Increased Serum Levels of the Specific AGE-Compound Methylglyoxal-Derived Hydroimidazolone in Patients With Type 2 Diabetes

B.K. Kilhovd, I. Giardino, P.A. Torjesen, K.I. Birkeland, T.J. Berg, P.J Thornalley, M. Brownlee, and K.F. Hanssen

A time-delayed fluorescence immunoassay was developed for the determination of serum levels of methylglyoxal (MG)-derived hydroimidazolone using a monoclonal antiserum raised against Nα-acetyl-Nδ-(5-hydro-5-methyl)-4-imidazolone, Europium-labeled anti-mouse IgG antiserum as indicator, and MG modified bovine serum albumin (BSA) as standard. Serum levels of hydroimidazolone were measured in 45 patients with type 2 diabetes aged 59.4 ± 6.1 (mean ± SD) years and with duration of diabetes of 7.3 ± 3.1 years, and in 19 nondiabetic controls aged 56.3 ± 4.3 years. The serum levels of hydroimidazolone were significantly higher in patients compared to controls: median, 3.0 (5-95 percentile, 1.6 to 5.4) U/mg protein versus 1.9 (1.2 to 2.8) U/mg protein ($P = .0005$). Significant positive correlations were observed between the serum levels of hydroimidazolone and serum levels of advanced glycation end products (AGEs), measured with a polyclonal anti-AGE antibody: $r = 0.59$ for patients ($P < .0001$), and $r = 0.65$ for controls ($P = .002$). Similarly, significant correlations were also found between serum levels of hydroimidazolone and N^{ϵ}-(carboxymethyl)-lysine (CML): $r = 0.36$ in patients and $r = 0.55$ for controls (both $P = .02$). Serum hydroimidazolone levels did not correlate with fasting plasma glucose or hemoglobin A_{1c} (HbA_{1c}) levels. The observed differences between patients with diabetes and nondiabetic controls seem to be comparable to differences measured for other AGE compounds.

CHRONIC HYPERGLYCEMIA leading to tissue damage is the primary etiological factor in the pathogenesis of diabetic microvascular disease,[1-4] and may contribute to macrovascular complications.[5,6] Hyperglycemia-induced tissue damage in diabetes may occur through different mechanisms. One putative mechanism is the formation of advanced glycation end products (AGEs). Different AGE compounds have been characterized, and some have been associated with diabetic late complications.[2,7-12]

Recently, there has been increasing focus on AGEs formed from biologically reactive dicarbonyls such as methylglyoxal (MG). MG is the most reactive AGE precursor by at least an order of magnitude, and the principal AGE precursor elevated in endothelial cells cultured in high-glucose media.[13] It is present in all biological systems,[14] the levels are increased in diabetes, and it has been linked to diabetic late complications.[15] MG is formed by nonenzymatic elimination of phosphate from the triosephosphates glyceraldehyde-3-phosphate and glycerone phosphate, as well as enzymatically from dihydroxyacetone phosphate by MG synthase.[16,17] It is also formed in ketone body metabolism from acetone, and in the metabolism of threonine.[18,19] MG reacts reversibly with arginine, lysine, and cysteine residues in proteins.[20] Further irreversible reactions utilize lysine to form glycosylamine protein crosslinks,[21] and arginine residues to form imidazolone derivatives,[22] of which some are fluorescent. MG is detoxified by the glyoxalase system which catalyses the conversion to D-lactate[23] using reduced glutathione as an essential cofactor. The AGE inhibitor aminoguanidine reacts mainly with dicarbonyl AGE precursors such as MG.[24] MG modification of proteins is also of interest due to individual variations in the detoxification enzyme glyoxalase.

An antibody against Nα-acetyl-Nδ-(5-hydro-5-methyl)-4-imidazolone, a MG-modified arginine compound (hydroimidazolone) has recently been raised. Using this antibody, Giardino et al have shown increased intracellular hydroimidazolone immunoreactivity in glomeruli from patients with diabetes.[25]

The aim of the present work was to develop an immunoassay for the measurement of MG-derived hydroimidazolone in human sera, and apply the assay to investigate whether the serum levels of hydroimidazolone in patients with type 2 diabetes differed from the levels in nondiabetic controls. We also wanted to study whether the serum levels of hydroimidazolone were related to the levels of other AGE compounds, such as AGEs measured with polyclonal anti-AGE antibodies and N^{ϵ}-(carboxymethyl)-lysine (CML). Furthermore, we aimed to examine possible correlations with indices of glycemic control, such as fasting plasma glucose and hemoglobin A_{1c} (HbA_{1c}).

MATERIALS AND METHODS

Methods

Preparation of MG-Modified Bovine Serum Albumin

Bovine serum albumin (BSA) was incubated in sodium phosphate buffer (100 mmol/L, pH 7.4, at 37°C) with 1 mmol/L ultra pure MG, provided by Paul J. Thornalley, for 4 days and then dialyzed against ammonium bicarbonate buffer (30 mmol/L, pH 7.9, at 4°C) and lyophilized to dryness. Lyophilized MG-modified BSA preparations were stored at −196°C.

This low-modified MG-BSA contained 17.8 (23%) modified arginine residues per molecule of serum albumin as determined by high-performance liquid chromatography (HPLC) amino acid analysis.[26]

From the Aker Diabetes Research Centre and the Hormone Laboratory, Aker University Hospital, Oslo, Norway; Albert Einstein College of Medicine, New York, NY; and the University of Essex, Essex, UK.

Submitted February 20, 2002; accepted September 3, 2002.

Supported by grants from the Norwegian Foundation for Health and Rehabilitation, Aker Diabetes Research Fund, and the Norwegian Diabetes Association, and in part by a grant from the National Institutes of Health, Bethesda, MD.

Address reprint requeststo Bente K. Kilhovd, MD, Aker Diabetes Research Centre, Aker University Hospital, N-0514 Oslo, Norway.

0026-0495/03/5202-0015$35.00/0

doi:10.1053/meta.2003.50035

Preparation of Monoclonal Anti–MG-Modified Arginine (anti-hydroimidazolone) Antibodies

Balb/c mice were injected with keyhole limpet hemocyanin (KHL) modified by incubating with 70 mmol/L of MG for 6 hours at 37°C. Culture supernatant from hybrid cells was screened by an indirect enzyme-linked immunosorbent assay (ELISA), and one anti–MG-AGE antibody-producing clone (IG7) was selected for further characterization. Epitope specificity was evaluated using both dot blots and an indirect competitive ELISA. The IG7 antibody reacted specifically with Nα-acetyl-Nδ-(5-hydro-5-methyl)-4-imidazolone, showed 1% cross-reaction against its oxidized form methyl-imidazolone, and to some extent recognized the analog glyoxal-derived arginine hydroimidazolone compound (data not shown). It did not react with Nα-acetyl-argpyrimidine, bis(Nα-acetyl)lys-4-methyl-imidazolium chloride or carboxyethyllysine. The IG7 antibody did not cross-react with the imidazolones produced by the reaction of 3-deoxyglucosone with arginine.[25] This shows that the antibody relatively specifically recognizes methylglyoxal-induced modifications of arginine residues. A report detailing the characterization of the antibody is in preparation.

Production of AGE-BSA and Polyclonal Anti–AGE-Modified Bovine Pancreatic Ribonuclease (RNase) Antibodies and CML-BSA and Monoclonal Anti-CML Antibodies

AGE-BSA and polyclonal anti–AGE-RNase antibodies were produced according to Makita et al.[27] The anti–AGE-RNase antibodies were a gift from Richard Bucala, The Picower Institute for Medical Research, New York, NY. CML-BSA and monoclonal anti-CML antibodies were a gift from Jes Clausen, Novo Nordisk AS, Bagsvaerd, Denmark. The CML-BSA preparation contained 20 CML-modified lysine residues per molecule of albumin corresponding to 34% of the available lysine residues.[28] Glycated BSA was purchased from Sigma, Stockholm, Sweden (Cat. no A8426).

DELFIA Immunoassays

Serum hydroimidazolone assay. Twelve-well microtiter strips (Pharmacia, Uppsala, Sweden, Cat. no 1244-550) were coated with 0.1 mL of MG-modified BSA (25 μg/mL) diluted in 0.05 mol/L carbonate buffer, pH 9.8, covered, and incubated overnight while shaking at room temperature. The strips were then kept at 4°C until washing. Immediately prior to assaying, the wells were washed 6 times in DELFIA washing solution (Wallac, Turku, Finland, Cat. no B117-100). Triplicates of 100 μL MG-modified BSA standard or serum (diluted 1/8) were added to each well together with 50 μL of anti-hydroimidazolone (IG7) antiserum diluted 1/5,000 in DELFIA assay buffer (Wallac, Cat. no 1244-106).

Seven standard solutions—0, 2.5, 5, 10, 20, 40, and 100 μg/mL—of MG-modified BSA were used in each assay. The strips were incubated while shaking in room temperature for 2 hours, and then washed 6 times in washing buffer. A quantity of 100 μL/well of Europium-labeled anti-mouse IgG antibodies (Wallac, Cat. no. 1244-130) weas then added in a final concentration of 0.1 μg/mL in DELFIA assay buffer. The strips were incubated while shaking for 1 hour at room temperature. Subsequently they were washed 6 times, and finally incubated for 5 minutes while shaking with DELFIA Enhancement solution (Wallac, Cat. no. 1244-105) prior to measurement of the Europium-ion chelate-specific fluorescence in a 1232 DELFIA Fluorometer (Wallac).

One hydroimidazolone unit was defined as the competitive activity of 1 μg of MG-modified BSA standard. The serum concentration of hydroimidazolone was adjusted for the total protein concentration in each sample, and is expressed as U/mg protein. In this way, possible systematic errors due to differences in protein content between groups were avoided.

Serum AGEs were measured by the polyclonal antibody according to the method described by Berg et al.[11] One AGE unit was defined according to Makita et al[27] as the competitive activity of 1 μg of AGE-BSA standard. The final serum concentrations of AGE were adjusted for total protein concentration according to Berg et al[11]: 1 U/mL adjusted for total protein = 1 U/mL × (sample protein concentration/mean protein concentration of all samples measured).

All serum samples were analyzed in the same run, and the intra-assay coefficient of variation (CV) was less than 12%. The polyclonal anti-AGE antibody did not react with MG-modified BSA in our assay.

Serum CML was measured according to a recently developed immunoassay[29] by using monoclonal anti-CML antibodies as detecting antibodies and CML-BSA as standard. Europium-labeled anti-mouse IgG antibodies were used as indicators. Results are expressed as CML units (1 U = 1 μg/mL CML-BSA standard) and are adjusted for total protein concentration in the same manner as for AGE. All analyses were performed in one run, and the intra-assay CV was less than 12%. The cross-reactivity with MG-modified BSA was 3% when calculated as MG-modified BSA protein against CML-BSA.

Other Analyses

Total protein concentrations in each serum sample was determined by the Biuret method (Boehringer, Mannheim, Mannheim, Germany) with a CV less than 2%. HbA_{1c} was analyzed using a HPLC method (Diamat analyzer, Bio-Rad, Richmond, CA), which has a normal range of 4.2% to 6.1% and an inter-assay CV of less than 3%. Fasting plasma glucose was measured by the glucose oxidase method using a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured enzymatically (Boehringer Ingelheim/Boehringer Mannheim, Germany), and low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula.[30]

Subjects

Forty-five patients with type 2 diabetes (19 women and 26 men), mean age 59.4 ± SD 6.1 years and duration of diabetes 7.3 ± 3.1 years included in the OCTOPUS study[31] were examined at baseline. All patients were treated with sulfonylurea and/or metformin. The mean HbA_{1c} was 8.6% ± 1.3%, mean fasting plasma glucose was 11.1 ± 2.8 mmol/L, and average HbA_{1c} in the last year before samples were drawn was 8.5% ± 1.1%. The patients had median total cholesterol levels of 6.4 (5-95 percentile, 4.9 to 9.2) mmol/L, triglycerides 1.72 (0.52 to 5.03) mmol/L, HDL cholesterol 1.15 (0.72 to 2.00) mmol/L, and LDL cholesterol 4.09 (2.92 to 6.92) mmol/L. None of the patients had renal failure. Nine of the 45 patients had microalbuminuria defined as a urinary albumin excretion rate between 20 and 200 μg/min in 2 of 3 overnight timed urine samples collected within 6 months prior to the investigation. Nineteen healthy blood donors (13 women and 6 men), mean age 56.3 ± 4.2 years, were used as controls. Blood samples were drawn in the fasting state, and stored at −40°C until assayed.

Statistical Analysis

Comparisons between groups were analyzed by the Mann-Whitney *U* test. Univariate correlations were tested with the use of Spearman correlations, as data are not normally distributed. Data given are medians (5-95 percentiles), except where otherwise stated. The 2-sided significance level was 5%; exact *P* values are given. Calculations were performed using SPSS version 9.0 (SPSS Inc, Chicago, IL) and intra-assay CV was calculated using the MedCalc version 5.00 (MedCalc Software, Mariakerke, Belgium).

RESULTS

Evaluations of the Hydroimidazolone Immunoassay

Dose-Response Curve

The fluorescence counts of the typical inhibition curve varied from 140,000 (no standard dose added) to 30,000 (100 μg/mL). The curve is depicted in Fig 1.

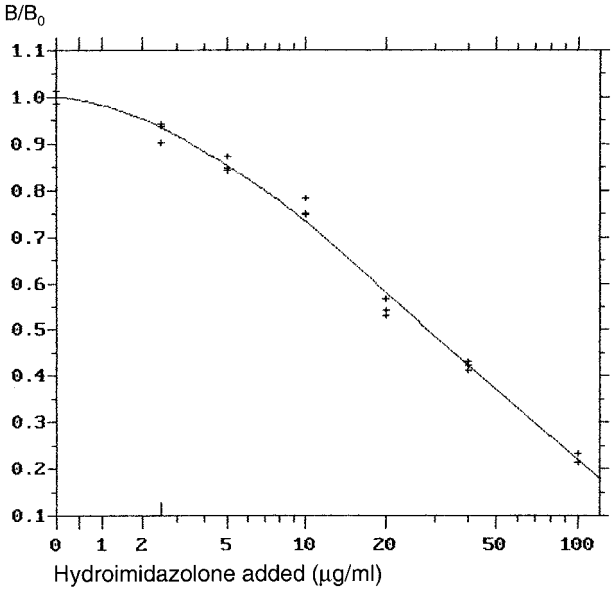

Fig 1. Dose-response curve for the immunoassay of hydroimidazolone. B_0 is defined as the fluorescence count without added MG-BSA standard. B represents fluorescence in the presence of added hydroimidazolone from standard or sample (mean of triplicate determinations).

Sensitivity

The mean least detectable concentration of hydroimidazolone, defined as twice the standard deviation of maximum binding, was 2.5 μg/mL.

Precision

Intra-assay CV of serum measurements varied between 9% and 15% depending on the hydroimidazolone content of the samples. Inter-assay CV was 21% at 1.5 U/mg protein.

Accuracy

Serum samples from patients with type 2 diabetes and controls were assayed following serial dilutions. Samples were diluted 2-, 4-, 8-, and 16-fold, and serum samples from diabetic patients as well as from controls produced parallel inhibition to hydroimidazolone standard.

Specificity

The cross-reactivity against AGE-BSA was 8% when calculated as AGE-BSA protein against MG-modified BSA protein. No cross-reactivity was found against CML-BSA or glycated albumin in the hydroimidazolone immunoassay.

Serum Levels of Hydroimidazolone, AGEs, and CML

The serum levels of hydroimidazolone were significantly increased in patients with type 2 diabetes compared to nondiabetic controls: median, 3.0 (1.6 to 5.4) versus 1.9 (1.2 to 2.8) U/mg protein ($P = .0005$). The difference between the 2 groups is clearly depicted in the box-plot in Fig 2. There was a significant correlation between serum levels of hydroimidazolone and AGEs measured with the polyclonal anti-AGE antibody: $r = 0.59$, ($P < .0001$), and between serum levels of hydroimidazolone and CML: $r = 0.36$ ($P = .02$), in patients with type 2 diabetes as well as in nondiabetic controls, $r = 0.65$ ($P = .002$) for AGEs, $r = 0.55$ ($P = .02$) for CML (Fig 3A and B).

The relative increase in hydroimidazolone levels found in diabetic subjects versus nondiabetic controls was comparable to previously reported differences in levels of CML, and slightly less than the increase in AGE levels measured with the polyclonal antibody. Hence, the median level of hydroimidazolone among patients was 158% of the median among nondiabetic controls, the median level of CML was 156%, and the median level of AGE was 166% of the control population.[12] There were no significant correlations between serum hydroimidazolone and fasting plasma glucose, HbA_{1c} or average HbA_{1c} assayed over the previous year. No significant difference in hydroimidazolone levels was shown between patients with or without microalbuminuria.

DISCUSSION

Significantly increased serum levels of hydroimidazolone in patients with type 2 diabetes were found in the present investigation. This is in agreement with the demonstration of increased levels of MG in diabetic complications,[15] and increased intracellular levels of MG-derived hydroimidazolone showed immunohistochemically in glomeruli from patients with diabetes.[25]

As the present assay has high sensitivity, serum may be assayed in high dilution. Thus, the likelihood of interference with nonspecific serum factors is reduced. Other researchers that have encountered difficulties in establishing assays for the measurement of AGEs in serum have suggested the presence of a factor in serum that interacts with AGEs immobilized on microtiter plates, and that this factor may be complement.[32]

We found a significant correlation between serum levels of hydroimidazolone and serum levels of AGEs determined by an immunoassay based on a widely used polyclonal antibody, and furthermore, between serum levels of hydroimidazolone and

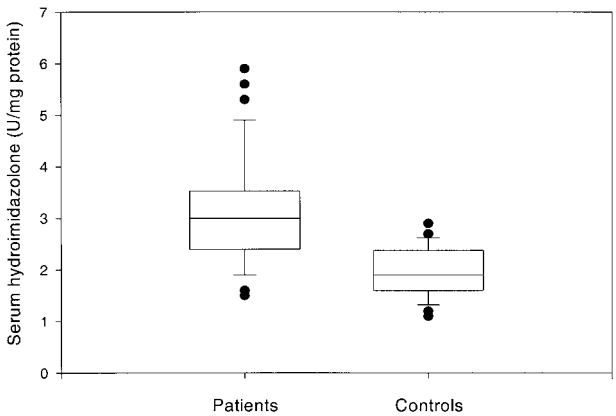

Fig 2. Box-plot (25th percentile, median, and 75th percentile) and whisker (10th and 90th percentiles) of the serum levels of MG-derived hydroimidazolone (U/mg protein) in patients with type 2 diabetes (n = 45) compared to nondiabetic controls (n = 19), $P = .0005$.

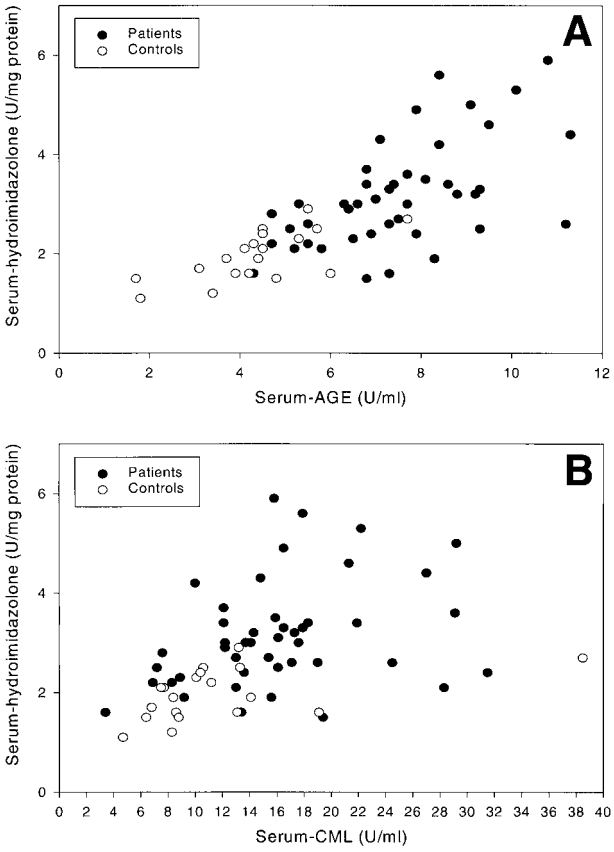

Fig 3. Scatter plot of serum levels of MG-derived hydroimidazolone and (A) AGEs measured by a polyclonal anti-AGE antibody ($r = 0.59$, $P < .0001$) in patients (●), and ($r = 0.65$, $P = .002$) in controls (○). (B) Serum levels of hydroimidazolone compared to serum levels of CML ($r = 0.36$, $P = .02$) in patients and ($r = 0.55$, $P = .02$) in nondiabetic controls. Serum levels of AGEs and CML are adjusted for total protein concentration (see text).

serum levels of CML. Thus, patients with diabetes may have increased levels of several AGEs. The recognition of MG-modified BSA by the monoclonal anti-CML antibody is due to simultaneous recognition of small amounts of N^{ϵ}-(carboxyethyl)lysine (CEL), a product of the reaction of MG with lysine residues in protein[33] as the "monoclonal" anti-CML antibody also recognizes CEL.[28]

The serum hydroimidazolone levels did not correlate with fasting plasma glucose or HbA_{1c} levels. This indicates that the serum levels may be influenced by several factors. First, fractions of MG may be derived from non–glucose-derived triose phosphates.[16] Second, environmental factors like diet may influence serum MG, as increased levels have been observed in sera of patients after a standardised meal, and they were positively correlated to postprandial blood glucose levels, but not to HbA_{1c}.[34] Furthermore, MG detoxification is enzymatically controlled by the glyoxalase system resulting in different MG levels in the presence of similar glucose levels. Differences in the efficiency with which individual patients detoxify MG may be crucial to individual susceptibility to complications. A previous study has shown correlations between HbA_{1c} and levels of MG-modified plasma protein[35] as measured with an immunoassay. However, this method included extensive enzymatic digestion and filtration of samples before assay, so the methods are not comparable.

Different AGE compounds have been linked to development of complications in diabetes mellitus,[10,11,36-38] but little is known about the in vivo effects of MG-derived hydroimidazolone. In vitro studies have shown arginine-derived hydroimidazolone formed from MG to be the recognition factor for the binding of human serum albumin minimally modified with MG (MG_{min}-HSA) to monocytic THP-1 cells.[22] MG_{min}-HSA induces synthesis and secretion of interleukin-1β and macrophage colony-stimulating factor from peripheral human monocytes and human monocytic THP-1 cells in vitro, as well as inducing tumor necrosis factor-α synthesis and secretion from THP-1 cells.[39] An indication of the possible importance of dicarbonyl AGE precursors such as MG is illustrated by the ability of the AGE-inhibitor aminoguanidine to reduce progression of retinopathy and overt nephropathy in patients with type 1 diabetes.[40,41] Aminoguanidine is known to prevent modification of plasma proteins by MG in vitro.[24]

In conclusion, the present immunoassay for measuring serum levels of MG-derived hydroimidazolone enabled the demonstration of increased levels of this hydroimidazolone in patients with type 2 diabetes. Thus the observed differences between patients with diabetes and nondiabetic controls seem to be comparable to differences measured for other AGE compounds. The hydroimidazolone assay provides a tool for investigating the relationship between hydroimidazolone and the development of complications in diabetes. Larger studies are needed to explore the exact nature of this relationship.

ACKNOWLEDGMENT

Rick Bucala, Picower Research Institution provided the anti-AGE antibodies. We thank Turi Arnesen Siegwarth for skilful technical assistance.

REFERENCES

1. Greene DA, Lattimer SA, Sima AAF: Sorbitol, phosphoinositides, and sodium-potassium-ATPase in the pathogenesis of diabetic complications. N Engl J Med 316:599-606, 1987

2. Baynes JW, Thorpe SR: Role of oxidative stress in diabetic complications. A new perspective on an old paradigm. Diabetes 48:1-9, 1999

3. Koya D, King GL: Protein kinase C activation and the development of diabetic complications. Diabetes 47:859-866, 1998

4. Brownlee M: Glycation and diabetic complications. Diabetes 43:836-841, 1994

5. Haffner SM, Lehto S, Rönnemaa T, et al: Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. N Engl J Med 339:229-234, 1998

6. Stratton IM, Adler AI, Neil AW, et al: Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes

(UKPDS 35): Prospective observational study. BMJ 321:405-412, 2000

7. Brownlee M, Cerami A, Vlassara H: Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 318:1315-1321, 1988

8. Vlassara H, Bucala R, Striker L: Pathogenetic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. Lab Invest 70:138-151, 1994

9. Nakamura Y, Horii Y, Nishino T et al: Immunohistochemical localization of advanced glycosylation endproducts (AGEs) in coronary atheroma and cardiac tissue in diabetes mellitus. Am J Pathol 143:1649-1656, 1993

10. Schleicher E, Wagner E, Nerlich AG: Increased accumulation of the glycoxdation product N^{ϵ}-(carboxymethyl)lysine in human tissues in diabetes and aging. J Clin Invest 99:457-468, 1997

11. Berg TJ, Bangstad H-J, Torjesen PA, et al: Advanced glycation end products in serum predict changes in the kidney morphology of patients with insulin-dependent diabetes mellitus. Metabolism 46:661-665, 1997

12. Kilhovd BK, Berg TJ; Birkeland KI, et al: Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease. Diabetes Care 22:1543-1548, 1999

13. Shinohara M, Thornalley PJ, Giardino I, et al: Overexpression of glyoxalase-1 in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. J Clin Invest 101:1142-1147, 1998

14. Thornalley PJ: The glyoxalase system: New developments towards functional characterization of a metabolic pathway fundamental to biological life. Biochem J 269:1-11, 1990

15. McLellan AC, Thornalley PJ, Benn J, et al: Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. Clin Sci 87:21-29, 1994

16. Phillips SA, Thornalley PJ: The formation of methylglyoxal from triose phosphates. Investigations using a specific assay for methylglyoxal. Eur J Biochem 212:101-105, 1993

17. Ray S, Ray M: Isolation of methylglyoxal synthase from goat liver. J Biol Chem 256:6230-6233, 1981

18. Koop D, Casazza JP: Identification of ethanol-inducible P-450 isozyme 3a as the acetone and acetol monooxygenase of rabbit microsomes. J Biol Chem 260:13607-13612, 1985

19. Lyles GA, Chalmers J: The metabolism of aminoacetone to methylglyoxal by semicarbazide-sensitive amine oxidase in human umbilical artery. Biochem Pharmacol 43:1409-1414, 1992

20. Lo TWC, Westwood ME, McLellan AC, et al: Binding and modification of proteins by methylglyoxal under physiological conditions. J Biol Chem 269:32299-32305, 1994

21. Frye EB, Degenhardt TP, Thorpe SR, et al: Role of the Maillard reaction in aging of tissue proteins. J Biol Chem 273:18714-18719, 1998

22. Westwood ME, Argirov OK, Abordo EA, et al: Methylglyoxal-modified arginine residues—A signal for receptor-mediated endocytosis and degradation of proteins by monocytic THP-1 cells. Biochim Biophys Acta 1356:84-94, 1997

23. Thornalley PJ: Glutathione-dependent detoxification of α-oxoaldehydes by the glyoxalase system: Involvement in disease mechanisms and antiproliferative activity of glyoxalase I inhibitors. Chem-Biol Interact 111-112:137-151, 1998

24. Lo TWC, Selwood T, Thornalley PJ: The reaction of methylglyoxal with aminoguanidine under physiological conditions and prevention of methylglyoxal binding to plasma proteins. Biochem Pharm 48:1865-1870, 1994

25. Giardino I, Thornalley PJ, Edelstein D, et al: Generation and characterization of a monoclonal antibody against AGEs that induce endothelial cell dysfunction. Diabetes 47:A123, 1998 (suppl 1, abstr)

26. Westwood ME, Thornalley PJ: Molecular characteristics of methylglyoxal-modified bovine and human serum albumins. Comparison with glucose-derived advanced glycation endproduct-modified serum albumins. J Prot Chem 14:359-372, 1995

27. Makita Z, Vlassara H, Cerami A, et al: Immunochemical detection of advanced glycosylation end products in vivo. J Biol Chem 267:5133-5138, 1992

28. Clausen JT, Christensen M, Skovsted I, et al: Antibodies against in vitro generated AGE: Specificity and applicability to assays for in vivo AGE. Diabetologia 40:A588, 1997 (abstr)

29. Berg TJ, Clausen JT, Torjesen PA, et al: The advanced glycation end product N^{ϵ}-(carboxymethyl)lysine is increased in serum from children and adolescents with type 1 diabetes. Diabetes Care 21:1997-2002, 1998

30. Friedewald WT, Levy RI, Fredrickson DS: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without the use of the preparative ultracentrifuge. Clin Chem 18:499-502, 1972

31. Birkeland KI, Rishaug U, Hanssen KF, et al: NIDDM: a rapid progressive disease. Results from a long-term, randomised, comparative study of insulin or sulphonylurea treatment. Diabetologia 39:1629-1633, 1996

32. Dorrian CA, Cathcart S, Clausen J, et al: Factors in human serum interfere with the measurement of advanced glycation endproducts. Cell Mol Biol 44:1069-1079, 1998

33. Ahmed MU, Brinkmann Frye E, Degenhardt TP, et al: N^{ϵ}-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. Biochem J 324:565-579, 1997

34. Beisswenger PJ, Howell SK, O'Dell RM, et al: α-Dicarbonyls increase in the postprandial period and reflect the degree of hyperglycemia. Diabetes Care 24:726-732, 2001

35. Shamsi FA, Partal A, Sady C, et al: Immunological evidence for methylglyoxal-derived modifications in vivo. J Biol Chem 273:6928-6936, 1998

36. Niwa T, Katsuzaki T, Ishizaki Y, et al: Imidazolone, a novel advanced glycation end product, is present at high levels in kidneys of rats with streptozotocin-induced diabetes. FEBS Letters 407:297-302, 1997

37. Monnier VM, Bautista O, Kenny D, et al: Skin collagen glycation, glycoxidation and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes. Relevance of glycated collagen products versus HbA_{1c} as markers of diabetic complications. Diabetes 48:870-880, 1999

38. Singh R, Barden A, Mori T, et al: Advanced glycation endproducts: A review. Diabetologia 44:129-146, 2001

39. Abordo EA, Thornalley PJ: Synthesis and secretion of tumour necrosis factor–α by human monocytic THP-1 cells and chemotaxis induced by human serum albumin derivatives modified with methylglyoxal and glucose-derived advanced glycation endproducts. Immunol Lett 58:139-147, 1997

40. Raskin P, Cattran D, Williams M, et al: Pimagedine (PG) reduces progression of retinopathy and lowers lipid levels in patients with type 1 diabetes mellitus (DM). J Am Soc Nephrol 10:A0914, 1999 (abstr)

41. Appel G, Bolton K, Freedman B, et al: Pimagedine (PG) lowers total urinary protein (TUP) and slows progression of overt diabetic nephropathy in patients with type 1 diabetes mellitus (DM). J Am Soc Nephrol 10:A0786, 1999 (abstr)