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Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry

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Glycation of proteins forms fructosamines and advanced glycation endproducts. Glycation adducts may be risk markers and risk factors of disease development. We measured the concentrations of the early glycation adduct fructosyl-lysine and 12 advanced glycation endproducts by liquid chromatography with tandem mass spectrometric detection. Underivatized analytes were detected free in physiological fluids and in enzymic hydrolysates of cellular and extracellular proteins. Hydroimidazolones were the most important glycation biomarkers quantitatively; monolysyl adducts (N_ε-carboxymethyl-lysine and N_ε-1-carboxyethyl-lysine) were found in moderate amounts, and bis(lysyl) imidazolium cross-links and pentosidine in lowest amounts. Quantitative screening showed high levels of advanced glycation endproducts in cellular protein and moderate levels in protein of blood plasma. Glycation adduct accumulation in tissues depended on the particular adduct and tissue type. Low levels of free advanced glycation endproducts were found in blood plasma and levels were 10–100-fold higher in urine. Advanced glycation endproduct residues were increased in blood plasma and at sites of vascular complications development in experimental diabetes; renal glomeruli, retina and peripheral nerve. In clinical uraemia, the concentrations of plasma protein advanced glycation endproduct residues increased 1–7-fold and free adduct concentrations increased up to 50-fold. Comprehensive screening of glycation adducts revealed the relative and quantitative importance of α-oxoaldehyde-derived advanced glycation endproducts in physiological modification of proteins – particularly hydroimidazolones, the efficient renal clearance of free adducts, and the marked increases of glycation adducts in diabetes and uraemia – particularly free advanced glycation endproducts in uraemia. Increased levels of these advanced glycation endproducts were associated with vascular complications in diabetes and uraemia.

Key words: diabetes, fructosamine, methylglyoxal, 3-nitrotyrosine, oxidative stress, uraemia.

INTRODUCTION

Glycation of proteins has been implicated in disease mechanisms. There is substantial evidence linking protein glycation to the development of vascular complications of diabetes and uraemia, and aging, with less well-established links to non-diabetic vascular disease, Alzheimer's disease and inflammatory disorders (reviewed in [1]). Glycation adducts are formed by the reaction of proteins with glucose, reactive α-oxoaldehydes such as glyoxal, methylglyoxal and 3-deoxyglucosone, and other saccharide derivatives. They are classified as early glycation adducts and AGEs (advanced glycation endproducts; Figure 1). Glycation adducts are also present in food and are absorbed as free adducts after digestion [2]. Glycation reactions are increased in diabetes mellitus where there is increased plasma glucose concentration and also increased concentrations of glyoxal, methylglyoxal and 3-deoxyglucosone [3–5], and in uraemia where there are increased concentrations of many α-oxoaldehydes [6].

AGEs have a wide range of chemical stabilities and varied biological effects [7–12]. In this report, we describe the quantitative screening of a comprehensive range of AGEs, protein-localized and free, in cellular and extracellular proteins and body fluids. We show for the first time that cellular proteins are more highly glycated than extracellular proteins, the relative and quantitative importance of α-oxoaldehyde-derived AGEs in physiological modification of proteins – particularly hydroimidazolones, tissue-specific accumulation of particular AGEs and renal clearance of all AGEs and an initial assessment of the derangement of AGE concentrations and clearance in diabetes and uraemia.

EXPERIMENTAL

Materials

[*guanidino*-$^{15}N_2$]L-Arginine, 4,4,5,5-[$^2H_4$]L-lysine and [$^{13}C_6$]L-lysine, [*methyl*-$^2H_3$]L-methionine and *ring*-[$^2H_4$]L-tyrosine (all > 98 % isotopic purity) were purchased from Cambridge Isotope Laboratories (Andover, MA, U.S.A.).

Abbreviations used: AGE, advanced glycation endproduct; CEL, N_ε-(1-carboxyethyl)lysine; CML, N_ε-carboxymethyl-lysine; 3DG-H, hydroimidazolones derived from 3-deoxyglucosone; 3DG-H1, N_δ-[5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl]ornithine; 3DG-H2, 5-[2-amino-5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-1-yl]norvaline; 3DG-H3, 5-[2-amino-4-hydro-4-(2,3,4-trihydroxybutyl)-5-imidazolon-1-yl]norvaline; DOLD, 3-deoxyglucosone-derived lysine dimer, 1,3-di(N^ε-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium salt; ESRD, endstage renal disease; FL, fructosyl-lysine; G-H1, N_δ-(5-hydro-4-imidazolon-2-yl)ornithine; GOLD, glyoxal-derived lysine dimer, 1,3-di(N^ε-lysino)imidazolium salt; HD, haemodialysis; LOD, limit of detection; MetSO, methionine sulphoxide; LC-MS/MS, liquid chromatography with tandem mass spectrometric detection; MG-H1, N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; MG-H2, 5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)norvaline; MG-H3, 5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)norvaline; MOLD, methylglyoxal-derived lysine dimer, 1,3-di(N^ε-lysino)-4-methyl-imidazolium salt; 3-NO_2Tyr, 3-nitrotyrosine; PD, peritoneal dialysis; RBC, red blood cell; STZ, streptozotocin; THP, N_δ-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydro-pyrimidin-2-yl)-ornithine.

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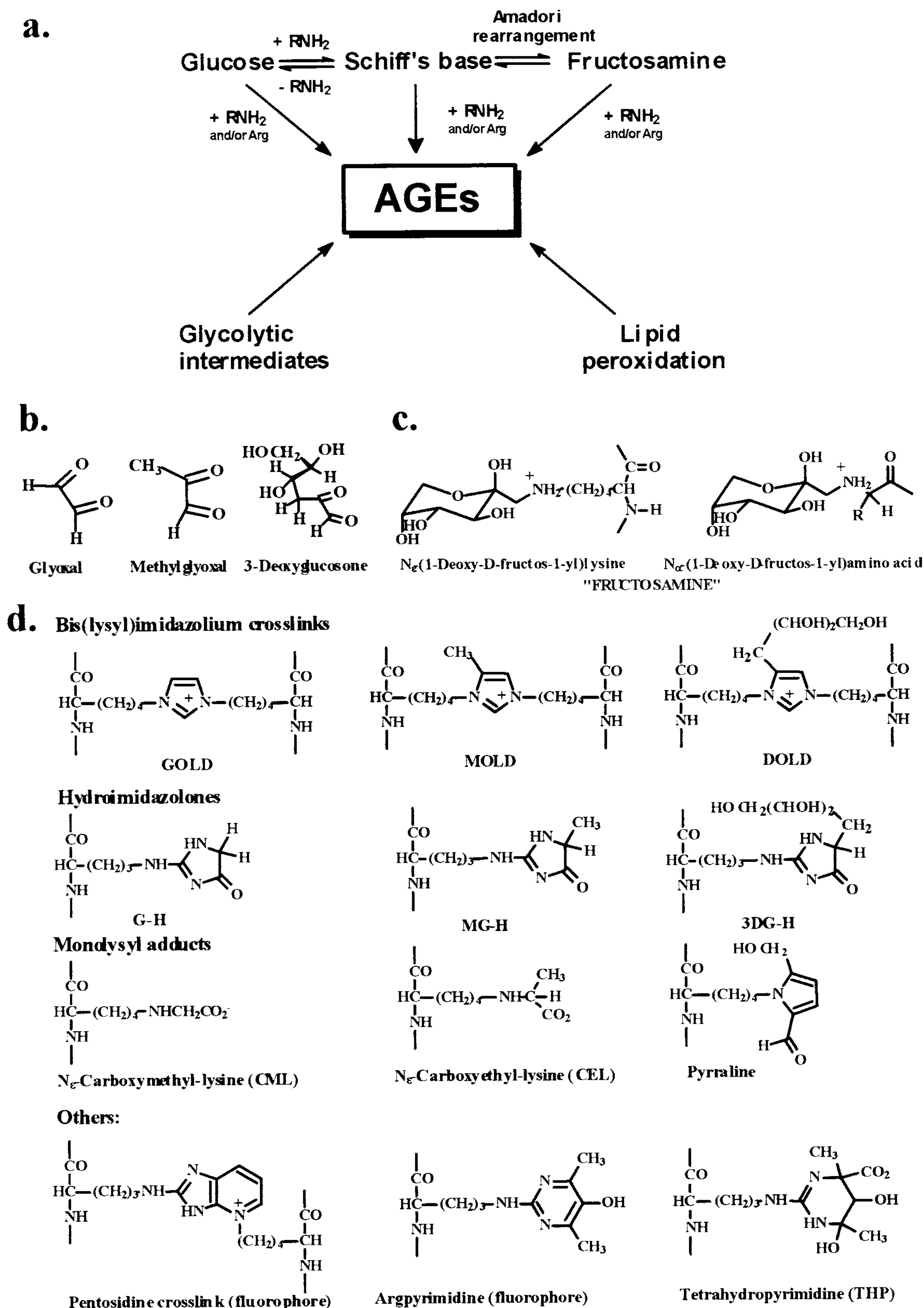

Figure 1 Protein glycation in physiological systems

(**a**) Pathways for the formation of AGEs; (**b**) α-oxoaldehyde-glycating agents; (**c**) early glycation adducts; (**d**) AGEs.

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Protein glycation adduct determination by LC-MS/MS (liquid chromatography with tandem mass spectrometric detection)

The following glycation adducts were determined: FL (fructosyl-lysine), methylglyoxal-derived AGEs {MG-H1 [N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine], MOLD [methylglyoxal-derived lysine dimer, 1,3-di(N^ε-lysino)-4-methyl-imidazolium salt], CEL [N_ε-(1-carboxyethyl)lysine], argpyrimidine and THP [N_δ-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydro-pyrimidin-2-yl)-ornithine]}, glyoxal-derived AGEs {G-H1 [N_δ-(5-hydro-4-imidazolon-2-yl)ornithine], GOLD [glyoxal-derived lysine dimer, 1,3-di(N^ε-lysino)imidazolium salt] and CML (N_ε-carboxymethyl-lysine)}, 3-deoxygluocosone-derived AGEs {3DG-H [hydroimidazolones derived from 3-deoxyglucosone], DOLD [3-deoxyglucosone-derived lysine dimer, 1,3-di(N^ε-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium salt] and pyrraline}, and pentosidine. The markers of oxidative stress, MetSO (methionine sulphoxide) and dityrosine, and the marker of nitrosative stress, 3-NO_2Tyr (3-nitrotyrosine), were also determined.

[*guanidino*-$^{15}N_2$]MG-H1, [*guanidino*-$^{15}N_2$]3DG-H, [*guanidino*-$^{15}N_2$]G-H1, [*guanidino*-$^{15}N_2$]argpyrimidine and [*guanidino*-$^{15}N_2$]THP were prepared from [*guanidino*-$^{15}N_2$]L-arginine after conversion to the N_α-t-butoxycarbonyl derivative [13]. [$^2H_8$]-GOLD, [$^2H_8$]MOLD and [$^2H_8$]DOLD were prepared from 4,4,5,5-[$^2H_4$]L-lysine after conversion to the N_α-formyl derivative [14]. [$^{13}C_6$]CEL, [$^{13}C_6$]CML and [$^{13}C_6$]pentosidine were prepared from [$^{13}C_6$]L-lysine after conversion to the N_α-formyl derivative. Synthetic methods for the preparation, purification and characterization of these AGE calibration standards were as described for their non-isotopically substituted analogues [7]. [*methyl*-$^2H_3$]MetSO was prepared from [*methyl*-$^2H_3$]L-methionine, and [$^2H_6$]dityrosine and 3-NO_2Tyr were prepared from *ring*-[$^2H_4$]L-tyrosine, by the methods described in [15–17].

Free glycation adducts were determined by assay of analytes in ultrafiltrate (12-kDa filter cut-off, 50-μl aliquots) of plasma and urine. Protein glycation adduct residues were determined in enzymic digests (50-μg protein equivalent) prepared from protein extracts of rat tissues, human cells and blood plasma [7]. Analytes released by self-digestion of proteases in assay blanks were subtracted from analyte estimates. Urine samples (100 μl) were incubated with acylase (10 units in 10 μl of 10 mM sodium phosphate buffer, pH 7.4, and 37 °C) for 0–6 h to enable detection of N-acetyl conjugates. Samples were assayed by LC-MS/MS using a 2690 Separation module with a Quattro Ultima triple quadrupole mass spectrometric detector (Waters-Micromass, Manchester, U.K.). Two 5-μm Hypercarb™ columns (Thermo Hypersil, Runcorn, Cheshire, U.K.) in series were used: 2.1 mm × 50 mm (column 1) and 2.1 mm × 250 mm (column 2). The mobile phase was 26 mM ammonium formate, pH 3.8, with a two-step gradient of acetonitrile (17–25 min, 0–31 % acetonitrile; 25–30 min, 31 % acetonitrile; 30–35 min, 31–50 % acetonitrile). The flow rate was 0.2 ml/min. The flow was diverted to bypass column 2 at 20 min to facilitate elution of hydrophobic analytes. Flow from the column in the interval 4–35 min was directed to the MS/MS detector. Glycation markers were detected by electrospray positive ionization-mass spectrometric multiple reaction monitoring. The ionization source temperature was 120 °C and the desolvation gas temperature 350 °C. The cone gas and desolvation gas-flow rates were 150 and 550 l/h, respectively. The capillary voltage was 3.55 kV and the cone voltage 80 V. Argon gas (2.7×10^{-3} mbar) was in the collision cell. Programmed molecular ion and fragment ion masses and collision energies were optimized to ± 0.1 Da and ± 1 eV for multiple-reaction-monitoring detection of analytes (Table 1). Amounts of internal standard used were: 10 nmol for amino acids, 250 pmol for FL and 10–50 pmol for AGEs and oxidation and nitrosation biomarkers.

Argpyrimidine, pentosidine and 3-NO_2Tyr were eluted and ionized more effectively in an acidic eluent using only column 1. The mobile phase was 0.1 % trifluoroacetic acid with a linear gradient of 10–50 % acetonitrile from 0 to 15 min and isocratic 50 % acetonitrile thereafter. THP was not quantified discretely since it co-eluted degraded to argpyrimidine during enzymic hydrolysis. Estimates of argpyrimidine concentration by LC-MS/MS, therefore, are the sum of argpyrimidine and THP concentrations.

Tissue and physiological fluid samples

Human blood cells were fractioned from venous blood (50 ml) drawn from normal healthy controls with heparin anticoagulant. RBCs (red blood cells) were sedimented by centrifugation (1000 ***g***, 10 min) and the buffy coat removed. They were washed three times with 4 vol. of 0.9 % NaCl, lysed with 4 vol. of ice-cold water and membranes sedimented by centrifugation (20000 ***g***, 30 min, 4 °C). The concentration of haemoglobin was determined by the Drabkin method. The buffy coat was suspended in RPMI 1640 (15 ml) and layered on top of a binary density gradient of Histopaque™-1077 and Histopaque™-1166 (15 ml of each density medium) and centrifuged (700 ***g***, 30 min). Mononuclear leucocytes and neutrophils were recovered from the density-medium interfaces, washed three times with PBS, lysed by sonication and membrane fragments sedimented by centrifugation (20000 ***g***, 30 min, 4 °C).

Male Sprague–Dawley rats, 250 g, were purchased from Charles River UK (Ramsgate, Kent, U.K.). They were kept at 2 rats/cage at 21 °C, under 50–80 % humidity and with a daily 14-h light period, and had free access to food and water. Diabetes was induced by intravenous injection with 55 mg/kg STZ (streptozotocin). Body weight and moderate hyperglycaemia were stabilized by subcutaneous injection of 2 units of Ultralente insulin every 2 days for 24 weeks. Plasma glucose concentration was determined by glucose oxidase method and glycated haemoglobin HbA_1 by boronate affinity chromatography (diagnostic kits 510 and 442; Sigma). The mean body weight of the normal healthy control rats increased from 278 ± 13 g at baseline to 712 ± 93 g at 24 weeks. The mean body weight of the diabetic rats increased from 268 ± 12 g at baseline to 351 ± 41 g at 24 weeks, which was significantly less than the normal controls ($P < 0.001$). After 24 weeks of diabetes, the plasma glucose concentrations were 6.0 ± 1.2 mM (controls) and 25.1 ± 4.3 mM (STZ diabetics; $P < 0.001$).

Venous blood samples with heparin anticoagulant and 24 h urine collections were taken from five normal healthy control subjects (age 43 ± 6 years, plasma creatinine 100 ± 16 μM) and 11 patients with ESRD (endstage renal disease) recruited from the nephrology and HD (haemodialysis) clinics at St Bartholomew's Hospital, London, U.K. Six patients (age 47 ± 15 years, plasma creatinine 666 ± 110 μM) were receiving peritoneal dialysis therapy and five patients had HD therapy (age 43 ± 15 years, plasma creatinine 838 ± 154 μM). Samples from patients on HD were collected immediately before a dialysis session. Blood cells were sedimented by centrifugation and plasma removed. Urine samples were collected at ambient temperature. Validation studies showed that <10 % of analyte amounts were lost during this period. Plasma and urine samples were stored at −80 °C prior to analysis; samples were analysed within 6 months of collection, storage conditions validated to give no significant change in analyte concentration. The study was approved by East London and The City Health Authority Research Ethics Committee

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Table 1 Chromatographic retention times and mass-spectrometric multiple reaction monitoring detection of protein biomarkers

Epimeric forms of MG-H are denoted as MG-H_A and MG-H_B. Estimates of the concentrations of hydroimidazolone AGEs in physiological samples are given as MG-H1, G-H1 and the sum of all structural isomers of 3-deoxyglucosone-derived hydroimidazolones, 3DG-H. Argpyrimidine, pentosidine and 3-NO_2Tyr were assayed using the method with mobile phase of 0.1 % trifluoroacetic acid. Estimates of argpyrimidine are the sum of argpyrimidine + THP in physiological samples. Isotopic purity of the stable isotope-substituted standards is the percentage of compound with the isotope substitution indicated; the value in parentheses is the percentage of the compound with no isotope substitution (impurity interference)–residual compound has partial isotope substitution. R_t, retention time; CE, collision energy; cv, coefficient of variation.

Analyte	R_t (min)	Parent ion (Da)	Fragment ion (Da)	CE (eV)	Neutral fragment loss(es)	Internal standard	Isotopic purity (%)	LOD (pmol)	Recovery (%)	Inter-batch cv (%)
Amino acids and FL										
Arg	14.2	175.2	70.3	15	H_2CO_2, $NH_2C(=NH)NH_2$	[$^{15}N_2$]Arg	96.4 (3.6)	0.52	94	1.8
Lys	6.0	147.1	84.3	15	H_2CO_2, NH_3	[$^{13}C_6$]Lys	83.9 (0.7)	0.31	94	2.7
Met	9.2	150.0	104.2	11	H_2CO_2	[*methyl*-$^2H_3$]Met	99.1 (0.5)	0.23	87	2.1
Tyrosine	26.9	182.1	136.2	13	H_2CO_2	[*ring*-$^2H_4$]Tyrosine	94.0 (0.5)	0.49	100	2.5
FL	9.7	291.0	84.3	31	H_2CO_2, fructosylamine	[$^2H_4$]FL	90.5 (0.6)	4.15	71	4.6
Monolysyl AGEs										
CEL	7.9	219.2	130.1	13	$NH_2CH(CH_3)CO_2H$	[$^{13}C_6$]CEL	82.5 (2.4)	0.075	100	7.2
CML	7.4	204.9	130.1	12	$NH_2CH_2CO_2H$	[$^{13}C_6$]CML	68.6 (1.4)	0.085	101	6.0
Pyrraline	28.7	255.2	84.3	23	2-CHO-5-$HOCH_2$-pyrrole, H_2CO_2	[$^2H_4$]Pyrraline	89.5 (0.5)	0.050	87	9.9
Bis(lysyl)imidazolium AGEs										
GOLD	26.3	327.1	198.3	21	$NH_2CH(CO_2H)CH_2CH_2CH=CH_2$	[$^2H_8$]GOLD	84.3 (3.1)	0.065	96	8.1
MOLD	27.1	341.2	212.3	21	$NH_2CH(CO_2H)CH_2CH_2CH=CH_2$	[$^2H_8$]MOLD	83.0 (0.3)	0.027	96	6.0
DOLD	26.8	431.2	302.4	26	$NH_2CH(CO_2H)CH_2CH_2CH=CH_2$	[$^2H_7$]DOLD	54.8 (2.9)	0.025	96	8.9
Hydroimidazolones										
G-H	24.9	215.0	100.2	14	$NH_2CH(CO_2H)CH_2CH=CH_2$	[$^{15}N_2$]G-H1	94.5 (2.7)	0.36	89	6.1
MG-H										
MG-H_A	23.7	229.2	114.3	14	$NH_2CH(CO_2H)CH_2CH=CH_2$	[$^{15}N_2$]MG-H1	96.1 (3.1)	0.19	83	3.8
MG-H_B	24.1									
3DG-H										
3DG-H3	23.8	319.1	114.8	20	$NH_2CH(CO_2H)CH_2CH=CH_2$	[$^{15}N_2$]3DG-H	97.5 (2.1)	0.49	70	3.6
3DG-H1	24.6									
3DG-H2	25.1									
AGE fluorophores										
Argpyrimidine	12.0	255.3	140.3	17	$NH_2CH(CO_2H)CH_2CH=CH_2$	[$^{15}N_2$]Argpyrimidine	92.4 (1.9)	0.45	84	9.4
Pentosidine	16.5	379.3	250.4	22	$NH_2CH(CO_2H)CH_2CH_2CH=CH_2$	[$^{13}C_6$]Pentosidine	83.1 (0.3)	0.23	101	2.3
Oxidative and nitrosative biomarkers										
MetSO	7.5	166.1	102.2	14	CH_3-SOH	[*methyl*-$^2H_3$]MetSO	99.5 (0.5)	0.15	102	4.1
Dityrosine	27.9	361.2	315.3	15	H_2CO_2	[*ring*-$^2H_6$]Dityrosine	80.9 (1.8)	0.17	94	7.7
3-NO_2Tyr	14.3	227.1	181.2	13	H_2CO_2	[*ring*-$^2H_3$]3-NO_2Tyr	95.7 (0.1)	0.022	88	10.5

(London, U.K.) and informed consent was given by all participants. Urinary and plasma creatinine were determined by colorimetric assay (diagnostic kit 510, Sigma).

Data and statistical analysis

Renal clearance of analytes (ml/min) was determined as $[\text{analyte}]_{\text{urine}} \times$ urine volume/($[\text{analyte}]_{\text{plasma}} \times$ urine collection time). The significance of differences between mean and median AGE concentrations was determined using Student's *t* test and the Mann-Whitney *U* test, respectively. LOD (limit of detection) is defined as the analyte concentration equivalent to 3 S.D. of the zero analyte control deduced from the analyte calibration curve.

RESULTS

Quantitative screening of AGEs by LC-MS/MS

AGE and other biomarker analytes were detected in cellular and extracellular protein of physiological systems (Figure 2 and Table 1). Hydroimidazolones were quantitatively important AGEs in proteins glycated *in vitro* [18]. Three structural isomers are formed by the reaction of α-oxoaldehydes with arginine residues [7] (Figure 3). For methylglyoxal-derived hydroimidazolones, the structural isomers MG-H1, MG-H2 [5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)norvaline] and MG-H3 [5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)norvaline] exist as a racemic mixture of two epimers [7]. The epimer pairs of MG-H structural isomers were partially resolved in the LC-MS/MS chromatogram but the structural isomers co-eluted (Figures 2g and 2h). The detector response to each isomer was similar and therefore MG-H determinations reflect the sum of all isomers, although MG-H1 is the major isomer formed in proteins modified minimally by methylglyoxal *in vivo* [18]. Analogous structural isomers of glyoxal-derived hydroimidazolones were also not resolved and gave one peak (R_t, retention time = 24.9 min). Under physiological conditions with a high arginine/glyoxal ratio, G-H1 was the major isomer formed [7] (Table 1 and Figures 2e and 2f). Hydroimidazolones derived from 3-deoxyglucosone, 3DG-H, were resolved into the three structural isomers, eluting in the order 3DG-H3 {5-[2-amino-4-hydro-4-(2,3,4-trihydroxybutyl)-5-imidazolon-1-yl]norvaline}, 3DG-H1 {N_δ-[5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl]ornithine} and 3DG-H2 {5-[2-amino-5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-1-yl]-norvaline} (Figures 2i and 2j). The detector responses for the structural isomers were similar; 3DG-H was calibrated against the detector response to a mixture of 3DG-H isomers. Hydroimidazolones are AGEs of moderate chemical stability under physiological conditions, degrading to the corresponding

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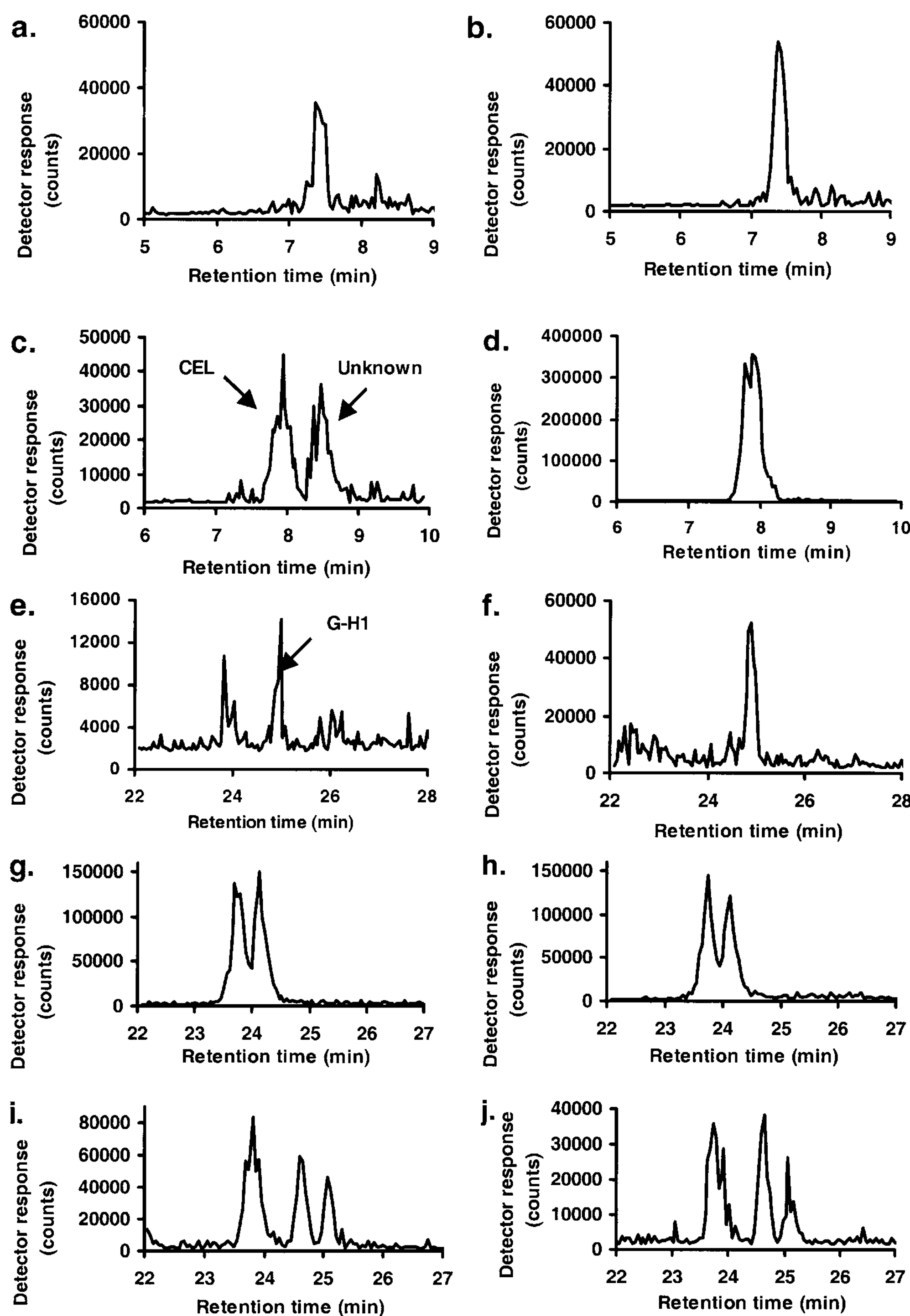

Figure 2 Specimen analytical chromatograms in the determination of protein glycation adducts by LC-MS/MS

(**a**) CML and (**b**) [$^{13}C_6$]CML (10 pmol) in plasma protein assay hydrolysate of a normal human subject, R_t (retention time) 7.4 min; (**c**) CEL and (**d**) [$^{13}C_6$]CEL (10 pmol) in haemoglobin assay hydrolysate of a normal human subject, R_t 7.9 min; (**e**) G-H1 and (**f**) [$^{15}N_2$]G-H1 (50 pmol) in rat sciatic nerve protein hydrolysate of a STZ diabetic rat, R_t 24.9 min; (**g**) MG-H and (**h**) [$^{15}N_2$]MG-H1 (50 pmol) in rat retinal protein hydrolysate of a STZ diabetic rat, R_t 23.7 (MG-H_A) and 24.1 (MG-H_B) min; (**i**) 3DG-H and (**j**) [$^{15}N_2$]3DG-H (50 pmol), isomers 1, 2 and 3 in rat renal glomerular protein hydrolysate of a STZ diabetic rat, R_ts 24.6, 25.1 and 23.8 min, respectively. Chromatographic conditions are described in the Experimental section.

α-oxoaldehyde and arginine and other products [7,19]. The half-lives of hydroimidazolones in 10 mM sodium phosphate buffer, pH 7.4 and 37 °C, were: MG-H1, 11.7 ± 2.7 days [7]; G-H1, 69 ± 16 days; 3DG-H1, 17.2 ± 2.3 days; 3DG-H2, 14.1 ± 1.6 days; 3DG-H3, 5.15 ± 0.65 days.

Cellular and extracellular AGE-modified proteins

The concentrations of AGEs in protein of human blood cells and blood plasma *in vivo* were determined (Tables 2 and 3). This revealed that for all AGEs, the concentration of the AGE

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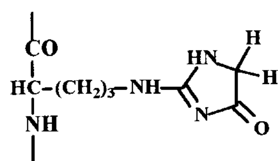
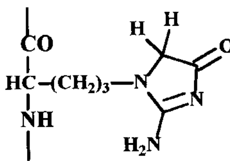
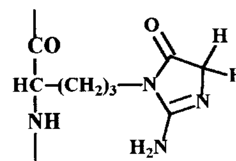
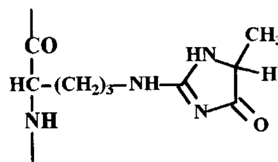
Glyoxal-derived hydroimidazolones

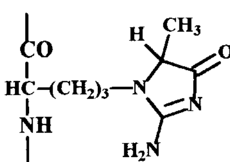


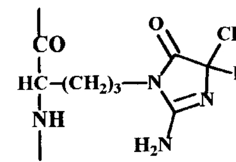




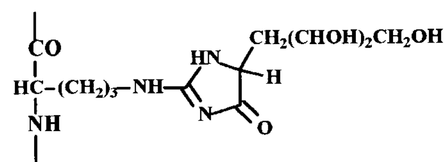
Methylglyoxal-derived hydroimidazolones

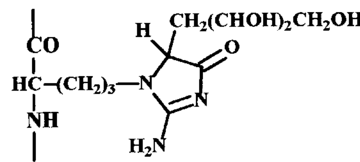






3-DG-derived hydroimidazolones





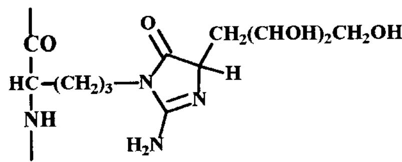


Figure 3 Structural isomerism of hydroimidazolone residues

G-H2, 5-(2-amino-5-hydro-4-imidazolon-1-yl)norvaline; G-H3, 5-(2-amino-4-hydro-5-imidazolon-1-yl)norvaline; other abbreviations are defined in the text.

Table 2 Protein AGEs in protein of human blood cells

A dash indicates that the analyte was below the LOD. Data are means $\pm$ S.D. ($n = 3$).

	AGE concentration		
Analyte	RBCs	Neutrophils	Mononuclear leucocytes
CML (mmol/mol of Lys)	0.068 ± 0.004	0.230 ± 0.004	0.078 ± 0.013
CEL (mmol/mol of Lys)	0.055 ± 0.004	0.118 ± 0.003	0.097 ± 0.009
MG-H1 (mmol/mol of Arg)	1.22 ± 0.41	4.70 ± 0.78	7.46 ± 1.13
3DG-H (mmol/mol of Arg)	7.12 ± 4.51	14.95 ± 3.36	6.25 ± 1.95
MOLD (mmol/mol of Lys)	0.0053 ± 0.0029	–	–
Argpyrimidine (mmol/mol of Arg)	–	1.17 ± 0.25	–
Pentosidine (mmol/mol of Lys)	0.022 ± 0.007	0.085 ± 0.011	0.023 ± 0.001
FL (mmol/mol of Lys)	1.74 ± 0.62	1.70 ± 0.33	0.789 ± 0.164
MetSO (mmol/mol of Met)	1.93 ± 0.19	0.81 ± 0.33	2.09 ± 0.31

residue was higher in cellular protein than in plasma protein: cf. estimates for CML and MG-H1. The concentration of FL residues in cellular protein was higher or lower than in plasma protein. The lysine content of cellular protein was in the range 200–700 nmol/mg of protein, and the lysine content of plasma protein was 820 ± 87 nmol/mg of protein. The arginine content of cellular protein was in the range 160–180 nmol/mg of protein, and the arginine content of plasma protein was 262 ± 20 nmol/mg of protein, respectively. MetSO residues were present at similar concentrations in cellular and plasma protein. The methionine content of cellular protein was in the range 90–180 nmol/mg of protein, and the methionine content of plasma protein was 146 ± 15 nmol/mg of protein. The highest levels of AGE residues were found for the hydroimidazolones MG-H1 and 3DG-H. These AGEs accounted for 1–3 % of total arginine residues. The protein cross-link pentosidine was found in the expected low concentrations; the highest concentration was in neutrophils. Bis(lysyl)imidazolium cross-linked AGEs were not detectable except for MOLD in plasma and RBCs. Argpyrimidine was detected in neutrophils but most argpyrimidine in blood plasma was present as the free adduct.

The concentration of dityrosine and 3-NO_2Tyr residues in cellular and plasma protein was generally lower than the LODs except dityrosine content of RBC protein and 3-NO_2Tyr of plasma protein. Free 3-NO_2Tyr was detected in blood plasma and urine of normal healthy control subjects. The concentration of hydroimidazolones MG-H1 and 3DG-H residues in plasma and tissue proteins was, therefore, usually at least 100-fold higher than dityrosine and 3-NO_2Tyr.

AGEs in blood plasma, urine and renal clearance

The localization of AGEs in blood plasma of normal healthy human subjects was investigated. AGEs may be present as residues

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Table 3 AGEs in blood plasma and renal clearance in normal healthy human subjects

The creatinine clearance was 108 ± 15 ml/min, the concentration of plasma protein was 64.4 ± 7.4 mg/ml and the concentration of protein in plasma ultrafiltrate was 0.41 ± 0.20 mg/ml ($n = 5$). Pentosidine residue concentration in plasma protein is given as mmol/mol of Lys. Data are means ± S.D. ($n = 5$–6).

Analyte	Plasma protein [mmol/mol of Arg, Lys, Met or Tyr] (nM)	Plasma 'free' [nM] (% of total)	Urine (nM)	Renal clearance (ml/min)
CML	0.021 ± 0.005 (1109)	23 ± 8 (2)	1607 ± 473	75 ± 22
CEL	0.011 ± 0.005 (581)	35 ± 14 (6)	2761 ± 879	93 ± 18
G-H1	0.057 ± 0.028 (962)	50 ± 17 (5)	1653 ± 426	44 ± 15
MG-H1	0.921 ± 0.120 (15 540)	110 ± 46 (1)	5281 ± 3273	38 ± 6
3DG-H	0.351 ± 0.083 (5922)	147 ± 19 (2)	4356 ± 2738	35 ± 6
MOLD	0.0008 ± 0.0002 (42)	15 ± 6 (26)	48 ± 28	4.2 ± 1.9
Argpyrimidine	< 0.03 (< 500)	47 ± 13 (> 18)	24 ± 4	0.90 ± 0.62
Pentosidine	0.0056 ± 0.0016 (474)	0.84 ± 0.50 (< 1)	14 ± 2	22 (7–67)
FL	0.767 ± 0.139 (40 504)	< 200 (< 1)	3956 ± 1962	> 30
MetSO	0.762 ± 0.151 (7404)	22 ± 8 (< 1)	48 ± 24	2.9 ± 0.8
3-NO_2Tyr	0.0018 ± 0.0014 (34)	6.5 ± 2.5 (16)	38 ± 9	8.3 ± 2.8

in plasma proteins, free in blood plasma and in putative 'AGE peptides'; peptide fragments of proteins enriched with AGEs [20]. Free AGEs undergo renal clearance and so the urinary concentration and renal clearance of major AGEs was investigated. Hydroimidazolones were the major AGEs in plasma protein. Approx. 2 % of human serum albumin contains MG-H1 residues, 1 % contains 3DG-H residues and 0.1 % contains G-H1 residues in normal control subjects. CML and CEL residues were present at approx. 10-fold lower concentrations than this. Free AGEs were detected in plasma at up to 6 % of the total plasma content of each respective AGE. Argpyrimidine, however, was only detectable in plasma ultrafiltrate (corroborated by both LC-MS/MS and fluorescence assays) and FL was only detectable in plasma protein. There was also significant oxidation of methionine residues in plasma protein, with MetSO residue levels equivalent to approx. 0.5 % of human serum albumin, whereas the concentration of 3-NO_2Tyr residues in plasma protein was equivalent to approx. 0.003 % of serum albumin. The concentrations of free MetSO and 3-NO_2Tyr in plasma were very low (Table 3).

The urinary concentrations of FL and major AGEs (hydroimidazolones, CML and CEL) were >1 μM and variable. Other AGEs, MetSO and 3-NO_2Tyr, were present at low concentration in urine (< 50 nM). The renal clearances of CML and CEL were similar to that of creatinine, and the renal clearance of FL may be also. The hydroimidazolone AGEs had renal clearances of approx. 40 ml/min. The renal clearance of pentosidine was similar to that of hydroimidazolones but variable. The renal clearances of argpyrimidine, MetSO and 3-NO_2Tyr were extraordinarily low (< 5 ml/min). Deacetylation of analytes with acylase prior to analysis did not increase the urinary concentrations of AGEs detected significantly. Analysis of AGEs in plasma ultrafiltrate with and without prior exhaustive enzymic hydrolysis was performed to detect the presence of AGE peptides. We found no significant increase in AGEs in the plasma ultrafiltrate after enzymic hydrolysis.

Concentrations of AGEs in renal glomeruli, retina, peripheral nerve, skeletal muscle and plasma protein of STZ diabetic rats and normal healthy controls

The accumulation of AGEs has been implicated in the biochemical dysfunction associated with diabetes mellitus linked to the chronic development of microvascular complications of diabetes – nephropathy, retinopathy and peripheral neuropathy [21]. We investigated the quantitative changes of AGEs and other biomarkers in protein extracts of renal glomeruli, retina, peripheral nerve, skeletal muscle and plasma protein of STZ-induced diabetic rats and normal healthy controls (Table 4). The diabetic rats had the characteristics of the diabetic state: increased plasma glucose concentration and increased glycated haemoglobin HbA_1. After 24 weeks of diabetes, glycated haemoglobin HbA_1 was 17.7 ± 1.6 %, which was increased with respect to normal controls (9.0 ± 0.8 %; $P < 0.001$). Most HbA_1 reflects the presence of fructosamine residues N_α-fructosyl-valine and FL [22]. FL residues were detected in protein extracts of rat tissues and plasma protein. FL residue concentration was increased markedly in diabetic rats in renal glomeruli (6-fold), retina (3-fold), sciatic nerve (7-fold), skeletal muscle (1-fold) and plasma protein (3-fold). There were significant increases of CML residues in diabetic rats in renal glomeruli (86 %), retina (162 %), sciatic nerve (216 %) and plasma protein (64 %) but no significant increases in skeletal muscle. The concentration of CEL residues in diabetic rats was increased in the renal glomeruli (115 %), sciatic nerve (351 %), skeletal muscle (104 %) and plasma protein (112 %) but no significant increase in the retina. The highest concentration of AGEs in rat tissues was found for the hydroimidazolones; MG-H1 and 3DG-H residues were found generally at much higher concentration than G-H1. There were tissue-specific increases in hydroimidazolone residue concentrations in diabetic rats, with respect to normal controls: G-H1 was increased in the retina (152 %), nerve (136 %) and plasma protein (105 %); MG-H1 was increased in renal glomeruli (195 %), retina (279 %), nerve (111 %) and plasma protein (54 %); and 3DG-H was increased in renal glomeruli (51 %), retina (110 %) and nerve (50 %). Argpyrimidine residues were detectable only in the retina, nerve and plasma protein and these concentrations did not increase significantly in diabetic rats. Very low concentrations of pentosidine residues were detected in the retina, nerve, muscle and plasma protein. The marker of oxidative stress, MetSO, was present at high levels in all tissues and plasma protein and was not changed significantly in diabetes.

Accumulation of free AGEs and AGE residues in plasma protein of human subjects with renal failure

The concentration of CML residues in plasma protein was increased 2-fold in PD (peritoneal dialysis) subjects and 3-fold in

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Table 4 AGEs in tissues, blood plasma and urine of control and streptozotocin-induced diabetic rats

A dash indicates that the analyte was below the LOD. Significance: *, **, *** and NS indicate $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P > 0.05$, respectively, with respect to normal healthy controls.

		AGE concentration				
Analyte	Study group	Renal glomeruli	Retina	Sciatic nerve	Skeletal muscle	Plasma protein
CML (mmol/mol of Lys)	Control	0.269 ± 0.111	0.172 ± 0.051	0.151 ± 0.087	0.188 ± 0.093	0.033 ± 0.004
	Diabetic	0.501 ± 0.186*	0.451 ± 0.291*	0.437 ± 0.077**	NS	0.062 ± 0.008***
CEL (mmol/mol of Lys)	Control	0.329 ± 0.102	0.339 ± 0.091	0.115 ± 0.069	0.050 ± 0.026	0.008 ± 0.003
	Diabetic	0.706 ± 0.047***	NS	0.519 ± 0.286**	0.102 ± 0.042*	0.017 ± 0.006**
G-H1 (mmol/mol of Arg)	Control	0.044 ± 0.029	0.552 ± 0.103	0.517 ± 0.238	0.119 ± 0.061	0.275 ± 0.041
	Diabetic	NS	1.39 ± 0.89**	1.22 ± 0.55*	NS	0.565 ± 0.206**
MG-H1 (mmol/mol of Arg)	Control	2.30 ± 0.25	1.88 ± 0.51	4.75 ± 2.74	1.70 ± 0.77	1.45 ± 0.39
	Diabetic	6.79 ± 0.19***	5.24 ± 2.34***	10.03 ± 0.66**	NS	2.24 ± 0.38**
3DG-H (mmol/mol of Arg)	Control	3.23 ± 0.90	0.20 ± 0.09	2.85 ± 1.24	2.11 ± 1.00	2.26 ± 0.89
	Diabetic	4.87 ± 0.32**	0.42 ± 0.15*	5.73 ± 0.72**	NS	NS
Argpyrimidine (mmol/mol of Arg)	Control	–	0.054 ± 0.005	1.11 ± 0.57	–	0.348 ± 0.161
	Diabetic	–	NS	NS	–	–
Pentosidine (mmol/mol of Lys)	Control	–	0.0016 ± 0.0011	0.0174 ± 0.0054	0.0072 ± 0.0027	0.0025 ± 0.0009
	Diabetic	–	NS	NS	NS	NS
FL (mmol/mol of Lys)	Control	0.233 ± 0.015	0.72 ± 0.21	0.49 ± 0.09	0.515 ± 0.290	1.77 ± 0.36
	Diabetic	0.974 ± 0.098***	2.59 ± 1.23**	3.69 ± 0.72***	0.962 ± 0.305*	7.35 ± 1.59***
MetSO (mmol/mol of Met)	Control	4.05 ± 0.83	3.56 ± 0.86	19.9 ± 3.4	1.93 ± 0.53	3.71 ± 1.55
	Diabetic	NS	NS	NS	NS	NS

HD subjects, with respect to normal controls. The concentration of CEL residues in plasma protein was increased 2-fold in PD subjects and 1-fold in HD subjects, with respect to normal controls. The concentration of free CML was increased approx. 4-fold in PD subjects and 8-fold in HD subjects. The concentration of free CEL was increased more markedly than free CML, however, increasing approx. 10-fold in PD subjects and 22-fold in HD subjects. Both plasma protein CML and CEL residues were increased in ESRD, therefore, and represent the major proportion of total plasma CML and CEL moieties in plasma but the concentrations of free CML and CEL increased severely in ESRD (Table 5).

The concentrations of hydroimidazolone AGE residues in plasma protein were also increased in PD and HD subjects. G-H1, MG-H1 and 3DG-H were increased 88 %, 59 % and 280 % in PD subjects and 33 %, 54 % and 368 % in HD subjects, respectively. Free hydroimidazolones were increased markedly and severely in PD and HD subjects. G-H1, MG-H1 and 3DG-H were increased 2-fold, 16-fold and 7-fold in PD subjects and 4-fold, 50-fold and 10-fold in HD subjects (Figures 4a–4c).

The concentration of pentosidine residues in plasma protein was increased 180 % in PD subjects and 333 % in HD subjects; plasma protein pentosidine concentration was significantly higher in HD subjects than in PD subjects ($P < 0.001$; Figure 4d). The concentration of free pentosidine was not increased significantly in PD subjects but was increased 5-fold in HD subjects. The concentration of FL residues in plasma protein was decreased significantly in PD and HD subjects, with respect to normal controls. The concentration of free FL remained below the LOD in both PD and HD subjects – as indeed it was in normal healthy control subjects. MetSO residues in plasma protein were increased in PD and HD subjects. Both PD and HD subjects are known to suffer oxidative stress [23]. Dityrosine was below the LOD in all subjects. The concentration of 3-NO_2Tyr residues in plasma protein was increased 2-fold in PD and HD subjects. Free 3-NO_2Tyr was also increased but not significantly in PD and HD subjects. The concentrations of lysine, arginine and methionine in plasma of normal healthy controls were 30.1 ± 8.6 μM, 15.0 ± 3.9 μM and 3.36 ± 0.80 μM, respectively, and were not changed significantly in PD subjects. In HD subjects, the concentrations of plasma arginine and methionine were decreased: the arginine concentration was 9.7 ± 0.8 μM (−35 %, $P < 0.05$) and the methionine concentration was 2.17 ± 0.52 μM (−35 %, $P < 0.05$). Therefore, the concentration of MetSO (mmol/mol of Met) was increased in HD subjects, with respect to normal control (controls, 6.6 ± 2.0; HD subjects, 12.1 ± 2.6; $P < 0.01$).

DISCUSSION

Advanced glycation of cellular protein

Protein AGEs were detected previously by immunoassay [24–27]. Quantifying AGEs by immunoassay is beset with doubts over the reliability of the data obtained, however, because of severe antibody adsorption on to AGE epitopes in proteins used to block non-specific antibody binding, uncertain specificity of antibodies employed, and the use of highly modified standard antigens dissimilar to the minimally modified antigens in physiological samples. For these reasons, AGE detection by immunoassay does not usually provide AGE levels in absolute concentrations or amounts but rather in arbitrary units with or without normalization to a reference AGE protein standard [25]. LC-MS/MS provides data of absolute AGE quantification and indicated that there was significant AGE modification of cellular proteins.

The concentrations of hydroimidazolone AGEs were remarkably high; up to approx. 1 % of total arginine. The predominance of hydroimidazolone AGEs is characteristic of advanced glycation by α-oxoaldehydes that are arginine-directed glycating agents [18]. The hydroimidazolone concentration, in some instances, exceeded that of FL. Human proteasome data indicate that the mean number of arginine residues per protein is 26 [28]. Therefore, up to 26 % of cellular proteins may have a hydroimidazolone modification *in vivo*. Rapid degradation of cellular protein [29] suppresses this and is an expected important source of free hydroimidazolones detected in plasma and urine. G-H1,

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Table 5 Accumulation of AGE residues in proteins and free in plasma in renal failure

A dash indicates that the analyte was below the LOD. Significance: P and P' values are significance levels with respect to control and PD subjects, respectively.

Analyte	Normal controls	Peritoneal dialysis	Haemodialysis
Glycation adduct residues in plasma protein			
CML (mmol/mol of Lys)	0.029 ± 0.006	0.073 ± 0.032 ($P < 0.01$)	0.109 ± 0.009 ($P < 0.001$)
CEL (mmol/mol of Lys)	0.011 ± 0.005	0.031 ± 0.011 ($P < 0.01$)	0.022 ± 0.005 ($P < 0.05$)
G-H1 (mmol/mol of Arg)	0.057 ± 0.028	0.107 ± 0.049	0.076 ± 0.049
MG-H1 (mmol/mol of Arg)	0.921 ± 0.120	1.45 ± 0.50 ($P < 0.05$)	1.39 ± 0.16 ($P < 0.001$)
3DG-H (mmol/mol of Arg)	0.35 ± 0.08	1.33 ± 0.72 ($P < 0.05$)	1.64 ± 0.26 ($P < 0.001$)
MOLD (mmol/mol of Lys)	0.0008 ± 0.0002	0.0069 ± 0.0002 ($P < 0.01$)	0.0051 ± 0.0006 ($P < 0.001$)
Argpyrimidine (mmol/mol of Arg)	–	–	–
Pentosidine (mmol/mol of Lys)	0.0056 ± 0.0016	0.0157 ± 0.0028 ($P < 0.001$)	0.0243 ± 0.0039 ($P < 0.001, P' < 0.01$)
FL (mmol/mol of Lys)	0.767 ± 0.139	0.281 ± 0.254 ($P < 0.05$)	0.166 ± 0.040 ($P < 0.01$)
MetSO (mmol/mol of Met)	0.76 ± 0.15	1.18 ± 0.33 ($P < 0.05$)	1.42 ± 0.11 ($P < 0.001$)
3-NO_2Tyr (mmol/mol of Tyr)	0.0018 ± 0.0014	0.0056 ± 0.0026 ($P < 0.05$)	0.0051 ± 0.0016 ($P < 0.01$)
Free glycation adduct			
CML (nM)	23 ± 8	108 ± 48 ($P < 0.01$)	200 ± 35 ($P < 0.001$)
CEL (nM)	35 ± 14	388 ± 180 ($P < 0.01$)	817 ± 263 ($P < 0.001$)
G-H1 (nM)	50 ± 17	167 ± 64 ($P < 0.01$)	237 ± 38 ($P < 0.001$)
MG-H1 (nM)	110 ± 46	1876 ± 676 ($P < 0.01$)	5496 ± 1138 ($P < 0.001$)
3DG-H (nM)	147 ± 19	1137 ± 531 ($P < 0.01$)	1541 ± 520 ($P < 0.01$)
MOLD (nM)	15 ± 6	13 ± 8	14 ± 5
Argpyrimidine (nM)	47 ± 13	71 (3–259)	90 (23–501)
Pentosidine (nM)	0.84 ± 0.50	2.35 ± 1.66	4.91 ± 2.76 ($P < 0.01$)
FL (nM)	–	–	–
MetSO (nM)	22 ± 8	19 ± 4	26 ± 9
3-NO_2Tyr (nM)	6.2 ± 2.5	13.5 ± 8.6	10.7 ± 4.9

CML and CEL residue concentrations were 10–100-fold lower than MG-H1 and 3DG-H residue concentrations.

MetSO was an important oxidative modification of cellular protein. It may be reduced to methionine by cytosolic MetSO reductase or further oxidized to sulphone, methionine sulphone [30], although further oxidation is usually a slow process. Unless MetSO reductase activity is overwhelmed by severe oxidative insult, the concentration of MetSO may change little; overwhelming oxidative insult may convert MetSO to methionine sulphone and decrease levels of MetSO. Dityrosine, an unrepairable marker of oxidative damage to proteins, was only detected in RBCs. Haemoglobin appears to be particularly sensitive to dityrosine formation where there is a readily oxidized tyrosine residue [31].

AGE residues in proteins and free in plasma, urinary AGEs and renal clearance

AGEs are considered to be a class of uraemic toxin [32]. Free AGEs were found in blood plasma in the range 0.8 nM (pentosidine) to 147 nM (3DG-H). This represented generally a minor proportion ($\leqslant 6\%$) of total AGE moieties in blood plasma, except for MOLD and argpyrimidine. Most AGE moieties were normally present, therefore, as AGE residues in plasma protein. The AGEs found in highest concentration in cellular and extracellular protein, when liberated as free AGEs, have high renal clearances, although there may be some re-absorption and/or renal tubular metabolism of hydroimidazolones. This indicates that there is efficient renal clearance of the major AGEs in physiological systems. We were unable to detect the presence of low-molecular-mass peptides enriched in AGEs, AGE peptides, in either normal controls or subjects with ESRD despite analysing sufficient protein of < 12-kDa mass to expect to do so. The normal high efficiency of proteasomal processing of cellular protein and intestinal digestion of ingested protein suggests that significant AGE peptide accumulation is unlikely. Previous estimates may have mistaken the extraordinarily marked changes of free AGEs in uraemia for 'AGE peptides' (see below).

The estimates of 3-NO_2Tyr residues in plasma protein and free 3-NO_2Tyr in plasma were similar to those of recent artifact-free estimates (see for example [33]). MetSO and 3-NO_2Tyr both had low renal clearances (< 10 ml/min). This reflects the efficient renal reduction of MetSO by MetSO reductase [30] and renal re-uptake and/or metabolism of 3-NO_2Tyr [34].

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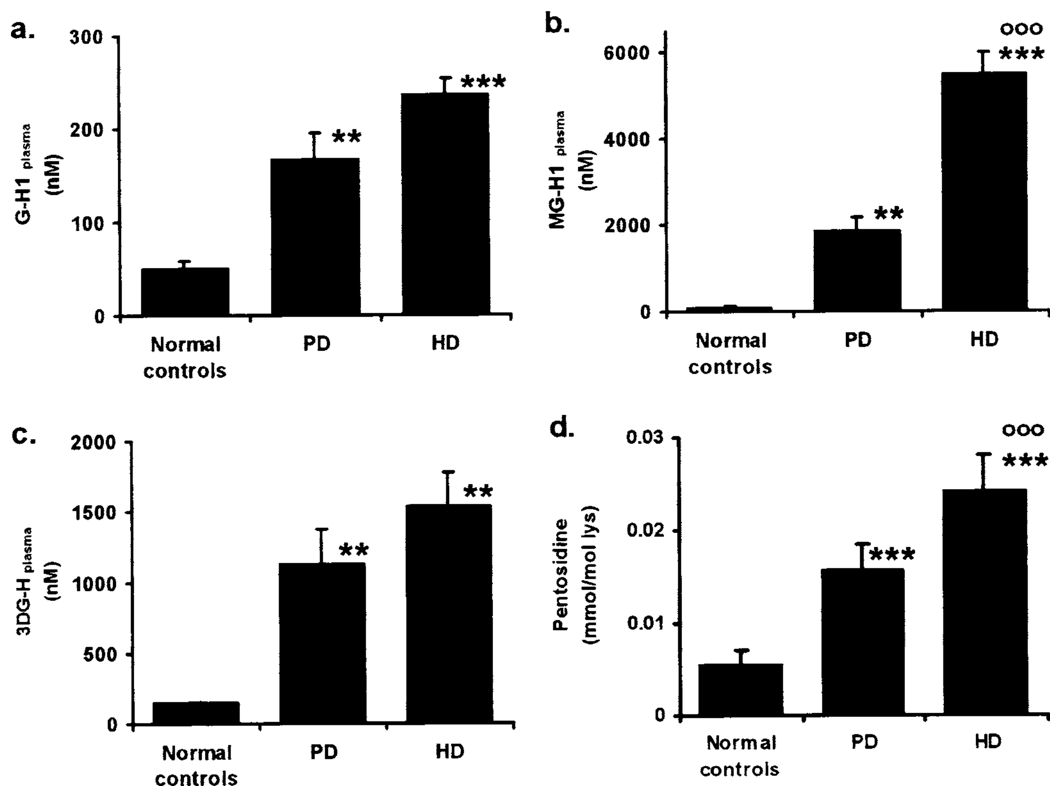

Figure 4 Accumulation of AGEs in uraemia

(**a**) G-H1, (**b**) MG-H1 and (**c**) 3DG-H free adducts in plasma and (**d**) pentosidine residues in plasma protein. **, *** and ○○, ○○○ indicate $P < 0.01$ and $P < 0.001$ with respect to normal controls and PD subjects respectively. Data are means ± S.E.M. ($n = 5$–6).

Concentrations of AGEs in renal glomeruli, retina, peripheral nerve and plasma protein of experimental diabetic rats and normal healthy controls

Herein, we presented the concentrations of AGEs in protein extracts of renal glomeruli, retina and sciatic nerve of normal control and diabetic rats. There were tissue-specific accumulations of AGE residues in diabetic rats: CML, CEL, MG-H1 and 3DG-H accumulated in renal glomeruli; CML, G-H1, MG-H1 and 3DG-H in the retina; CML, CEL, G-H1, MG-H1 and 3DG-H in the nerve; and CML, CEL, G-H1 and MG-H1 in plasma protein. The marked increases of FL residues at all these sites were suggested previously by immunoblotting detection [35]. This accumulation of AGE and FL residues may be linked to the development of retinopathy, nephropathy, neuropathy and generalized angiopathy. The STZ diabetic rats on insulin-maintenance therapy for 24 weeks developed incipient nephropathy [36] but they do not usually develop retinopathy or neuropathy in this period – more severe, untreated diabetes is required. The AGE accumulation is generally preceding the development of complications in this model of diabetes (including overt nephropathy), therefore, and AGEs may be causally linked to the development of complications rather than indicators of complications status. Surprisingly, there was no significant change in MetSO residue concentration in diabetic rats although some tissues had high levels of MetSO residues in the normal control rats – particularly the retina and nerve. Mild oxidative stress was present in the diabetic rats – plasma protein thiol concentration was decreased by 31 % with respect to controls [36] but it was not sufficient to overwhelm MetSO reductase activity. Therapeutic intervention with high-dose thiamine and Benfotiamine decreased the AGE accumulation and prevented diabetic nephropathy and retinopathy without change in FL [36,37]. Overall, the quantitative screening of glycation adducts in STZ diabetic rats was consistent with a role of advanced glycation in the development of vascular complications of diabetes.

Accumulation of plasma protein and free AGEs in renal failure

There were 1–7-fold increases of AGE residues in plasma proteins of PD subjects and similar increases in HD subjects except for the glycoxidation adduct pentosidine which was increased more markedly in HD than PD subjects. HD subjects suffer more pronounced oxidative stress than PD subjects [23]. Herein, the oxidative marker MetSO residues in plasma protein were increased by 55 % in PD subjects and 87 % in HD subjects, consistent with this. Pentosidine residue measurement in plasma protein, despite being of very low concentration, was confirmed as a sensitive marker of ESRD, particularly to glycoxidative processes. The concentration of FL in plasma protein was decreased in both PD and HD subjects. This may also be attributed to decreased stability of FL residues in oxidative stress where the oxidative degradation of FL contributes to the formation of CML [1].

The extraordinary new finding reported here is the marked accumulation of free AGEs in PD and HD subjects – particularly MG-H1 and 3DG-H that accumulated to micromolar levels. MG-H1 accumulated most markedly, attaining levels similar to those found in urine of normal control subjects. Free AGE accumulation

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probably originates mainly from the degradation of protein glycated endogenously and AGEs absorbed from food. The increased endogenous glycation in uraemia arising from increased α-oxoaldehydes will exacerbate this effect, particularly when glyoxalase I activity is low [6,38,39]. High levels of free AGEs were found in HD and PD dialysate [40] but lack of effective clearance of AGEs leads to their accumulation. It was surprising that FL did not accumulate in the plasma of PD and HD subjects given the substantial urinary excretion of FL in normal control subjects. FL is metabolized by the enzyme fructosamine-3-phosphokinase and is thereby converted to 3-deoxyglucosone [41]. This may account for the accumulation of 3-deoxyglucosone in uraemia and formation of related AGEs (3DG-H and others).

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