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Evidence of Glucuronidation in the Glycation Product LW-1: Tentative Structure and Implications for the Long-Term Complications of Diabetes

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Abstract

LW-1 is a collagen-linked blue fluorophore whose skin levels increase with age, diabetes and end-stage renal disease (ESRD), and correlate with the long-term progression of microvascular disease and indices of subclinical cardiovascular disease in type 1 diabetes. The chemical structure of LW-1 is still elusive, but earlier NMR analyses showed it has a lysine residue in an aromatic ring coupled to a sugar molecule reminiscent of advanced glycation end-products (AGEs). We hypothesized and demonstrate here that the unknown sugar is a *N*-linked glucuronic acid. LW-1 was extracted and highly purified from ~99 g insoluble skin collagen obtained at autopsy from patients with diabetes/ESRD using multiple rounds of proteolytic digestion and purification by liquid chromatography (LC). Advanced NMR techniques (¹H-NMR, ¹³C-NMR, ¹H-¹³C HSQC, ¹H-¹H TOCSY, ¹H-¹³C HMBC) together with LC-mass spectrometry (MS) revealed a loss of 176 *amu* (atomic mass units) unequivocally point to the presence of a glucuronic acid moiety in LW-1. To confirm this data, LW-1 was incubated with β -glycosidases (glucosidase, galactosidase, glucuronidase) and products were analyzed by LC-MS. Only glucuronidase could cleave the sugar from the parent molecule. These results establish LW-1 as a glucuronide, now named glucuronidine, and for the first time raise the possible existence of a “glucuronidation pathway of diabetic complications”. Future research is needed to rigorously probe this concept and elucidate the molecular origin and biological source of a circulating glucuronidine aglycone.

Keywords

collagen; skin; autofluorescence; type 1 diabetes; uremia; NMR

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Conflict of Interest The authors declare that they have no conflict of interest.

Introduction

Reducing sugars can react non-enzymatically with long-lived proteins to form insoluble, cross-linked, sometimes fluorescent molecules referred to as advanced glycation end-products (AGEs). For many years, protein autofluorescence has been widely used as a surrogate marker for the accumulation of AGEs, both *in vitro* and *in vivo* [1] despite that most AGEs are not fluorescent [2] and that the chemical nature of the fluorescence is poorly understood [3]. Recently, a pragmatic interest into the biochemical nature of autofluorescence has resurfaced with the introduction onto the commercial market of optical readers capable of measuring autofluorescence non-invasively in human forearm skin [4]. In short, these readers have shown that autofluorescence intensity is associated with the risk of both micro- and macrovascular complications of diabetes [4] as well as mortality in patients undergoing renal dialysis with and without diabetes [5].

Over the years the quest by us and others to understand the biochemical nature of AGE fluorescence led to the molecular discovery and/or *in vivo* validation of acid stable fluorescent AGEs such as pentosidine, argpyrimidine, versperlysine or triosidines [6–9], some of which are associated with the complications of type 1 and/or type 2 diabetes. However, their fluorescence properties did not sufficiently match or quantitatively accounted for the typical excitation/emission (ex/em) 370/440 nm tissue autofluorescence that is measured by noninvasive fluorescence readers until the discovery of the *collagen-linked long-wave fluorophore* LW-1 in our laboratory [10].

Earlier studies showed LW-1 has a mass of 623 Da and fluorescent maxima at ex/em 348/463 nm. NMR experiments including ^1H NMR, ^1H - ^{13}C HSQC, ^1H - ^1H TOCSY showed it had a lysine residue in an aromatic ring. While the HSQC showed a number of signals characteristic of sugars, it was not possible to draw definitive structural conclusions due to small quantities of LW-1 isolated from skin collagen. However, mass spectrometry (MS) evidence showed a transition at m/z 623 (parent/molecular ion) $\rightarrow$ m/z 447 (product) with the loss of 176 amu (atomic mass unit) tentatively indicative of a glycosidic cleavage fragment reported for mass spectrometric studies on the metabolism of drugs [11] and toxins [12] by glucuronidation. Such transition was also reported in MS studies on post-translational modification of human acidic proline-rich salivary protein PRP-1 by *O*-linked conjugation of a serine residue with glucuronic acid [13].

In the present investigation, we isolated bulk quantities of highly purified LW-1 to investigate the nature of the sugar moiety, specifically testing the hypothesis that glucuronic acid is the molecule responsible for the NMR signals and the loss of 176 in the mass spectrum of LW-1. Since the sugar was cleaved by β -glucuronidase, it was now renamed glucuronidine. A tentative “best match” structure is proposed and the potential role of glucuronidation in diabetic complications is discussed.

Materials and methods

Chemicals, enzymes and substrates

Otherwise noted, all chemicals, enzymes, and substrates were purchased from Sigma-Aldrich (St. Louis, MO). β -Glucosidase was from almonds (specific activity, 2.03 units/mg solid, EC 3.2.1.21), β -glucuronidase from bovine liver (1.64 units/mg solid, EC 3.2.1.31) and β -galactosidase from *E. coli* (271 units/mg solid, EC 3.2.1.23).

Skin donors

Skin samples were obtained by autopsy from 13 human donors at age range 38–83 yrs from either the University Hospitals Cleveland Medical Center, Cleveland, Ohio or the National Disease Research Interchange (NDRI, Philadelphia, PA). Major diagnoses at death were diabetes and/or chronic renal failure including some patients with end-stage renal disease (ESRD).

Tissue processing

Insoluble collagen was prepared from skin by extraction according to procedures previously published by us [10]. The yield of insoluble collagen was ~ 99 g.

LW-1 purification from large batch digestion of collagen

Because of the expense of the proteolytic enzymes and that some enzymes were discontinued by the vendors, the digestion protocol was modified from that previously published by us [10]. Insoluble collagen was solubilized by an initial digestion for 24 hrs at 37°C with collagenase (type CLSPS, SA $\approx$ 1.316 KU/mg solid, Worthington Biochemical Corp., Lakewood, NJ) consisting of 1.431 KU collagenase in 1.262 L buffer H (0.02 M HEPES, 0.1 M calcium chloride, pH 7.5). The buffer was degassed and purged with argon before use. Chloroform/toluene were used as the anti-microbial agents (1:1 at 6 μ l/ml buffer). The undigested residue from the first collagenase digestion was washed with buffer H, minced using the blade of a scalpel, and re-digested with collagenase same $\approx$ 503 KU in 252 ml buffer H. The *percent solubilization* of the insoluble collagen by collagenase was ~ 91.1% based upon the freeze-dried residual pellet (8.9 g) leftover at the end of the collagenase digestion (per initial collagen weight ~ 99 g). The collagenase digests from the above were combined ($\approx$ 2.44 L) followed by determination of the collagen content by the hydroxyproline (hyp) assay [14] assuming a collagen content of 14% hyp by weight [15]. The freeze-dried digest, in turn, was re-digested for 24 hrs at 37°C (pH 7.5) with each of 5% pronase (i.e., wt/wt collagen by hyp, Roche Diagnostics/Roche Applied Science, Indianapolis, IN; SA 7 KU/g) and 1% achromopeptidase (Wako Chemicals USA, Richmond, VA; SA 1.2 KU/mg). After the latter enzyme treatment, the digestion efficiency; i.e., percent digestion, was determined to be $81 \pm 6\%$ by the ninhydrin assay [16]. However, hyp was *not* detected without acid hydrolysis of the digest.

The digest totaling 3.94 L was acidified with trifluoroacetic acid (TFA) to pH ~ 3 and applied at 1 L aliquots to a preparative 5×64 cm glass column loaded with Sep-Pak tC18 resin (trifunctional silane bonded-phase sorbent, custom bulk, 37–55 μ m, 125Å; Waters, Milford, MA) which ran at 4°C in a cold room. The mobile phase was 0.1% TFA in water

followed by the elution of the highly fluorescent brown-colored material with isocratic steps of 30% and 60% acetonitrile in water. Fractions ~ 20 ml each were collected with a Pharmacia fraction collector (FRAC-200, GE Healthcare Bio-Sciences, Pittsburgh, PA) as monitored by UV350 nm and fluorescence at excitation (ex)/emission (em) 348/463 nm, as well as assayed for collagen (hyp assay) and ninhydrin. Fractions containing the fluorescent peak were combined and freeze-dried. The total yield of highly brown material was ~ 13.3 g.

This material was reconstituted with ~ 212 ml buffer H and sequentially digested for 24 hrs at 37°C (pH 7.5) with each of 1% enteropeptidase /enterokinase (Sigma E0885, ~ 6.4 KU, SA 63 U/mg) and 1% prolidase (Sigma P6675, ~ 14.75 KU, SA 116 U/mg). Each enzyme was added in ~ 212 ml buffer H using chloroform/toluene as the anti-microbial agent (0.5 ml each). These digestions were also followed by both the hyp and ninhydrin assays which showed 78±1% and 51±3% digestion efficiencies, respectively. This digestion procedure yielded ~ 460 ml of digest.

The digest was desalted by passing it over a 5 × 50 cm glass column loaded with Sep-Pak tC18 resin equilibrated and eluted as that previously described in the above. Fractions were collected and monitored for UV350 nm, fluorescence, collagen, and ninhydrin. The fluorescent peak was collected and freeze-dried which yielded ~ 8 g brown material.

LW-1 was purified from the highly fluorescent fraction of the secondary digest by HPLC using a repeated injection/collection technique similar to that previously described by us [10]. The digest was reconstituted with 125 ml water containing 0.01 M heptafluorobutyric acid (HFBA). Aliquots ~ 1 ml were injected by HPLC onto a 25 cm × 10 mm, 5 µm semi-preparative C18 column (Discovery BIO Wide-Pore C18, Sigma-Aldrich) followed by collecting the LW-1 peak with a fraction collector (FRAC-100, see above). The HPLC setup was as follows: Waters Model 515 pumps, Model 717 plus autosampler and Model 680 gradient controller (Waters, Milford, MA). The flow was 2 ml/min which was diverted over to the fraction collector using a fluid solenoid switch valve (12VDC, Parker Hannifin Corp., General Valve Division, Fairfield, NJ) as synchronized and programmed by the gradient controller. The linear gradient program was as follows: 0–5 min, 100% A; 5.1–75 min, 5–34%B; 76–87 min, 100%B, where solvent A: 96% water, 4% acetonitrile with 0.01 M HFBA; solvent B: 60% acetonitrile in water (water, Millipore; acetonitrile, Fisher). LW-1 eluted at ~ 68 min as monitored at excitation/emission 348/463 nm by a JASCO Model 821-FP fluorescence detector (Jasco Inc., Easton, MD).

The collected peaks containing LW-1 were combined, freeze-dried, and re-injected onto a 25 cm × 10 mm, 5 µm Discovery HS C18 column (Sigma-Aldrich) using the following linear gradient: 0–5 min, 100% A; 5.1–40 min, 1–25% B; 41–52 min, 100% B; where solvent A: water with 0.1% trifluoroacetic acid (TFA); solvent B: 60% acetonitrile, 40% water with 0.1% TFA. The column was ran at flow rate 2 ml/min of which the eluate was monitored for fluorescence at ex/em 348/463 nm (JASCO) and absorbance at 210 and 348 nm (Waters Model 2996 photodiode array detector). LW-1 eluting at ~ 34 min was collected and freeze-dried. Since preliminary analysis showed that contaminants were present in the ¹H NMR spectrum, LW-1 was repurified using a 50 × 2.1 mm Hypercarb HPLC column (Thermo Fisher). The linear gradient was as follows: 0–60 min, 0–100% B; 60–70 min, 100% B;

where solvent A: 95% water, 5% acetonitrile with 0.1% TFA; solvent B: 60% acetonitrile, 40% water with 0.1% TFA. The column was ran at flow rate 0.5 ml/min of which LW-1 eluted at $\approx$ 28.4 min when monitored by the fluorescence and absorbance detectors wavelengths same.

Procedures for NMR experiments

A total of $\approx$ 5 mg purified LW-1 was exchanged with deuterium oxide (99.990 atom % D, Sigma-Aldrich), reconstituted to a final volume of 325 μ l D₂O, and placed in a BMS-3 NMR microtube (Shigemi Inc., Allison Park, PA). Experiments were conducted at the Cleveland Center for Membrane and Structural Biology (Case Western Reserve University, Cleveland, OH) using a 900 MHz NMR instrument (Bruker BioSpin Corporation, Billerica, MA). The following experiments were conducted: ¹H NMR (proton NMR), ¹³C NMR (carbon-13 NMR direct), ¹H-¹H TOCSY (total correlation spectroscopy), ¹H-¹³C HSQC (heteronuclear-single quantum coherence), ¹H-¹³C HMBC (heteronuclear multiple-bond correlation).

Cleavage of LW-1 by glycosidases

Assays were conducted in volumes of 50 μ l using a 384-well cell culture plate (Costar 3702, Costar-Corning, Corning, NY). To each well was added 5 μ l ($\sim$ 40 nmol) LW-1 purified standard that was incubated for 16 h at 37°C with 1.7 μ l of either β -glucosidase (0.67 units), β -glucuronidase (16 units) or β -galactosidase (0.67 units) in buffer consisting of either 10 mM citrate, pH 5.0 (glucosidase and glucuronidase) or phosphate buffered saline (PBS), pH 7.2 (galactosidase). Positive controls consisted of incubations of each enzyme with its marker substrate (4-nitrophenyl β -D-glucopyranoside, phenolphthalein β -D-glucuronide, 4-nitrophenyl- β -D-galactopyranoside) at conditions same. Negative controls were incubations of substrates in buffer without the respective enzyme, or incubations of the enzyme without their substrates same. Other controls included the incubation of LW-1 in buffer (citrate, PBS) without the enzyme. All incubations were in duplicate.

At the end of the incubation, verification was made for enzyme activity in the positive controls either by scanning the plate for absorbance at 405 nm using a TECAN microplate reader (Tecan, Inc., Morrisville, NC) (glucosidase, galactosidase) or injecting a sample onto the LC-MS/MS and measuring the cleavage of phenolphthalein β -D-glucuronide (glucuronidase) (see below). Additionally, the level of LW-1 in each well was measured by LC-MS (see below) and total autofluorescence. For the latter, a 10 μ l aliquot of each well contents was diluted with 1.5 ml water and placed in a quartz cuvette (45 mm $\times$ 12.5 mm $\times$ 12.5 mm). This was followed by measuring fluorescence at ex/em 348/363 nm (i.e., fluorescence maxima for LW-1) with a JASCO Model 821-FP fluorescence detector modified with a rectangular cell holder (Jasco, Inc., Easton, MD).

Analyses by liquid chromatography-mass spectrometry (LC-MS)

LW-1 was assayed by LC-MS consisting of a Waters 2690 Alliance separation module operated and connected by MassLynx software (v4.1) to a Micromass Quattro Ultima triple-quadrupole MS detector with electro-spray ionization (ESI) operating in the positive ion mode (Waters, Milford, MA, USA). The content of each well $\sim$ 50 μ l (see above) was placed into an injection vial and acidified with $\sim$ 1.7 μ l formic acid followed by injection of $\sim$ 10 μ l

of each sample onto a Hypercarb guard column: 10×2.1 mm, $5 \mu\text{m}$ contained in an Uniguard holder (Thermo Fisher). LW-1 was isocratically eluted at flow rate 0.2 ml/min with a 80:20 mixture of solvents A:B where solvent A: 0.1% formic acid/water titrated to pH 3.6 with ~ 14.8 M ammonium hydroxide PLUS (Thermo Fisher); solvent B: 90% acetonitrile/water (Burdick & Jackson, Muskegon, MI). The eluate was directed onto the MS programmed for full MS mode and scanned at m/z 50–650 between 0.4 to 10 min at cone voltage 60 V. Under these conditions, the molecular/parent ion of LW-1 at m/z 623 eluted ≈ 2.8 min in the chromatogram.

The positive control for β -glucuronidase was assessed by specific activity measured by the molecular/product ion transition for phenolphthalein β -D-glucuronide $\rightarrow$ phenolphthalein at m/z 495/319. The setup for the LC-MS system was the same as described for LW-1 except scanning was at m/z 50 to 550. Phenolphthalein β -D-glucuronide and phenolphthalein eluted at ≈ 2.2 and 1.5 min, respectively.

Results

Evidence for glucuronic acid in LW-1 by NMR

^1H NMR showed the same major signals for LW-1 as previously published by us [10]. However, the present spectrum was achieved with 32 scans (~ 15 min) compared with 400 scans ($\sim$ overnight) for the previous one which was obtained with an 800 MHz instrument [10]. Additionally, fewer signals were observed in the aliphatic region at < 2.5 ppm (Fig. 1) indicating that the present preparation of LW-1 was more concentrated and better purified. As with the previous ^1H spectrum, there were notable signals (ppm) in the aromatic region at 6.95 (d), 8.02 (d) and 8.63 (s) as well as a cohort of signals in the aliphatic region including the alpha proton at ~ 3.7 ppm, and most notably a doublet at 5.03 ppm as detailed below (Fig. 1).

The previous HSQC spectrum showed a cluster of signals indicative of a possible sugar moiety as a part of the LW-1 structure; however, no definite conclusion could be made because a lack of data in the noncontinuous HSQC [10]. In the present study, four equivalent signals were again observed in the aliphatic region at (^1H , ^{13}C) ppm: (3.64, 76.2), (3.67, 72.6), (3.69, 77.9), (3.76, 74.2) (Fig. 2A). More interestingly, however, due to increased window width of the aromatic spectrum, the present HSQC showed a proton at 5.03 ppm coupled to a carbon at 106 ppm indicative of an anomeric proton-carbon of a sugar (Fig. 2B). This conclusion was based upon NMR data reported by other investigators on ^1H , ^{13}C chemical shifts for sugars and their conjugates [17–22]. In support, the splitting and coupling constant for the anomeric proton at 5.03 ppm; i.e., doublet, $J=7.9$ (Fig. 1) is in agreement with those previously published for glycoconjugates [19,21], particularly those containing glucuronic acid [11,20,22]. In further support, the TOCSY showed that the sugar protons at 3.64 – 3.76 ppm were coupled to the anomeric proton at 5.03 ppm (Fig. 3).

The anomeric carbon signal at 106 ppm in the HSQC (Fig. 2B) was also observed in the ^{13}C NMR (direct) spectrum (Fig. 4). This latter spectrum also showed four sugar carbon signals at 72.2, 73.9, 76, 76.8 ppm (Fig. 4) $\sim$ HSQC (Fig. 2A) as well as a carbon signal at ~ 175 ppm (Fig. 4) indicative of a carboxylic acid group in support of our proposition that LW-1 is

a sugar conjugate containing glucuronic acid. The signals observed in the ^{13}C NMR spectrum for LW-1 (Fig. 4) are listed in Table 1 in reference to signals from other NMR experiments made for LW-1 in Figs. 1–2.

The ^1H - ^{13}C HMBC spectrum showed long-range couplings (i.e., 2–4 bonds) between the anomeric proton at 5.03 ppm with carbon atoms at 130.8 ppm and 129 ppm, but failed to see a signal at ~ 175 ppm (Fig. 5). In contrast, the sugar protons at 3.68–3.72 ppm showed couplings with a carbon at 174.6 ppm indicative of the carboxylate group of glucuronic acid (Fig. 5). These results are in agreement with the ^1H - ^{13}C HMBC spectrum reported for the glucuronide conjugate of valproic acid [23] which also showed that the anomeric proton of glucuronic acid failed to show a long-range coupling with its carboxylate group despite a distance of 4 bonds in the sugar's closed chain configuration.

Evidence for glucuronic acid by mass spectrometry

Repeat analysis of the new LW-1 preparation by MS was the same as that previously published by us [10] and showed a molecular (parent) ion at m/z 623 with a major fragment at m/z 447; i.e., a loss of 176 amu which is diagnostic for glucuronides [24]. Other fragments were at m/z 272, 318 and 402 (data not shown).

Evidence for glucuronic acid by treatment with β -glycosidases

Further evidence for the presence of glucuronic acid was by treatment of purified LW-1 with three different glycosidases with the specific objective of cleaving the sugar from the molecule. In this experiment, β -glucosidase was chosen as one of the glycosidases because some of the cross-links in collagen are reportedly enzymatically glycosylated with glucose [25]. Secondly, since some hydroxylysine residues of collagen are glycosylated with galactose [26], as well as a galactosylated form of pyridinoline is known to exist in collagen [27], LW-1 was also treated with β -galactosidase. LW-1 levels were monitored by MS at m/z 623 (i.e., parent ion of LW-1) and fluorescence at ex/em 348/463 nm. The results showed that cleavage occurred with β -glucuronidase, but not with β -glucosidase or β -galactosidase (Figs. 6, 7A). Most notably, the autofluorescence of LW-1 was greatly reduced, but not totally abolished, after treatment with β -glucuronidase (Fig. 7B).

Tentative structure for glucuronidine, the glucuronide adduct of LW-1

Based on a meticulous analysis of similar NMR data from the literature, we provide in Fig. 8 a tentative and only partial structure for glucuronidine/LW-1 relying on the NMR experiments in Figs. 1–5 and Table 1.

The aromatic proton signals with chemical shifts at 6.94 and 8.03 ppm are coupled to each other ($J=2.8$ Hz, Fig. 3) as well as directly coupled to carbon atoms at 95 and 139 ppm, respectively (Figs. 2B, 8). These protons also have long range couplings with quaternary carbons at 129.9, 137.8 and 129.9, 137.5, 174.5 ppm, respectively (Fig. 5, Table 1). Taken together, we propose that these atoms at (^1H , ^{13}C) (6.94, 95), (8.03, 139) comprise a pyrrolic ring structure shown in Fig. 8. The long-range carbon signal at 174.5 ppm coupled to the proton at 8.03 ppm (Fig. 5) is a carboxylic acid group which we suggest is bonded to a (heteroatom) nitrogen of the pyrrolic ring (Fig. 8). The reason for this assignment is that the

signal at 8.03 ppm is the only proton which correlates with a carbon signal at 174.5 ppm in the aromatic region of the HMBC plot (Fig. 5).

Likewise, the aromatic proton signal at 8.63 ppm (Fig. 1) is directly coupled to a carbon at 110 ppm (Figs. 2 & 8, Table 1) and has long-range couplings with quaternary carbons at 128.5, 130.8, 136.5 and 143.3 ppm (Fig. 5). It is assigned a member of a middle ring structure; i.e., pyridine ring, shown in Fig. 8. This ring is further comprised of the ϵ -amino group of a lysine residue (Fig. 8). In this, protons at 4.43 ppm which are directly coupled to the ϵ -carbon of lysine at 48 ppm (Fig. 2, Table 1) have long-range couplings with the quaternary carbons at 129 and 138 ppm (Fig. 5), but surprisingly not with the quaternary carbons at 130.8 and 143.3 ppm (Figs. 5 & 8). We suggest that the latter two carbons comprise a third outer ring; i.e., pyridine ring, which is conjugated with glucuronic acid (Fig. 8).

Both the anomeric carbon-proton signal of glucuronic acid at (^1H , ^{13}C) (5.02, 106) ppm and the methyl group at (2.69, 13) ppm (Fig. 2, Table 1) have long-range couplings with a quaternary carbon at 130.4 ppm (Fig. 5). In turn, this methyl group has a long-range coupling to an aromatic carbon signal at 157 ppm (Fig. 5). However, the anomeric carbon of glucuronic acid is not coupled to the quaternary carbon at 157 ppm (Fig. 5). These results suggests that the glucuronic acid moiety is not directly bound to an aromatic carbon, but instead likely *N*-linked to a tricyclic heteroaromatic ring involving an ϵ -*N*-lysine protein residue and an incompletely understood R-residue as shown in Fig. 8. Attempts to further refine these assignments using computerized NMR programs were not successful.

Discussion

The above results raise several issues that pertain to the structure and mechanism of formation of glucuronidine, the role of glucuronidine and glucuronidation in the pathogenesis of diabetic complications, and the involvement of glucuronidation in other conditions such as uremia.

Structure and mechanism of formation of glucuronidine/LW-1

The analytical data presented above unequivocally show the presence of glucuronic acid in LW-1. Since glucuronic acid conjugates are traditionally regarded as detoxification reactions of exogenous and endogenous small molecular weight compounds, this raises the question of how does glucuronic acid become attached to the extracellular matrix (ECM)? One scenario is a gut derived xenobiotic or bacterial metabolite, or an endogenous meta/catabolite becomes glucuronidated in the liver or kidney, and eventually finds its way to the ECM to which it covalently binds to a free or pre-glycated lysine residue. Evidence of such modification were presented by Presle et al. [28] who found that glucuronidated ketoprofen could bind lysine residues. On the other hand, glucuronic acid is a substrate for ECM components such as glycosaminoglycan and proteoglycan (mucopolysaccharide) synthesis [29] and while evidence for presence of glucuronates in the ECM has been previously reported, mechanistic details of their formation are lacking. Has the glucuronide been intracellularly added to the ECM component or via a circulating aglycone? Yet, it appears from current knowledge of the structure of glucuronidine that the latter is more compatible

with a mechanism involving attachment of a circulating reactive glucuronidated substrate (aglycone) to lysine residues.

Interestingly, a recent study involving a small number (n=16) of adolescent patients with type 1 diabetes of median age 17 yrs showed a strong positive correlation ($P=0.91$, $P<0.0001$) between noninvasive skin autofluorescence/SAF measured by the AGE Reader and glucuronic acid levels in skin [30]. A significant source for glucuronic acid is thought to originate from turnover and degradation of mucopolysaccharides [31] whereby glucuronic acid is derived from heparan sulfate originating from the breakdown of mucopolysaccharides by heparanase [32,30], an endo-glucuronidase that cleaves heparan sulfate in a variety of mammalian cells and tissues [33]. Heparanase is upregulated by diabetes [34] and levels are elevated in urine and plasma in diabetic patients directly proportional with glycemia [32]. Furthermore, levels are elevated by nephropathy [35] and hemodialysis [36].

Roles of glucuronidine and glucuronidation in diabetic complications

We recently reported that glucuronidine/LW-1 levels in insoluble skin collagen obtained from skin biopsies were significantly associated with the long-term progression of diabetic complications in DCCT/EDIC patients with type 1 diabetes, including progression of background retinopathy, albumin excretion rate, intima-media thickness of the carotid artery and cardiac hypertrophy [37]. These findings raise the critical question of whether and how would excess glucuronidation of a glucuronidine precursor play a role in these complications, if at all.

Years ago Eisenburg et al. [38] pioneered work on the role of glucose in the glucuronic acid pathway/cycle of glucose metabolism. This pathway is a minor alternative to the Embden-Meyerhof pathway of glycolysis, but upregulated during diabetes as measured by increased serum concentrations of the ketopentose, xylulose [39]. Both glucuronic acid [40] and xylulose [41] significantly increase in the serum of patients with diabetes, although with considerable variability especially for xylulose [41]. Conversely, Fishman's group concluded that neither free glucuronic acid nor its glucuronide conjugates increase in serum of diabetic patients [42]. Furthermore, there was no relationship between serum levels of glucuronic acid and xylulose with glycemia [40,43].

The effect of diabetes on glucuronidation of *exogenous* compounds including drugs has been extensively studied with equivocal results. Diabetes either significantly impairs [44], significantly accelerates [45], or does not significantly affect [46] glucuronidation depending upon the compound being tested and the UDP-glucuronyltransferase (UGT) isoform involved in the catalysis. Undoubtedly, the technique used to measure UGT plays an important role in these results and their interpretation: e.g., (a) the use of isolated microsomes *vs.* whole tissue homogenates; (b) the use of native microsomes *vs.* microsomes that have been pre-activated/permeabilized with Triton X-100 or alamethicin; and (c) the rodent model used to induce diabetes: streptozotocin (STZ) *vs.* alloxan *vs.* genetic models of spontaneous diabetes. Interestingly, Price and Jollow [47] reported that increased resistance of diabetic rats (STZ) to acetaminophen toxicity was due in part to increased capacity to eliminate the drug by glucuronidation.

In contrast, the glucuronidation of *endogenous* compounds is a grossly under-investigated area of study [48]. Of these, glucuronidated bilirubin, the endproduct of heme catabolism, has been most widely studied [48]. In short, rat models of STZ [49] and spontaneous diabetes [46] have shown a significant increase in the conjugation of bilirubin as well as the conjugation of the steroid estradiol [49]. UGTs, particularly isoform UGT1A1 associated with the glucuronidation of bilirubin, are significantly activated and upregulated by diabetes [46,49]. In human studies, there was a significant, approximate 2.5-fold increase in the conjugation of circulating bilirubin in diabetes [50]. Additionally, glucuronidated androgen metabolites are associated with metabolic risk factors for diabetes in men [51].

In view of these results, considering our own data on the association between glucuronidine and the long-term complications of diabetes [37], as well as the fact that the glucuronidation pathway is UDP-glucuronic acid formation-dependent, we propose that the glucuronidation pathway should be added to the Brownlee scheme of glucotoxicity [52] shown in Fig. 9. While there is at this time a paucity of data in support of this association, work by Sallustio et al. [53] point to a link between DNA damage, glycooxidation and glucuronidation-mediated bioactivation of xenobiotics to acyl glucuronide formation that is decreased by the glucuronidation inhibitor borneol or aminoguanidine. These studies revealed an important role for UDP-glucuronyltransferases in this phenomenon [54]. Excess glucuronidation may conceivably also lead to lower circulating levels and cellular uptake of food-derived antioxidants that are substrates for glucuronidation, partially explaining why antioxidant therapy has failed to prevent complications [55]. Similarly, excess diversion of UDP-glucose toward glucuronidation would make it more limiting for other pathways, such as the synthesis of glycogen [56] and contribute to hypoglycemia [57]. Finally, since the pathway is consuming 2 moles of NAD^+ , excess glucuronidation would conceivably worsen NAD^+ depletion, the consequences of which are widely understood as being deleterious for ROS homeostasis, histone acetylation and methylation reactions, as well as gene expression [58]. These considerations clearly suggest that new research is needed to understand the potential role of the glucuronidation pathway in diabetic complications.

Glucuronidation in uremia

Because of diminished renal clearance, a plethora of metabolites accumulate in uremia including those of the aromatic amino acids; i.e., tryptophan, phenylalanine, tyrosine [59], as well as amino-carbonyl reactions/glycooxidation products of reducing sugars reaction with proteins [60]. In further complexity, mounting evidence suggests that microbial metabolism in the gut contributes substantially to uremic retention solutes [61].

Like for the studies on glucuronidation in diabetes described above, rodent studies have shown that glucuronidation is either decreased, increased, or unaffected by renal failure [62]. In humans, glucuronide conjugation in renal failure has not been extensively studied. However, two recent independent studies have shown that p-cresyl glucuronide formation from p-cresol is significantly increased in patients with chronic kidney disease (CKD) [63,61]. p-Cresol is a toxic uremic retention solute that originates from tyrosine by colonic microbial metabolism. Serum levels of p-cresyl glucuronide progressively and significantly ($P<0.0001$) increase with the severity of CKD [63]. In both studies, levels were significantly

($P = 0.002$) associated with the outcomes CVD mortality and overall mortality even after adjustment for a host of covariates including age, diabetes, blood pressure, cholesterol and smoking [63,61]. In that regard, glucuronidine/LW-1 is highly elevated in ESRD [10].

Ultrafiltrates collected from uremic patients contain appreciable amounts of oligosaccharides and glycopeptides. In attempt to elucidate the chemical makeup of these “middle molecules”, le Moël et al. [64] found that the majority of the acidic oligosaccharides consisted of glucuronoconjugates. Patients with ESRD have plasma levels of free and conjugated glucuronic acid up to ten times higher vs. healthy subjects [64]. Thus, glucuronic acid and glucuronoconjugates are the components of ultrafiltrates. Nevertheless, the glucuronoconjugates are removed extensively by peritoneal and hemodialysis [64]. From these observations, Le Moel et al. [64] suggests that middle molecules are eliminated by glucuronidation similar to that observed for the detoxification of drugs and their metabolites by phase II metabolism catalyzed by UGTs.

Conclusions

A partial but key breakthrough was achieved with the discovery of a glucuronide in the structure of LW-1, now named glucuronidine. Additional structural studies are needed to understand the origin of the aglycone, and hence the full structure of glucuronidine. Crossing these milestones will open the door for ELISA based clinical assays of cumulative glucuronidation activity in diabetes. At this time there is extreme paucity of data on glucuronidation and diabetic complications. Cellular and molecular studies on the role of hyperglycemia in the activation of the glucuronidation pathway in diabetes are needed to help break new grounds on the biochemical and genetic interface between nutrition, the microbiome and the progression of diabetic complications.

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Fig. 1. ¹H-NMR spectrum for LW-1. Chemical shifts (ppm) and splitting are given in parenthesis

Fig. 2. ^1H - ^{13}C HSQC spectrum for region (A) aliphatic (B) aromatic of LW-1. The HSQC shows direct couplings (i.e., one bond) between ^1H and ^{13}C atoms. For each signal, the chemical shifts (^1H , ^{13}C) are given in parenthesis. x-axis: ^1H ; y-axis: ^{13}C

Fig. 3. ^1H - ^1H TOCSY for LW-1. TOCSY shows protons at 3.71 ppm (range ≈ 3.59 – 3.84) are correlated with the anomeric proton at 5.05 ppm

Fig. 4.
 ^{13}C -NMR (direct) spectrum for LW-1. The chemical shift is indicated above each ^{13}C signal (ppm)

Fig. 5.
 ^1H - ^{13}C HMBC spectrum for LW-1. The HMBC shows long-range couplings (i.e., 2–4 bonds) between ^1H and ^{13}C atoms. For each signal, the chemical shifts (^1H , ^{13}C) ppm are given in parenthesis

Fig. 6. Reprocessed ion chromatograms of LW-1/glucuronidine at m/z 623 after treatment of purified LW-1 for 16 hrs at 37°C with glycosidases as indicated (see Methods)

Fig. 7.

(A) levels of LW-1/glucuronidine measured by LC-MS expressed as integration area at m/z 623 and (B) relative fluorescence at excitation/emission 348/463 nm of LW-1/glucuronidine after treatment of purified material for 16 hrs at 37°C with glycosidases, as indicated (see Methods)

Fig. 8. Propose structure for glucuronidine/LW-1 consisting of three heterocyclic aromatic rings conjugated with glucuronic acid. Tentative hydrogen and carbon atom assignments match those NMR signals (ppm) in Table 1

Fig. 9. Pathways of glucotoxicity in the complications of diabetes as proposed by Brownlee (highlighted in green). Our working hypothesis is that activation of the Uronic Acid pathway (highlighted in orange) could become an important contributor of cellular toxicity during hyperglycemia when GAPDH activity is compromised in cells capable of glucuronidation, such as liver, kidney, skin, retina and brain. The strong association between glucuronidine and the long-term complications of Type 1 diabetes tentatively support this hypothesis

Table 1

Summary of signal assignments for NMR experiments performed on LW-1/glucuronidine

¹³ C NMR signal	¹ H NMR signal	Short-Range ¹ H- ¹³ C connectivities one bond (¹ H, ¹³ C) HSQC signals	Structural Assignment	Long-Range ¹ H- ¹³ C connectivities 2-4 bonds (¹ H, ¹³ C) HMBC signals
ppm	ppm	ppm	attribute/moiety	ppm
13	2.69	(2.69, 13)	CH ₃	(2.69, 130.4) (2.69, 156.6)
22.4	1.46	(1.35, 22.7) (1.4, 22.7) (1.46, 22.6) (1.51, 22.6)	γ-CH ₂ lysine	-
29.2	2.10	(2.10, 29.4)	δ-CH ₂ lysine	-
29.9	1.98	(1.98, 30)	?	-
30.7	1.98	(1.87, 30.9)	?	-
30.8	1.87	(1.91, 31)	β-CH ₂ lysine	-
48	4.43	(4.43, 47.8)	ε-CH ₂ lysine	-
52	4.96	(4.96, 52) (5.29, 52)	?	(4.96, 130.8) (4.96, 136.5) (5.29, 130.8) (5.29, 136.5)
55	3.7	(3.7, 55.4)	α-CH lysine	-
72.2	3.67	(3.67, 72.6)	CH sugar	-
73.9	3.76	(3.76, 74.2)	CH sugar	-
76	3.64	(3.64, 76.2)	CH sugar	-
76.8	3.69	(3.69, 77.9)	CH sugar	-
95	6.94	(6.94, 95)	CH aromatic ring	(6.91, 128.7) (6.94, 137.8)
106	5.03	(5.02, 106)	anomeric CH sugar	(5.04, 130.3)
110	8.63	(8.63, 110)	CH aromatic ring	(8.64, 128.5) (8.64, 130.8) (8.64, 136.5) (8.64, 143.3)
129.9	-	-	quaternary C	(8.64, 128.5) (8.03, 128.9) (6.91, 128.7) (5.04, 130.3) (4.45, 129) (2.69, 130.4)
131.1	-	-	quaternary C	(8.64, 130.8) (5.29, 130.8) (5.04, 130.3) (4.96, 130.8)
131.7 ?	-	-	-	-
137.5	-	-	quaternary C	(8.64, 136.5) (8.03, 136.6) (6.94, 137.8) (5.29, 136.5) (4.96, 136.5) (4.43, 138)
139	8.01	(8.01, 139)	CH aromatic ring	(8.03, 128.9) (8.03, 136.6) (8.03, 174.5)
144	-	-	quaternary C	(8.64, 143.3)
157	-	-	quaternary C	(2.69, 156.6) (3.67, 156.8) (3.68, 155.2) (3.75, 155.2)
174.6, 174.8, 174.9	-	-	quaternary C-COOH	(8.03, 174.5) (3.72, 174.5)