Glycated and Oxidized Protein Degradation Products Are Indicators of Fasting and Postprandial Hyperglycemia in Diabetes

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OBJECTIVE — To assess the relative importance of fasting and postprandial hyperglycemia to vascular dysfunction in diabetes, we have measured indicators of glycation, oxidative and nitrosative stress in subjects with type 1 diabetes, and different postprandial glucose patterns.

RESEARCH DESIGN AND METHODS — Plasma and urinary levels of specific arginine- and lysine-derived advanced glycation end products, as well as oxidative and nitrosative products, were measured by liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS) after 2 months of treatment with insulin lispro or human regular insulin in 21 subjects participating in a cross-over study. Hb-bound early glycation (Amadori) products were also measured after each treatment period by high-performance liquid chromatography (fructosyl-valine Hb or HbA_{1c} [A1C]:Diamat) and fructosyl-lysine Hb by LC-MS/MS (A1C: fructosyl-lysine).

RESULTS — In diabetic patients, the concentrations of protein glycation and oxidation-free adducts increased up to 10-fold, while urinary excretion increased up to 15-fold. Decreasing postprandial hyperglycemia with lispro gave 10–20% decreases of the major free glycation adducts, hydroimidazolones derived from methylglyoxal and 3-deoxyglucosone, and glyoxal-derived *N*ε-carboxymethyl-lysine. No differences were observed in A1C:Diamat or A1C: fructosyl-lysine with lispro or regular insulin therapy in spite of significant decreases in postprandial glycemia with lispro.

CONCLUSIONS — We conclude that the profound increases in proteolytic products of proteins modified by advanced glycation endproducts in diabetic patients are responsive to changes in mean hyperglycemia and also show responses to changes in postprandial hyperglycemia.

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Abbreviations: 3-DG, 3-deoxyglucosone; 3DG-H, 3-DG–derived hydroimidazolone *N*δ-(5-hydro-5-[2,3,4-trihydroxybutyl]-4-imidazolon-2-yl)ornithine; 3-NT, 3-nitrotyrosine; AGE, advanced glycation end product; CEL, *N*ε-carboxyethyl-lysine; CML, *N*ε-carboxymethyl-lysine; FPG, fasting plasma glucose; G-H1, glyoxal-derived hydroimidazolone *N*δ-(5-hydro-4-imidazolon-2-yl)ornithine; LC-MS/MS, liquid chromatography with triple quadrupole mass spectrometric detection; MetSo, methionine sulfoxide; MG-H1, methylglyoxal-derived hydroimidazolone *N*δ-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine; MPG, mean plasma glucose; NFK, *N*-formylkynurenine; PPG, postprandial glucose.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Vascular complications of diabetes, including retinopathy, neuropathy, nephropathy, and macrovascular disease, are the major cause of morbidity and mortality in diabetic patients, with macrovascular disease being a major cause of premature death (1,2). Dysfunction of the key cells responsible for vascular function, including endothelial cells, pericytes, and vascular smooth muscle cells, can be induced by increased cellular concentrations of glucose during hyperglycemia. This can activate multiple pathways of biochemical dysfunction (3,4) leading to increased glycation of proteins (5,6). Oxidative stress can also lead to cellular protein modification and damage and can be initiated by mitochondrial dysfunction, activation of vascular NADPH oxidase, and uncoupling of endothelial nitric oxide synthase (3,7,8).

The relative importance of fasting and postprandial hyperglycemia to vascular dysfunction in diabetes, including protein glycation and oxidative stress, remains controversial. In a number of prospective clinical trials, postprandial glucose (PPG) excursions have been linked to increased risk of mortality from cardiovascular disease (9–12), and decreasing PPG by intensive therapy with therapeutic agents such as the fast-acting insulin lispro or with oral hypoglycemic agents decreased the progression of retinopathy, neuropathy, and nephropathy in diabetic subjects (13,14). It is crucial, therefore, to quantify the contributions of increased fasting plasma glucose (FPG) and PPG to biochemical dysfunction linked to the development of macrovascular and microvascular complications of diabetes (15).

High plasma glucose concentrations in the postprandial periods can further increase the levels of glycation and oxidative damage to cellular and plasma proteins in diabetes. Glycation of proteins is a complex series of reactions where early-stage reactions lead to the formation of the early glycation adduct, fructosyllysine and NH_2-terminal fructosyl-amino acids, and later-stage reactions form ad-

vanced glycation end products (AGEs) (5). Fructosyl-lysine and its Schiff's base degrade slowly to form AGEs. Physiological α-dicarbonyls, including glyoxal, methylglyoxal, and 3-deoxyglucosone (3-DG), are also potent glycating agents that are formed by the degradation of glycated proteins, glycolytic intermediates, and lipid peroxidation and react with proteins to form AGEs directly. Important AGEs quantitatively are hydroimidazolones derived from arginine residues modified by glyoxal, methylglyoxal, and 3DG-*N*δ-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1), *N*δ-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine (MG-H1), and *N*δ-(5-hydro-5-[2,3,4-trihydroxybutyl]-4-imidazolon-2-yl)ornithine (3DG-H) and related structural isomers, respectively. Other important and widely studied lysine-derived AGEs are *N*ε-carboxymethyl-lysine (CML), *N*ε-carboxyethyl-lysine (CEL), and pentosidine (6). Major quantitative markers of oxidative damage to proteins are methionine sulfoxide (MetSO) and *N*-formylkynurenine (NFK), formed by the oxidation of methionine and tryptophan, respectively (16,17), and a widely studied marker of nitration damage to proteins is 3-nitrotyrosine (3-NT) (18). Detection of protein glycation, oxidation, and nitration adducts by liquid chromatography with triple quadrupole mass spectroscopy (LC-MS/MS) has been reported previously in the quantitative screening of protein glycation, oxidation, and nitration adducts, measured as adduct residues in proteins and free adducts in physiological fluids (6). In diabetes, protein glycation adducts residues have been shown to be increased in cytosolic proteins at sites of development of vascular complications including renal glomeruli, retina, and nerves (6,19). Cells maintain the quality and functional integrity of proteins by degradation and replacement of proteins damaged by oxidation and glycation (6,20). This occurs by proteolysis, liberating the oxdized, glycated, and nitrated amino acids as free adducts, which in turn are released into blood plasma and excreted in urine (6).

We have previously shown that decreasing postprandial hyperglycemia with insulin lispro therapy decreases postprandial glucose, methylglyoxal, and 3-DG levels, but not HbA_{1c} (A1C) (21) in subjects with type 1 diabetes, suggesting that specific AGEs related to these chemically reactive precursors may also be decreased. To investigate the impact of reducing postprandial levels of glucose and dicarbonyls on a number of related AGEs, we have investigated the levels of the arginine-derived AGEs G-H1, MG-H1, and 3DG-H, the lysine-derived AGEs CML and CEL, and the levels of oxidative products (3-NT, NFK, and MetSO) in subjects with lower postprandial glucose excursions secondary to insulin lispro therapy.

RESEARCH DESIGN AND METHODS

— The recruitment of diabetic patients for this study has been described previously (21). Briefly, 21 type 1 diabetic patients on multiple insulin injections were studied using a randomized cross-over design. There were 15 males and 6 females with mean age 40 ± 10 years and mean diabetes duration of 16.6 ± 9.4 years. Insulin lispro or biosynthetic human regular insulin (Humulin; Eli Lilly, Indianapolis, IN) combined with intermediate (NPH or Lente) or long-acting (Ultralente) insulin was given over an initial 2-month period before switching to the alternate rapid-acting insulin. Subjects performed self-monitoring of blood glucose and recorded their results on a daily basis. Glucose values were determined five times daily (before breakfast, lunch, and supper and 2 h post breakfast and supper) to facilitate adjustments of insulin dosage, and seven blood glucose readings per day (before each meal, 1 and 2 h after breakfast, and 1 and 2 h postsupper) were measured once weekly to assess long-term postprandial glucose excursions. Postprandial glycemic excursions are expressed as the mean of the sum of the difference between preprandial glucose values and the postbreakfast and postsupper plasma glucose values utilizing the seven blood glucose profiles during the 2nd month of each study period (21). Fasting glucose levels were determined for each subject as the mean of all recorded fasting glucose values. Mean glucose levels were calculated based on all of the glucose determinations on the days that seven values were determined during the 2nd month of each study period.

As described in our prior study, we also determined plasma levels of 3-DG, methylglyoxal, and the methylglyoxal metabolite, D-lactic acid, at the end of each 2-month study period in the fasting state and 1 and 2 h after a standard test meal. Their postprandial excursions were calculated based on fasting, 1-, and 2-h values. A1C was measured at the end of each 2-month study period during which the subject received insulin lispro or regular insulin. Twelve normal healthy control subjects were also studied: six men and six women with mean age 52 ± 12 years.

Blood and urine samples. Venous blood samples with heparin anticoagulant and 24-h urine collections were taken from diabetic patients and normal healthy control subjects. 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 before analysis. Urinary and plasma creatinine were determined by colorimetric assay (diagnostic kit 510; Sigma, St. Louis, MO). The study protocol conformed to the ethical guidelines of the latest Declaration of Helsinki and was approved by the local Committee for the Protection of Human Subjects. Informed consent was obtained from all participants.

Assessment of A1C. At baseline and at the end of each 2-month study period, A1C was measured by high-performance liquid chromatography (Diamat method; BioRad, Irvine, CA) in the clinical laboratory at the Dartmouth-Hitchcock Medical Center (22). This determines the percentage of Hb with a fructosyl-valine residue on the NH_2-terminus of the β-chain and will be referred to as A1C:Diamat. This accounts typically for 60% of fructosamine residues in Hb (23), the remaining 40% being fructosyl-lysine residues, mostly on lys-61 of the α-chain (24). Total fructosyl-lysine residues in Hb (and not fructosyl-valine) are determined by the LC-MS/MS technique described below and will be referred to as A1C: fructosyl-lysine.

Protein glycation, oxidation, and nitrosation-free adduct determination by LC-MS/MS. The following glycation adducts were determined: fructosyl-lysine, MG-derived AGEs (MG-H1, CEL, argpyrimidine, and methylglyoxal-derived lysine dimer), glyoxal-derived AGEs (G-H1 and CML), 3-DG–derived AGEs (3DG-H), and pentosidine. Protein oxidation adducts, MetSO, NFK, and dityrosine, the marker of nitrosative stress 3-NT, and amino acids lys, arg, tyr, met, and trp were also determined. Stable isotope standards for internal standardization were purchased and synthesized as described (6). Free glycation adducts were determined by assay of analytes in

Table 1—*Concentrations of protein glycation and oxidation-free adducts in plasma filtrate*

AGEs	Control	Regular	Lispro	*P* (lispro versus regular)
Fructosyl-lysine (nmmol/l)	96 ± 36	177 ± 84*	153 ± 58*	
CML (nmmol/l)	26.6 ± 9.0	113.6 ± 42.0†	97.1 ± 41.3†	<0.01 (−19%)
CEL (nmmol/l)	25.4 ± 13.6	77.7 ± 29.4†	72.2 ± 24.3†	
G-H1 (nmmol/l)	15.1 ± 8.7	41.3 ± 17.9†	29.5 ± 9.7†	
MG-H1 (nmmol/l)	43 ± 18	421 ± 238†	331 ± 161†	
3DG-H (nmmol/l)	51 ± 15	122 ± 50†	106 ± 54†	<0.01 (−23%)
MetSO (nmmol/l)	94 ± 22	491 ± 145†	497 ± 177†	

Data are means ± SD for diabetic patients ($n = 21$). *P* = significance of the differences between the mean for diabetic patients with regular insulin or lispro therapy. Where significant, percentage reversal of the increase in diabetes with respect to control values are given. Plasma-free adduct levels of argpyrimidine, methylglyoxal-derived lysine dimer, pentosidine, NFK, 3-NT, and dityrosine were below line of distinction. *P* values relative to control subjects: * and † indicate significance at the 1 and 0.1 % levels, respectively. Data on control and regular insulin therapy were previously reported in ref. 39.

ultrafiltrate (12-kDa filter cutoff, 50 μl aliquot) of plasma and urine. Analytes released by self-digestion of proteases in assay blanks were subtracted from analyte estimates.

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.) as described (6). The only modification was a change of acetonitrile gradient (linear gradient of 10–50% acetonitrile from 0 to 15 min and isocratic 50% acetonitrile thereafter) for the detection of argpyrimidine, pentosidine, dityrosine, and 3-NT. Pentosidine, NFK, and trp were determined by liquid chromatography with fluorimetric detection using a Waters 2475 multichannel fluorescence detector (Waters-Micromass). The mobile phase was 0.1% trifluoroacetic acid with isocratic 10% acetonitrile from 0 to 20 min and then a linear gradient of 10–50% acetonitrile from 20 to 50 min eluted through column one only at a flow rate of 0.4 ml/min. The retention time, detection excitation and emission wavelengths ($\lambda_{ex}/\lambda_{em}$), and limits of detection for the analytes were pentosidine 25.7 min, 320/385 nm, and 6 fmol; NFK 43.7 min, 330/437 nm, and 13 pmol; and tryptophan 46.2 min, 286/400 nm, and 29 pmol. Authentic standard pentosidine and NFK were prepared as described (6,25).

Statistical analysis. Significance of difference between means and medians of diabetic patients on regular insulin and lispro therapy were assessed by a paired *t* test and Wilcoxon signed rank, respectively. Correlation analysis was performed by calculating Spearman's ρ statistic.

RESULTS

The impact of decreased postmeal glucose excursions from insulin lispro treatment, on glycation, oxidation, and nitration-free adduct levels in plasma

The concentration of fructosyl-lysine–free adduct in plasma was decreased by lispro therapy, relative to regular insulin, although this change did not achieve statistical significance. The concentration of CML-free adduct was decreased significantly (19%) by lispro therapy (Table 1), however, whereas CEL-free adduct concentration was decreased 11% by lispro therapy, and this decrease was not statistically significant. The increase in 3DG-H–free adduct concentration seen in diabetes was significantly reversed (by 23%) by lispro therapy (Table 1), and the concentrations of G-H1– and MG-H1–free adducts were decreased by 29 and 24%, respectively, although these changes did not quite achieve statistical significance (Table 1). The MetSO-free adduct level in plasma was not changed significantly by the switch to lispro therapy (Table 1).

The impact of decreased postmeal glucose excursions from insulin lispro treatment on glycation, oxidation, and nitration-free adduct excretion in urine

Urinary excretion of fructosyl-lysine–free adducts was not changed significantly by the switch to lispro therapy. The urinary excretion of CML-free adducts in diabetes was significantly reversed (19%) by lispro therapy, however, and the urinary excretion of CEL was reversed by 42% by the switch to lispro therapy, although this change did not quite achieve significance (Table 2). The urinary excretion of G-H1 was not changed significantly by lispro therapy. The urinary excretions of MG-H1– and 3DG-H–free adducts were significantly decreased (13 and 19%) by the switch to lispro therapy. Argpyrimidine, methylglyoxal-derived lysine dimer, pentosidine, NFK, 3-NT, and dityrosine-free adducts in urine were not significantly decreased by lispro therapy.

Renal clearance of protein glycation and oxidation-free adducts of diabetic subjects treated with insulin lispro or regular insulin

The renal clearance of creatinine in the diabetic patients in this study was not significantly different from normal healthy control subjects. The only difference observed between therapy with lispro and regular insulin was that lispro increased the renal clearance of CML significantly ($P < 0.01$), reversing the decrease in clearance of CML found in diabetes by 11%. Control renal clearances of analytes are from Thornalley et al. (6).

Fractional excretion of protein glycation and oxidation-free adducts were deduced to assess the efficiency of renal elimination. Extreme values of fractional excretions were for fructosyl-lysine (>100%) and MetSO (<1%). The efficiency of renal elimination in diabetes was increased for MG-H1– and 3DG-H–free adducts and decreased for CML, CEL, and G-H1 (regular insulin therapy only). Therapy with lispro in diabetes increased the efficiency of renal elimination of CML-free adduct.

Levels of standard indicators of glycemic control associated with regular and lispro therapy. We investigated the levels of five indicators of glycemic control (A1C determined by the Diamat method [A1C:Diamat] and fructosyl-lysine in Hb [A1C:fructosyl-lysine], mean FPG, mean overall plasma glucose values [MPG], and mean PPG excursions) achieved in subjects on regular insulin or lispro treatment. As is seen in Table 3, no significant difference was observed in levels of A1C:Diamat, A1C:fructosyl-lysine, or mean fasting glucose in the face of highly significant differences in mean PPG levels and

Table 2—*Urinary excretion of protein glycation, oxidation, and nitration-free adducts*

Analyte	Control	Regular	Lispro	*P* value (lispro versus regular)
Fructosyl-lysine	4.3 ± 2.6	22.3 ± 7.9‡	21.6 ± 8.1‡	
CML	1.19 ± 0.53	2.56 ± 1.37‡	2.30 ± 0.91‡	<0.05 (−19%)
CEL	2.64 ± 1.10	3.26 ± 1.80	3.00 ± 1.14	
G-H1	1.54 ± 0.59	1.22 ± 0.39	1.16 ± 0.45*	
MG-H1	2.3 ± 1.2	34.8 ± 24.9‡	30.6 ± 13.0‡	<0.05 (−13%)
3DG-H	4.40 ± 1.82	8.33 ± 1.52‡	7.60 ± 2.29‡	<0.05 (−19%)
Arg-pyrimidine	0.70 ± 0.40	0.80 [0.16–5.18]	0.88 [0.13–7.20]	
Methylglyoxal-derived lysine dimer	0.027 ± 0.011	0.040 ± 0.014†	0.045 ± 0.015†	
Pentosidine	0.022 ± 0.014	0.118 [0.033–0.654]‡	0.108 [0.005–0.336]‡	
MetSO	0.018 ± 0.009	0.080 ± 0.035‡	0.070 ± 0.029‡	
NFK	3.6 ± 1.7	20.3 ± 10.8‡	26.6 ± 16.2‡	
3-NT	0.034 ± 0.014	0.036 ± 0.018	0.047 ± 0.024	
Dityrosine	0.186 ± 0.086	0.267 ± 0.066†	0.247 ± 0.059*	
Arg	3.0 ± 1.8	19.0 ± 11.2‡	14.6 ± 6.4‡	
Lys	60.6 ± 30.6	98.4 ± 38.9*	102.4 ± 54.7*	
Met	5.0 ± 2.3	35.3 ± 13.8‡	34.6 ± 14.2‡	
Tyr	44.8 ± 25.4	87.3 ± 45.1†	86.7 ± 42.8†	
Trp	16.0 ± 11.2	31.7 ± 10.5†	28.7 ± 13.2*	
Fractional excretion of free adducts (%)§				
Fructosyl-lysine	61 ± 4	137 ± 13†	126 ± 16†	
CML	77 ± 7	15 ± 2‡	20 ± 2‡	<0.01 (+33%)
CEL	85 ± 15	35 ± 4‡	37 ± 3‡	
G-H1	55 ± 9	25 ± 4†	43 ± 7	
MG-H1	41 ± 7	79 ± 11*	102 ± 19*	
3DG-H	38 ± 5	63 ± 6*	69 ± 6†	
MetSO	2.80 ± 0.53	0.15 ± 0.02‡	0.13 ± 0.02‡	

Data are means ± SD for diabetic patients (n = 21). Values are nmol/mg creatinine. P = significance of the differences between the mean for diabetic patients with regular insulin or lispro therapy. Where significant, percentage reversal of the increase in diabetes with respect to control values are given. P values relative to controls: *, †, and ‡ indicate significance at the 5, 1, and 0.1 % levels, respectively. §Data are means ± SE. Fractional excretion of analyte was calculated as follows: ([analyte]$_{urine}$ [nmmol/l] × 24-h urine volume) × 100/([analyte]$_{plasma}$ [nmmol/l] × glomerular filtration rate [ml/min] × 1440). Control and regular excretion data were reported previously in ref. 39.

modest decreases in overall mean glucose levels with lispro therapy.

Correlations between indicators of glycemic control and plasma and urinary levels of free adducts in diabetic subjects

We investigated correlations between three indicators of glycemic control (FPG, MPG, and mean PPG excursions) and glycation, oxidation, and nitration residues–free adducts in diabetic subjects, and several correlations were observed (Table 3). Highly significant positive correlations were found between FPG and MPG and the plasma concentration of fructosyl-lysine–free adduct, while a significant negative correlation was found between PPG and fructosyl-lysine–free adduct. The concentration of MG-H1– and CEL–free adduct in plasma also showed a significant correlation with FPG, and both of these products just failed to reach significance with MPG. Plasma levels of 3DG-

Table 3—*Levels of standard indicators of glycemic control associated with regular and lispro therapy and their correlations with A1C and glycation, oxidation, and nitration-free adducts*

Insulin therapy	Regular	Lispro	*P**
A1C:fructosyl-lysine	8.63 ± 3.38	7.89 ± 3.43	
A1C:Diamat	7.83 ± 0.86	7.59 ± 0.90	
FPG	178 ± 68	193 ± 79	
MPG	175 ± 39	153 ± 27	<0.05
PPG excursion	98 ± 108	−45 ± 135	<0.001
Standard glycemic indicators	Correlation with plasma-free adducts and A1C§		
FPG	Fructosyl-lysine (0.55)‡, MG-H1 (0.37)*, CEL (0.33)*		
MPG	A1C:Diamat (0.34)*, A1C: fructosyl-lysine (0.64)‡, fructosyl-lysine (0.54)‡		
PPG excursion	3DG-H (0.35, P = 0.02), FL (−0.43)†		
	Urinary excretion of free adducts		
MPG	Fructosyl-lysine (0.44)†, 3-NT (−0.35)*, dityrosine (0.60)‡,		
PPG excursions	3-NT (−0.35)*		

Data are means ± SD for diabetic patients (n = 21). P = significance of the mean with respect to diabetic patients with regular insulin and lispro therapy, respectively. §Correlation coefficients (Spearman's ρ) are given in parentheses. *, †, and ‡ indicate significance at the 5, 1, and 0.1% level, respectively.

H–free adducts also showed a significant correlation with PPG ($P = 0.02$).

MPG also correlated with 24-h urinary excretion of fructosyl-lysine–free adduct, as well as with dityrosine, while a significant negative correlation was seen between MPG and 3-NT (Table 3). PPG also showed a significant negative correlation with excretion of urinary-free 3-NT (Table 3).

Correlations between indicators of glycemic control and A1C in diabetic subjects

As shown in Table 3, we observed statistically significant relationships between A1C (Diamat and fructosyl-lysine) with MPG, while mean PPG showed no such relationship (*P* values of 0.73 and 0.27) for A1C Diamat and fructosyl-lysine, respectively.

CONCLUSIONS — Protein glycation was viewed originally as a posttranslational modification that accumulated mostly on extracellular proteins. AGEs were thought to be formed slowly throughout life, and the concentrations of AGEs found represent a life-long accumulation of the glycation adducts. This applies to chemically stable AGEs formed on long-lived proteins. However, fructosyl-lysine and some AGEs (hydroimidazolones for example) have relatively shorter chemical half-lives under physiological conditions (2–6 weeks), and they may also be formed on cellular and short-lived extracellular proteins. The concentrations of these glycation adducts, therefore, reflect the rate of the respective glycation process in the 2–6 weeks before sampling. Proteins modified by glycation, oxidation, and nitration are degraded by cellular proteolysis, releasing free protein glycation, oxidation, and nitration glycation adducts (6,20).

The switch from regular insulin to lispro therapy of type 1 diabetic patients and the associated decrease in PPG produced significant decreases in protein glycation–free adducts of MG-H1, 3DG-H, and CML in plasma and urine. This confirms that decreasing postprandial glucose concentrations, as well as the previously observed decreases in methylglyoxal and 3-DG levels by therapy with lispro, can also decrease the levels of their specific AGEs (21). In this study population, we previously observed that the mean postprandial excursions of glucose, methylglyoxal, and 3-DG were ~55, 15, and 13% of the corresponding fasting concentrations. The limited time period over which these elevations occur in the postprandial period may explain why the diabetes-associated increases in AGEs derived from these glycating agents were reversed by only 10–20% with lispro therapy. The observed reduction in AGEs with lispro treatment appears to relate to the decreased area under the curve for the meal-related variation of methylglyoxal, 3-DG, and glycoxidation (assuming oxidative degradation of fructosyl-lysine is the major precursor of CML in vivo). The modest changes of protein glycation and glycoxidation adducts found herein, therefore, are in keeping with the extent to which therapy with lispro decreases daily glucose exposure in our study. The hydroimidazolones MG-H1 and 3DG-H emerged as sensitive indicators of this because the concentrations of their precursors increase rapidly (21) in response to increased glucose concentration. Hydroimidazolones are also the major quantitative glycation adducts formed from these precursors, with diabetes-associated MG-H1–free adduct concentrations achieving levels 3- to 10-fold higher than other free AGE adducts, as shown in the present and prior studies (26,39). CML probably emerged as an indicator sensitive to PPG because oxidative stress enhanced in the postprandial period increased the rate of degradation of fructosyl-lysine to CML. Although we observed a strong relationship between urinary levels of dityrosine and mean glucose levels, the limited data on oxidative stress in this study, including measurement of 3-NT, dityrosine, and MetSO, did not confirm this hypothesis, although many other independent studies have shown that postprandial hyperglycemia and hyperlipidemia enhances oxidative stress (27–31). These findings are in contrast to the relative insensitivity of the Amadori product, represented by fructosyl-lysine residues of Hb or A1C (Nα-fructosyl-valine + fructosyl-lysine residues), which were not significantly decreased by lispro treatment (Table 3 and [21]). It is of interest that most other conventional markers of glycemic control were not sensitive to significant decreases in postprandial glycemia. These include markers such as mean fasting blood glucose levels as well as A1C:Diamat and A1C:fructosyl-lysine, all of which were unchanged by reducing glucose levels in the postprandial state. We did observe a modest change in mean daily glucose concentrations in association with lispro therapy however, although this was not sufficient to decrease A1C levels.

We did observe significant correlations between FPG and MPG levels and fructosyl-lysine–free adducts in plasma and urine and MPG with A1C:fructosyl-lysine and A1C:Diamat, confirming that the formation of Amadori products are strongly related to overall glycemia. Fasting glucose levels also correlated with MG-HI and CEL, demonstrating that methylglyoxal-related AGEs are indicators of mean blood glucose as well as postprandial glycemia. The only plasma AGE–free adduct that correlated with PPG was 3DG-H, while plasma fructosyl-lysine–free adduct demonstrated a significant negative relationship with PPG, confirming that it is an insensitive marker for postprandial glucose fluctuations.

The fractional excretion of fructosyl-lysine–free adduct was >100% in diabetic patients. In diabetes, there may be increased fructosyl-lysine–free adduct released by tubular epithelial cell proteolysis of protein in the glomerular filtrate (32). In contrast, the fractional excretion of MetSO was very low, reflecting the efficient conversion of MetSO to methionine by methionine sulfoxide reductase in the kidney (33). Lispro therapy increased the fractional excretion of CML-free adduct in diabetes, suggesting that the decreased urinary excretion of CML-free adduct was due to decreased formation of CML-free adduct rather than a decline in the efficiency of excretion.

Although the effect of decreasing postprandial hyperglycemia on protein glycation was modest, this study demonstrates that some AGEs, unlike fructosamines, are responsive to control of PPG. Since we have shown that some of the largest increases of protein glycation–free adducts in diabetes occurs for plasma concentration and urinary excretion of CML, MG-H1, and 3DG-H, it is not surprising that these glycation-related variables are most sensitive to change in glycemic control with lispro therapy. It was somewhat surprising, however, that lispro produced significant decreases in plasma and urinary excretion of glycation-free adducts but no significant reduction of glycation adduct residues of plasma protein or Hb. The latter observation could be related to our prior observation that increases in AGE residues in Hb tended to be modest in diabetes, possibly secondary to protection and repair of Hb from glycation damage by glyoxalase I and fructosamine 3-phosphokinase activities in erythrocytes (34–36). Plasma proteins damaged by AGE forma-

tion may suffer preferential cellular proteolysis and hence the modest changes in glycation damage are more readily detected in proteolysis products than in glycation adduct residues of plasma protein. The changes observed in this study also occurred in a relatively short 2-month time period, and longer periods of reduction in postprandial glucose levels could be associated with greater changes in AGE levels. In recent studies we have found that postprandial methylglyoxal levels are significantly decreased by treatment with nateglinide in type 2 diabetes (37), suggesting that decreases in methylglyoxal-derived AGEs may also occur with other treatments targeting postprandial glycemia. Since postprandial increases in α,β-dicarbonyls and their associated AGEs can lead to direct cellular damage, decreasing their levels with lispro as well as decreasing postprandial oxidative lipemia and oxidative stress (27–29,31,38) could potentially lead to a reduction in long-term vascular complications.

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