Fig. 1A) and 4.7-fold (Fig. 5) per mg bladder weight in two different experiments, compared with control mice. It is unlikely that the difference in urothelial surface area between diabetic and control mice, estimated at approximately 1.5-fold greater than the difference in mean bladder weights, is the main cause of those differences in adherence. Increased AGE formation in the presence of a hyperglycemic and oxidative environment, and the resulting nearly irreversible modifications of proteins, lipids and nucleic acids, have been implicated in many complications of diabetes and aging, via tissue damage caused by receptor-independent and -dependent mechanisms (Goldin et al. 2006; Ramasamy et al. 2012). However, a possible role for AGEs in host–pathogen interactions has not been reported previously.

AGE accumulation has been shown in various tissues and organs in diabetic animals and humans, including connective tissue, skin, serum, erythrocytes, hepatocytes, glomerular endothelial cells, lung, intestine, intervertebral discs and atherosclerotic plaques (Schleicher et al. 1997; Ling et al. 2001; Goldin et al. 2006). In bladder specimens from non-diabetic patients who underwent radical cystectomy for bladder cancer, common AGE CML and pentosidine were detected within the extracellular matrix between muscle layers, but not in smooth muscle fibers or urothelial cells (Matsumoto et al. 2009). In whole bladder and other pelvic tissues from eight-week STZ-diabetic female Sprague Dawley rats, significantly increased levels of CML and furosine, but not CEL, were found in comparisons with normal and 5% sucrose-induced diabetic rats (Pan et al. 2010). Our findings that the common AGE CML and CEL accumulate on the superficial urothelial layer and urine of STZ-diabetic mice at concentrations per μg protein several times higher than in non-diabetic mice are in general agreement with the study in diabetic rats. Further support for increased amounts of AGEs in diabetic urothelium came from our observations that the lectins GNA and RCA-1 bound to two different AGE-modified proteins (Fig. 3) and bound to urothelium from diabetic mice more than from control mice (Fig. 1B).

We provide further evidence that AGEs can enhance binding of type 1 fimbriated UPEC to the bladder in diabetic mice, as shown by in vitro binding of type 1 fimbriae to several AGE products (Fig. 4), as well as inhibition of in vivo bacterial adherence by treatment of mice with the AGE inhibitor pyridoxamine (Fig. 5). UPEC elaborate type 1 fimbriae on the surface, and the oligomannose-binding FimH adhesin at the fimbria tip is essential for binding to the urothelium, invasion, the distinct process of intracellular bacterial colony formation and colonization of the bladder in chronic infection (Mulvey et al. 1998; Hannan et al. 2010). In clinical isolates of type 1 fimbriated UPEC, several amino acid residues of FimH have been found to be under positive selection, suggesting that FimH plays an important role in human UTI (Chen et al. 2009). Our finding that the binding of UPEC type 1 fimbriae to different AGEs was inhibited to similar extents by preincubation with either the FimH competitor mannose or with the corresponding AGE-modified BSA suggests that FimH is involved in type 1 fimbriae binding to AGEs, although further work is required to determine if AGEs are competitive with mannose.

Antibiotics that have been used to successfully treat UTI have also led to increasing bacterial resistance to those drugs, resulting in antibiotic resistant recurrent and chronic UTI. In recent years, many attempts have been made to develop alternative treatment modalities to supplant or complement current antibiotic regimens in the treatment or prevention of UTI (Cusumano et al. 2011; Dielubanza and Schaeffer 2011). Rationally designed mannose derivatives (biarylmannosides) have been developed as FimH inhibitors and shown to be orally active in preventing and treating UTI in mice (Cusumano et al. 2011). If increased AGE levels on bladder mucosa are found to be common in human diabetes, AGE inhibition could be another viable alternative treatment in diabetic and elderly individuals. Among AGE inhibitors, the natural B6 vitamer pyridoxamine is relatively nontoxic and has been shown to inhibit complications of diabetes in mice, including retinopathy in type 1 diabetes (Stitt et al. 2002) and nephropathy in type 1 and type 2 diabetes (Degenhardt et al. 2002; Tanimoto et al. 2007). In a retrospective analysis of two Phase II studies of patients with type 1 or type 2 diabetic nephropathy, pyridoxamine was found to have some efficacy, especially in type 2 diabetes patients (Williams et al. 2007).

In conclusion, we have provided multiple lines of evidence that together suggest strongly that UPEC adherence increases in diabetic mice by virtue of increased accumulation of AGEs on the urothelial surface. These data add to the understanding of
mechanisms that contribute to differences in UPEC adherence to mucosal surfaces in diabetic compared with healthy individuals, and may lead to development of alternative or complementary therapeutic modalities including AGE inhibition in diabetic or elderly patients with chronic or recurrent UTI.

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