2-Aminoadipic acid is a marker of protein carbonyl oxidation in the aging human skin: effects of diabetes, renal failure and sepsis

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We hypothesized that the ε-amino group of lysine residues in long-lived proteins oxidatively deaminates with age forming the carbonyl compound, allysine (α-aminoadipic acid-δ-semialdehyde), which can further oxidize into 2-aminoadipic acid. In the present study, we measured both products in insoluble human skin collagen from $n = 117$ individuals of age range 10–90 years, of which $n = 61$ and $n = 56$ were non-diabetic and diabetic respectively, and a total of $n = 61$ individuals had either acute or chronic renal failure. Allysine was reduced by borohydride into 6-hydroxynorleucine and both products were measured in acid hydrolysates by selective ion monitoring gas chromatography (GC)-MS. The results showed that 2-aminoadipic acid ($P < 0.0001$), but not 6-hydroxynorleucine ($P = 0.14$), significantly increased with age reaching levels of 1 and 0.3 mmol/mol lysine at late age respectively. Diabetes in the absence of renal failure significantly ($P < 0.0001$) increased 2-aminoadipic acid up to < 3 mmol/mol, but not 6-hydroxynorleucine (levels < 0.4 mmol/mol, $P = 0.18$). Renal failure even in the absence of diabetes markedly increased levels reaching up to < 0.5 and 8 mmol/mol for 6-hydroxynorleucine and 2-aminoadipic acid respectively. Septicaemia significantly ($P < 0.0001$) elevated 2-aminoadipic acid in non-diabetic, but not diabetic individuals, and mildly correlated with other glycoxidation markers, carboxymethyl-lysine and the methylglyoxal-derived products, carboxyethyl-lysine, argpyrimidine and MODIC (methylglyoxal-derived imidazolium cross-link). These results provide support for the presence of metal-catalysed oxidation (the Suyama pathway) in diabetes and the possible activation of myeloperoxidase during sepsis. We conclude that 2-aminoadipic acid is a more reliable marker for protein oxidation than its precursor, allysine. Its mechanism of formation in each of these conditions needs to be elucidated.

Key words: glycation, lysine, methylglyoxal, myeloperoxidase, redox-active metals, semicarbazide-sensitive amine oxidase.

INTRODUCTION

Aging in human skin is characterized by a progressive loss of elasticity and increased stiffness, processes which ubiquitously occur in all connective tissues with age [1]. The exact cause for the deterioration is not fully understood, but it is commonly observed that solar exposure may greatly accelerate these processes including the induction of elastosis and wrinkle formation suggesting that oxidation is involved. However, even in non-exposed areas of skin, chronological aging still occurs as characterized by atrophy, reduced resilience and fine wrinkle formation [2].

Histologically, skin is composed of a dermal layer of ECM (extracellular matrix) consisting predominately of type I collagen [3]. Type I collagen is structurally a fibrillar protein which provides tensile strength, mechanical stability and a protective barrier. With age, skin collagen becomes progressively more insoluble, yellow, fluorescent and cross-linked. Markers for processes of oxidation, lipoxidation, glycoxidation and glycation are all found to increase with chronological age in human skin collagen. These include orthotyrosine, methionine sulfoxide, CML (N^{ε}-carboxymethyl-lysine), CEL (N^{ε}-carboxyethyl-lysine), pentosidine, glucosepane and fructose-lysine [4]. Undoubtedly, the decrease in collagen turnover with age is the single most important factor in determining the age-related accumulation of these products with a reported half-life of 15 years for human skin collagen [5].

Fibrillar collagen, such as that from skin, is characterized by an uninterrupted helical region with alternating non-helical polar and non-polar domains, permitting lateral alignment of molecules in staggered arrays, a prerequisite for stabilization by lysine-derived cross-links. This alignment pattern is thought to be necessary for initiation of physiological cross-linking by LOX (lysyl oxidase) in the development and maturation of collagen [3]. In this mechanism, LOX, a copper-dependent enzyme, selectively and oxidatively deaminates the lysine residue forming α-aminoadipic acid-δ-semialdehyde, referred as allysine (Scheme 1A).

Several years ago, Stadtman [6] hypothesized that the ε-amino group of lysine residues in proteins may undergo deamination by MCO (metal-catalysed oxidation) reactions to form allysine. The conversion was postulated to be stochastic, spontaneous, metal-catalysed and a contributor to total protein oxidation with age, as measured by non-specific carbonyl formation *in vivo* [7]. More importantly, MCO was implicated in a number of age-related disease processes including Alzheimer's disease, atherosclerosis, cataractogenesis and diabetes [7]. Requena et al. [8] subsequently concluded that allysine was a major product formed during protein oxidation in model systems and cultured cells subjected to MCO *in vitro*.

However, various investigations indicate that allysine probably undergoes a further oxidative reaction to form 2-aminoadipic acid *in vivo* [9–11] (Scheme 1A). Interestingly, Bailey et al. [12]

Abbreviations used: AGE, advanced glycation end-product; CEL, N^{ε}-carboxyethyl-lysine; CML, N^{ε}-carboxymethyl-lysine; d_4-CML, N^{ε}-carboxymethyl-lysine-4,4,5,5-d_4; d_8-lysine, DL-lysine-3,3,4,4,5,5,6,6-d_8; ECM, extracellular matrix; LC, liquid chromatography; LOX, lysyl oxidase; MCO, metal-catalysed oxidation; MODIC, methylglyoxal-derived imidazolium cross-link; MPO, myeloperoxidase; SKD, Strecker degradation; SSAO, semicarbazide-sensitive amine oxidase; VAP-1, vascular adhesion protein-1.

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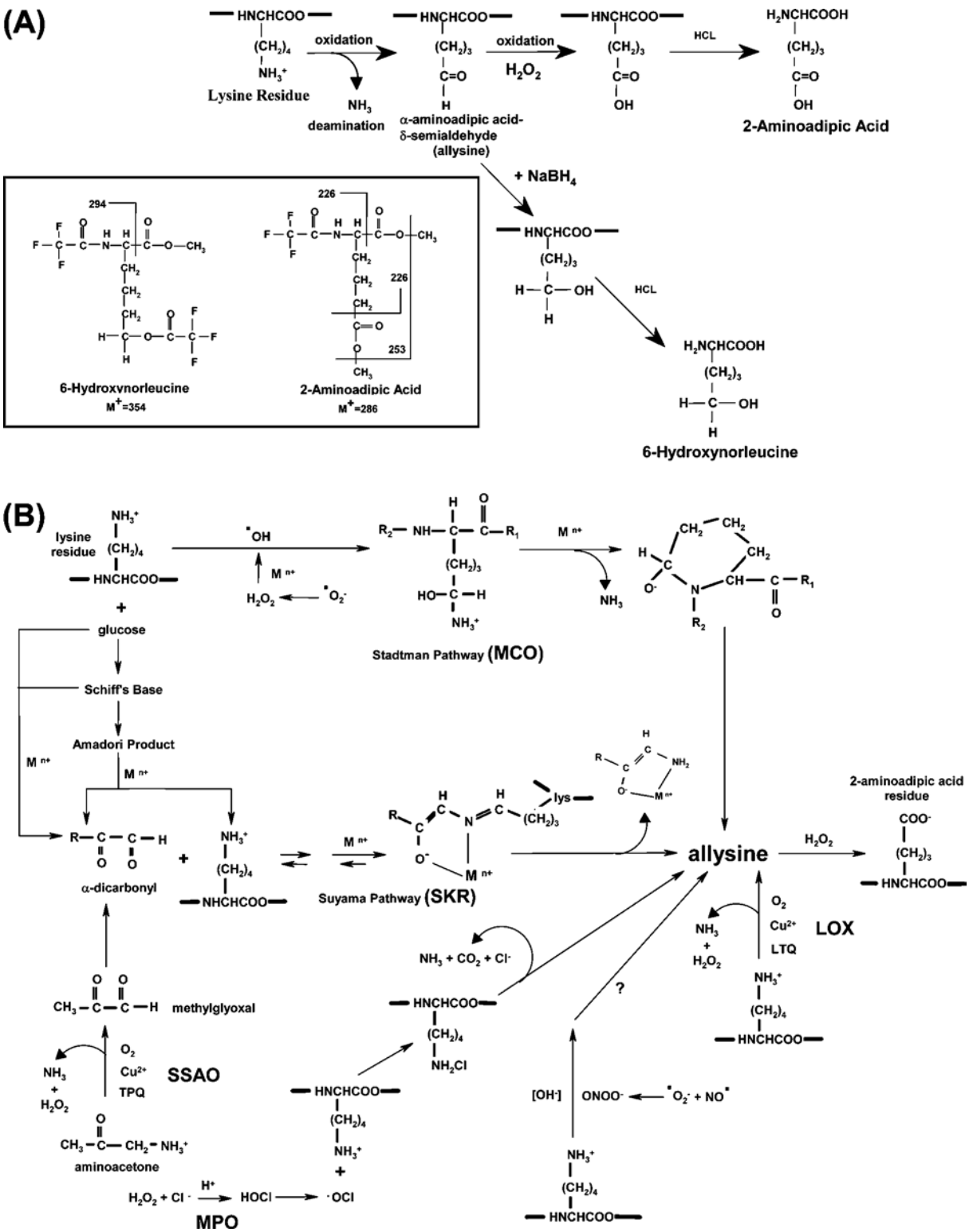


Scheme 1 Oxidation of lysine residues in skin collagen

(**A**) Formation of 6-hydroxynorleucine and 2-aminoadipic acid as markers for protein oxidation. Insert: fragmentation patterns for trifluoroacetyl methyl ester derivatives of 6-hydroxynorleucine and 2-aminoadipic acid used for quantification by selected ion monitoring GC-MS. (**B**) Proposed mechanisms for deamination of lysine residues in proteins forming allysine which converts into 2-aminoadipic acid by oxidation: TPQ, 2,4,5-trihydroxyphenylalanine quinone; LTQ, lysine tyrosyl quinone.

reported many years ago the presence of significant amounts of elevated levels of 2-aminoadipic acid in old (i.e. 10–14 yrs) compared with fetal bovine skin collagen. The authors proposed a scheme for the formation of 2-aminoadipic acid by oxidation of the reducible cross-links, dehydrohydroxyl-lysinonorleucine and dehydrolysinonorleucine, as a physiological basis for maturation

of connective tissues *in vivo*. However, levels of these reducible cross-links, as well as the activity of LOX, decline with age [3]. Thus the importance of LOX-derived cross-links in explaining the observed age-related deterioration of connective tissues *in vivo* is in question.

In light of the lack of an association between allysine and aging, we hypothesized that the latter would be converted into the more stable 2-aminoadipic acid, and thus accumulate in the aging skin. In the present study, we have measured 2-aminoadipic acid and allysine as the borohydride-reduced product, 6-hydroxynorleucine, in acid hydrolysates of insoluble human skin collagen as a function of chronological age, diabetes and renal failure. The data were then correlated with a number of AGEs (advanced glycation end-products).

MATERIALS AND METHODS

Chemicals

Standards of L-2-aminoadipic acid and d_8-lysine (DL-[3,3,4,4,5,5,6,6-$^2H_8$]lysine) were purchased from Sigma–Aldrich; CEL and d_4-CML (N^{ε}-carboxymethyl-lysine-4,4,5,5-d_4) were from Dr Susan Thorpe and Dr John Baynes (Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC, U.S.A.); argpyrimidine and CML were from Dr Marcus Glomb (Institute of Food Chemistry, Technical University of Berlin, Berlin, Germany); and MODIC (methylglyoxal-derived imidazolium cross-link) was from Dr Klaus Biemel (Institute of Food Chemistry, University of Hohenheim, Stuttgart, Germany). 6-Hydroxynorleucine was synthesized as follows: a total of 2.57 g of Nα-CBZ-L-lysine (Sigma) was refluxed for 6 h in 200 ml of 100 % ethanol containing 2.16 g of concentrated sulfuric acid and then dried by rotor-evaporation after adjusting to pH 6 with 1 M sodium hydroxide. The residue was reconstituted with 9 ml of 15 M acetic acid upon which 3 ml of 8 mM sodium nitrite was added very slowly over a 1 h period while maintaining the solution at 0 °C. The solution was then stirred for 30 min at 90 °C, dried by a rotor-evaporator, and deprotected by stirring for 6 days in 6 M HCl at room temperature (20 °C). The acid was removed by rotor-evaporation and 6-hydroxynorleucine was purified by cation-exchange chromatography (Dowex 50 WX8, Fisher); the column was equilibrated with 1 M HCl and the product was eluted with 1 M ammonium hydroxide. 6-Hydroxynorleucine was considered pure by TLC and by satisfactory ^{1}H- and ^{13}C-NMR, and MS.

Tissue donor information

Tissue collection was done according to the guidelines and approval from the Institutional Review Board for Human Investigation at University Hospitals of Cleveland (UHC, Cleveland, OH 44106, U.S.A.) using the Human Tissue Procurement Facility at UHC for most samples and the National Disease Research Interchange (NDRI, Philadelphia, PA 19103, U.S.A.) for additional samples ($n = 6$). Human skin was obtained at autopsy as previously described in [13] from $n = 117$ individuals of age range 8–90 yrs, of which $n = 69$ and $n = 48$ were of the Caucasian and African-American races respectively. There were $n = 60$ males and $n = 57$ females. Diagnoses as cause of death and other major pathologies including diabetes and chronic renal failure were obtained from the medical and anatomical records of each patient at autopsy as previously detailed [13]. Additionally, diagnosis of acute renal failure and sepsis were obtained. In most cases, these diagnoses were unequivocal and were specifically stated in each patient's record. However, in some cases, it was necessary to consult charts for elevated levels of blood urea nitrogen and creatinine as parameters for acute renal failure, and white blood cell count and growth of micro-organisms in cultures taken from blood, lung and spleen during clinical and post-mortem evaluations as parameter for sepsis. Each individual was categorized into one of twelve groups according to the presence or absence of diabetes, acute and chronic renal failure and sepsis, as summarized in Figure 3. There were $n = 19$ individuals diagnosed without diabetes, renal failure and sepsis; however, $n = 3$ were considered outliers for 2-aminoadipic acid levels as described in the Results section (Figures 1A, 1B and 3). Although levels for these three individuals are clearly identified in the graphs (Figures 1A, 1B and 3), they were not included in the statistical analysis. Totals of $n = 34$ and $n = 27$ patients had acute and chronic renal failure respectively. Of this latter group, $n = 16$ had end-stage renal disease requiring haemodialysis, of which $n = 8$ were diabetic.

Preparation of insoluble collagen

Insoluble collagen was prepared as previously described in [13] and included: delipidation with 2:1 chloroform/methanol; extraction with 1 M sodium chloride and 0.5 M acetic acid at 4 °C; and digestion with 0.1 % pepsin in 0.5 M acetic acid at 4 °C followed by freeze-drying.

Borohydride reduction and acid hydrolysis

Approximately 2 mg of each collagen sample was weighed and placed into a 12 × 75 mm borosilicate glass test tube followed by reduction with 0.1 M sodium borohydride as previously described in [14]. After freeze-drying, the collagen was placed into a 13 × 100 mm borosilicate glass tube fitted with a PTFE (polytetrafluoroethylene)-faced, rubber-lined screw cap. Each sample was acid hydrolysed for 18 h with 2 ml of 6 M HCl as detailed elsewhere [13]. In order to minimize discolouration during acid hydrolysis, the concentration of tissue to acid was maintained at approximately 1 mg/ml. In addition, the acid was degassed under vacuum followed by purging with nitrogen by bubbling for at least 15 min. The acid was immediately pipetted into each tube followed by thoroughly purging the tube with nitrogen before sealing with the screw cap.

Analytical assays

Amounts of 6-hydroxynorleucine and 2-aminoadipic acid were determined in acid hydrolysates of processed skin samples, derivatized as their trifluoroacetyl methyl esters, by selected ion monitoring gas chromatography (GC)/MS as previously described in [15]. In these assays, analytes were monitored for their largest, but specific product ion signals determined to be at $m/z = 294$ for 6-hydroxynorleucine and $m/z = 226$ and 253 for 2-aminoadipic acid (Scheme 1A). Their molecular ions were observed but not used in these evaluations. These two compounds eluted at approximately 20 and 21.4 min respectively, using the temperature programme described in [15]. CEL and the internal standards, d_8-lysine, and d_4-CML, were simultaneously determined as monitored for their ion signals at $m/z = 379$, 328 and 396 which eluted at 31.3, 24.9 and 31 min respectively. Argpyrimidine and MODIC were determined by liquid chromatography (LC)/MS as previously described [13,16].

Statistical methods

Statistical analysis was performed according to methods previously described in detail in [15]. In brief, regression analysis, Spearman's correlations and the Mann–Whitney Test were computed using SPSS software. Testing for homogeneity of variance was done using either the F-test or the Burr–Foster Q-Test as previously described [15]. Data were transformed with either the

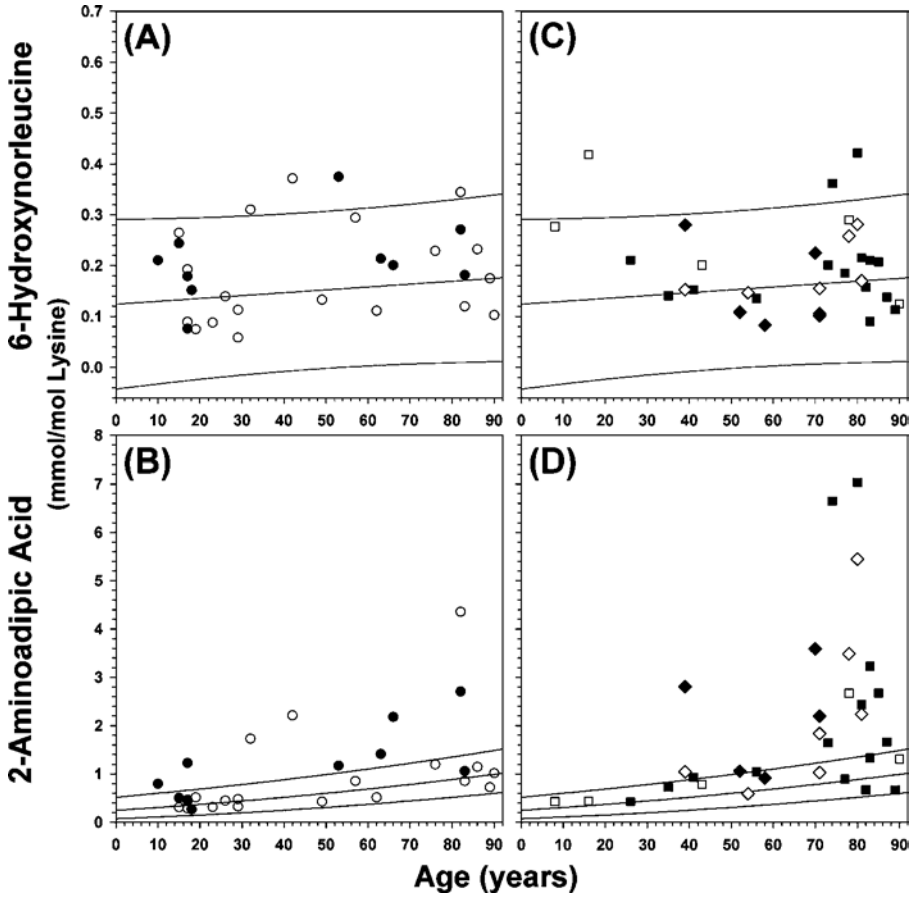


Figure 1 Plots of 6-hydroxynorleucine and 2-aminoadipic acid versus chronological age in non-diabetic individuals

Levels determined in relationship to sepsis and acute and chronic renal failure in human insoluble skin collagen. For each graph, regression line and 95 % confidence intervals of prediction are shown. Regression line equations where x = age and y = parameter: (**A**) and (**C**): 6-hydroxynorleucine, $y = 5.7 \times 10^{-4}x + 0.124$, $n = 16$, $r = 0.23$, $P = 0.39$ (NS); (**B**) and (**D**): 2-aminoadipic acid, $y = 3.1 \times 10^{-5}x^2 + 0.006x + 0.246$, $n = 16$, $r = 0.86$, $P < 0.0001$. ○, non-diabetic; ●, non-diabetic + sepsis; ◇, non-diabetic + acute renal failure; ■, non-diabetic + acute renal failure + sepsis; ◇, non-diabetic + chronic renal failure; ◆, non-diabetic + chronic renal failure + sepsis.

square-root or log y transformations. Significance was considered $P < 0.05$.

RESULTS

Preliminary analysis identifies 2-aminoadipic acid associated with chronological aging and sepsis in skin collagen

A preliminary analysis of 6-hydroxynorleucine and 2-aminoadipic acid was made in a total of $n = 117$ human insoluble skin collagen samples as a function of donor age. It initially excluded individuals with diabetes and chronic renal failure since these factors are known to catalyse both oxidative and glycoxidative processes in skin collagen [4]. With these exclusions ($n = 68$), the initial plots surprisingly showed a significant increase with age for 2-aminoadipic acid ($P = 0.003$), but not 6-hydroxynorleucine ($P = 0.80$) (results not shown). However, it was noted that a wide spread occurred for 2-aminoadipic acid levels especially at old age. Subsequently, a follow-up of these outlying values ($n = 13$) showed that many of them ($n = 9$) were from patients diagnosed with sepsis at autopsy either as a major or accessory cause of death.

Evaluation of levels in relationship to age, sepsis, diabetes and renal failure

In further study, the anatomical record of each patient was thoroughly evaluated for evidence of sepsis and acute renal failure, i.e. a common complication of sepsis [17]. Diabetes and chronic renal failure in these patients have previously been described in other studies utilizing the same material [13]. All factors were statistically analysed by two methods, multivariate regression and correlation analyses.

Multivariate regression analysis

Plots of 6-hydroxynorleucine and 2-aminoadipic acid levels versus age in relationship to sepsis and renal failure were made for a total of $n = 61$ non-diabetic and $n = 56$ diabetic patients, as shown in Figures 1 and 2 respectively. For clarity, levels in patients with acute renal failure were co-plotted with those having chronic renal failure (Figures 1C, 1D, 2C and 2D). In these analyses, three outliers were identified for non-diabetic patients without sepsis (Figure 1B). Of these, one died from complications due to sickle cell disease (age 32 years), another from gastrointestinal bleeding secondary to cirrhosis of the liver (age 42 years), and a third from severe pneumonitis and fibrosis of the lung without evidence of septicaemia (age 82 years). These outliers were deleted from further statistical analysis.

Non-diabetic individuals. Initial plots were made for levels versus age for $n = 19$ non-diabetic individuals diagnosed without renal failure and sepsis (Figures 1A and 1B). Excluding the $n = 3$ outliers just described, a regression line and 95 % confidence intervals were computed and plotted for the remaining $n = 16$

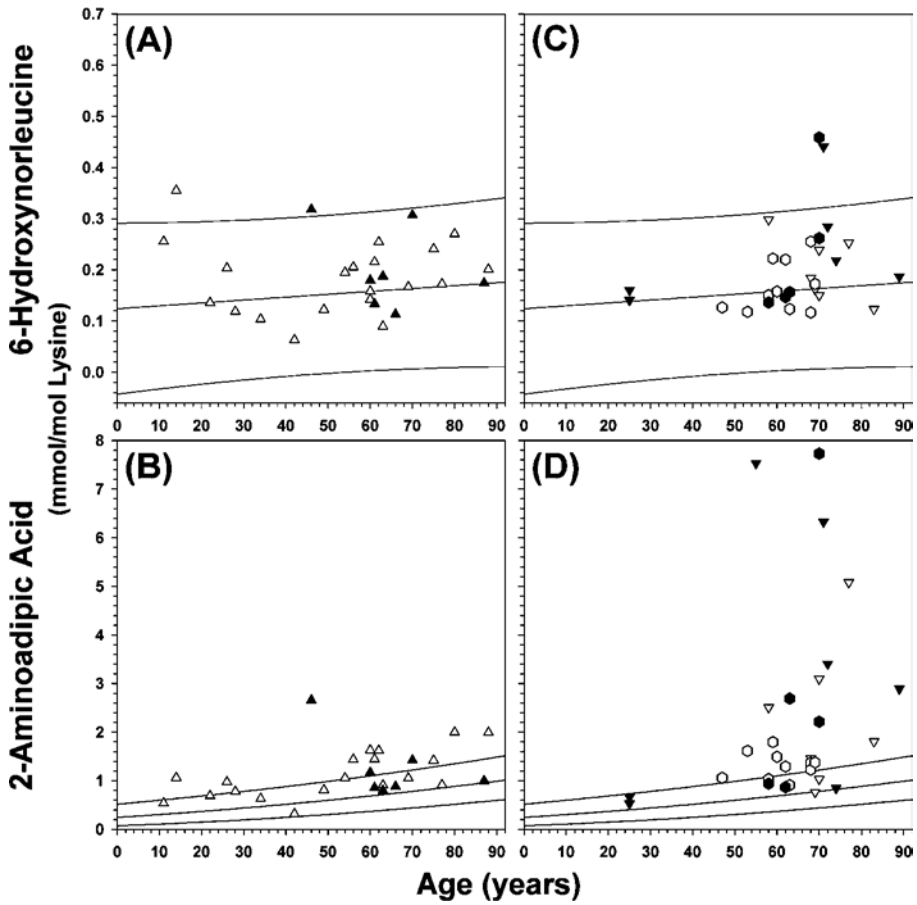


Figure 2 Plots of 6-hydroxynorleucine and 2-aminoadipic acid versus chronological age in diabetic individuals

For details see the legend for Figure 1. △, diabetic; ▲, diabetic + sepsis; ▽, diabetic + acute renal failure; ▼, diabetic + acute renal failure + sepsis; ⬡, diabetic + chronic renal failure; ⬢, diabetic + chronic renal failure + sepsis).

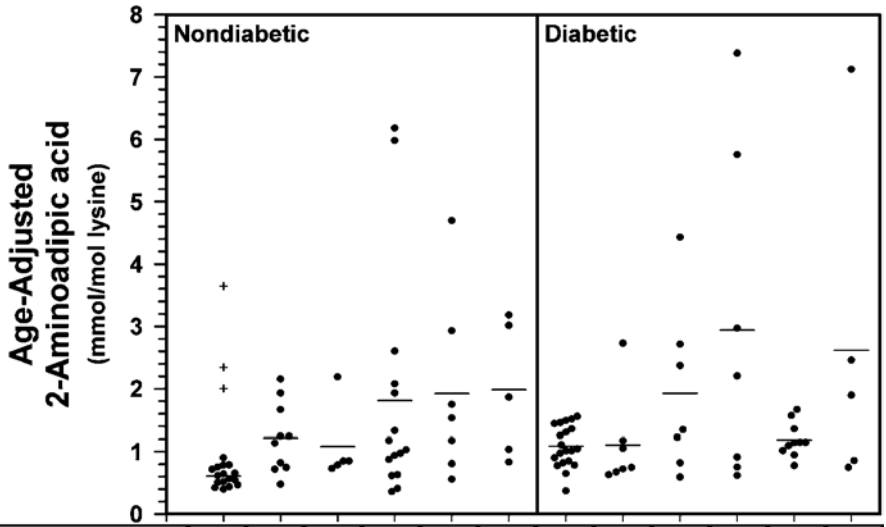
individuals for both 6-hydroxynorleucine (Figure 1A) and 2-aminoadipic acid (Figure 1B). Furthermore, these regression lines and confidence intervals were reproduced in all other plots of this study as a reference point for statistical comparisons (Figures 1C, 1D and 2A–D).

The results showed that 6-hydroxynorleucine increased with both age and sepsis with levels reaching approximately 0.3 mmol/mol lysine at age 82 years (Figure 1A). By regression analysis, the increase was significant for sepsis ($P = 0.038$), but not age ($P = 0.14$). In comparison, 2-aminoadipic acid levels were significantly and dramatically increased due to both age ($P < 0.0001$) and sepsis ($P < 0.0001$) reaching levels greater than 1 mmol/mol lysine at old age (Figure 1B).

Diabetic individuals. Levels of 6-hydroxynorleucine for almost all diabetic patients were within the confidence intervals determined for non-diabetic individuals without sepsis (Figure 2A). In contrast, 2-aminoadipic acid levels for the majority of these patients were elevated either above the regression line or the confidence intervals as shown in Figure 2(B). Subsequently, regression analysis showed that 6-hydroxynorleucine in these patients was not affected by age ($P = 0.5$), diabetes ($P = 0.18$) or sepsis ($P = 0.68$) (Figure 2A). In contrast, 2-aminoadipic acid was increased by age ($P < 0.0001$) and diabetes ($P < 0.0001$), but not by sepsis ($P = 0.87$) (Figure 2B).

Renal failure. 6-Hydroxynorleucine and 2-aminoadipic acid increased to 0.4 and 7 mmol/mol lysine respectively in some non-diabetic patients with renal failure (Figures 1C and 1D). Regression analysis revealed that 6-hydroxynorleucine was not significantly elevated by acute ($P = 0.22$) and chronic renal failure ($P = 0.98$) nor by sepsis ($P = 0.72$). In comparison, 2-aminoadipic acid was significantly elevated by chronic renal failure ($P = 0.002$), but not by acute renal failure ($P = 0.27$). Additionally, these levels were significantly ($P = 0.01$) elevated by sepsis during renal failure, but only when all non-diabetic data ($n = 58$) were tested. However, within renal failure patients themselves ($n = 32$), sepsis did not significantly ($P = 0.56$) elevate 2-aminoadipic acid, although there was a significant ($P = 0.001$) age effect. These differences may in part be due to the small number of patients represented in some of these groups as well as the large variation in levels (Figure 3).

In diabetic patients with renal failure, 6-hydroxynorleucine and 2-aminoadipic acid were elevated to levels of approximately 0.5 and 8 mmol/mol lysine respectively in some patients (Figures 2C and 2D). For all diabetics ($n = 56$), the presence of acute renal failure in association with diabetes significantly ($P = 0.014$) elevated levels of 2-aminoadipic acid, but not 6-hydroxynorleucine ($P = 0.12$). Chronic renal failure did not significantly affect levels for either parameter ($P > 0.05$). Within diabetic patients with renal failure ($n = 29$), levels were significantly



Group (Gp)*†	1	2	3	4	5	6	7	8	9	10	11	12
Diabetes							+	+	+	+	+	+
Sepsis		+		+		+		+		+		+
Acute Renal Failure			+	+					+	+		
Chronic Renal Failure					+	+					+	+
mean (age-adj.)	0.61	1.21	1.08	1.82	1.92	1.99	1.08	1.1	1.93	2.94	1.19	2.62
± S.D.	0.15	0.56	0.62	1.85	1.45	1.09	0.33	0.75	1.35	2.66	0.27	2.62
Gp x *vs.* Gp 1 (prob.)‡	-	0.001	0.006	0.003	0.001	<0.0001	<0.0001	0.015	0.001	0.001	<0.0001	0.002
total individuals (n)	16†	10	5	15	7	5	20	7	7	7	10	5

Figure 3 Levels of 2-aminoadipic acid age-adjusted to 50 years and categorized as to the presence of diabetes, sepsis, and acute and chronic renal failure

*The age-adjusted level for each individual is indicated by a black dot. †Three outliers (ages 32, 42 and 82 years) as indicated by crosshair symbols have not been included in the statistical analysis. ‡Statistical probability (*P*) by the Mann–Whitney Test of a significant difference in the comparison of Gp *x* versus Gp 1 where $x = $ Gp 2–12. Gp, group.

elevated by sepsis for both 2-aminoadipic acid ($P = 0.037$) and 6-hydroxynorleucine ($P = 0.041$). However, large variations in levels were noted for both parameters (Figures 2C and 2D) whereby homogeneity of variance was rejected ($P < 0.01$) for 2-aminoadipic acid even with statistical transformation.

Thus these results suggested that further statistical testing was needed.

Differential effects of sepsis, diabetes and renal failure on 2-aminoadipic acid levels after age-correction. Because age was strongly associated with 2-aminoadipic acid levels in the above results, these levels, in turn, were age-adjusted to 50 years and categorized into one of twelve groups according to the presence or absence of diabetes, sepsis and renal disease as indicated in Figure 3. Levels in each of these groups were statistically compared with levels of the non-diabetic group diagnosed without sepsis and renal failure (Figure 3). First, the results showed that age correction removed approximately 17 % and 9 % of the variation in levels for non-diabetic and diabetic data respectively. Secondly, significance was reached for each of these comparisons as indicated (Figure 3), i.e. 0.61 versus 1.21 ($P = 0.001$), 0.61 versus 1.08 ($P = 0.006$) etc. Thirdly, mean levels within non-diabetic and diabetic groups tended to be cumulatively higher when sepsis was diagnosed in association with either acute or chronic renal failure compared with sepsis alone; i.e. 1.08 versus 1.82, 1.92 versus 1.99, 1.93 versus 2.94, 1.19 versus 2.62 (Figure 3). However, each of these comparisons did not reach statistical significance ($P > 0.45$). Fourthly, the diagnosis of sepsis by itself significantly increased levels in non-diabetic individuals (0.61 versus 1.21, $P = 0.001$), but not diabetics (1.08 versus 1.1, $P = 0.29$, Figure 3).

Correlation analyses

Other oxidative markers and AGEs previously measured in this laboratory were screened for significant ($P < 0.05$) associations with sepsis in reference to the effects of age, diabetes and renal failure (i.e. acute and chronic renal failure) by Spearman's correlation analyses (Table 1). These results showed that across all data ($n = 114$), sepsis significantly and positively correlated with acute renal failure ($r = 0.29$, $P = 0.002$) but not chronic renal failure ($r = -0.07$, $P > 0.05$) (results not shown). For non-diabetics, the following parameters significantly correlated with sepsis (Table 1): 2-aminoadipic acid ($r = 0.34$, $P = 0.01$), CEL ($r = 0.32$, $P = 0.013$), CML ($r = 0.30$, $P = 0.023$), argpyrimidine ($r = 0.29$, $P = 0.033$) and MODIC ($r = 0.27$, $P = 0.048$). For diabetics, CEL was the only parameter associated with sepsis, but with borderline significance ($r = 0.27$, $P = 0.04$). Similarly, as noted in the above results, age was significantly and strongly associated with all parameters except for argpyrimidine and MODIC in diabetic subjects ($P > 0.05$; Table 1).

The incidence of acute renal failure significantly ($r = 0.25$, $P = 0.008$) increased with age, an observation not made with chronic renal failure ($r = 0.04$, $P > 0.05$) (results not shown). Acute renal failure was not significantly ($P > 0.05$) associated with any of these parameters except for CEL in diabetic individuals where levels were significantly elevated by acute renal failure ($r = 0.29$,

Table 1 Oxidative and glycoxidative markers correlating significantly with sepsis in insoluble human skin collagen in relationship to age, renal failure and diabetes

For each parameter, the correlation coefficient (r) and statistical probability (P) are stated on the first and second lines respectively. Correlation coefficients with $P < 0.05$ are considered significant. Analysed using Spearman's correlations.

		Non-diabetic ($n = 58$)				Diabetic ($n = 56$)			
Marker		Age	Sepsis	Acute renal failure	Chronic renal failure	Age	Sepsis	Acute renal failure	Chronic renal failure
2-Aminoadipic acid	(r)	0.62	0.34	0.17	0.35	0.45	0.08	0.23	0.15
	(P)	< 0.0001	0.01	0.21	0.007	0.001	0.56	0.09	0.28
CEL	(r)	0.73	0.32	0.21	0.32	0.45	0.27	0.29	0.27
	(P)	< 0.0001	0.013	0.11	0.015	< 0.0001	0.04	0.028	0.042
CML	(r)	0.82	0.30	0.20	0.21	0.38	0.22	0.04	0.35
	(P)	< 0.0001	0.23	0.14	0.12	0.003	0.10	0.79	0.008
Argpyrimidine	(r)	0.24	0.29	0.09	0.01	0.01	− 0.09	0.013	− 0.06
	(P)	0.08	0.033	0.54	0.97	0.99	0.55	0.93	0.67
MODIC	(r)	0.65	0.27	0.05	0.15	0.13	− 0.02	− 0.26	0.21
	(P)	< 0.0001	0.048	0.74	0.26	0.38	0.89	0.07	0.13

$P = 0.028$). In comparison, chronic renal failure significantly correlated with 2-aminoadipic acid ($r = 0.35$, $P = 0.007$) and CEL ($r = 0.32$, $P = 0.015$) in non-diabetic individuals and CML ($r = 0.35$, $P = 0.008$) and CEL ($r = 0.27$, $P = 0.042$) in diabetic individuals (Table 1).

DISCUSSION

Proteins with a slow turnover like skin collagen accumulate a variety of oxidative modifications with age including glycation products [7]. In this regard, protein carbonyls have been a hallmark for these oxidative modifications and are widely used as an indicator for protein damage [18]. Thus there has been considerable interest in understanding the source and mechanism for formation of protein carbonyls, whether from glycation, lipid peroxidation or MCO.

A major surprise in the present study is the finding that 2-aminoadipic acid accumulated with age to large levels while confirming that allysine levels, measured as 6-hydroxynorleucine, did not increase. This suggests that allysine is continuously oxidized into the stable 2-aminoadipic acid (Scheme 1A). A second surprise was that 2-aminoadipic acid levels were associated with the presence of diabetes, renal disease and especially sepsis, i.e. a disease associated with high morbidity and mortality [19].

Mechanistically, the deamination of lysine residues forming allysine may orginate from six pathways (Scheme 1B). One involves MCO as hypothesized by Stadtman [7]. In this pathway (Scheme 1B), the superoxide radical ($^{\bullet}O_2^-$) is catabolized by SOD (superoxide dismutase) into hydrogen peroxide (H_2O_2) which can decompose in the presence of redox-active metals (M^{n+}) into the hydroxyl radical ($^{\bullet}OH$). The latter species leads to the abstraction of a hydrogen atom from the side-chain of a lysine residue and intermediates involved in allysine formation (Scheme 1B) [8]. In short, other than inflammation, the validity of this hypothesis would depend upon the reactive oxygen species originating from sources other than the ECM or the skin itself, since cellularity declines with age [20].

Another potential source of allysine is from the reaction of $ONOO^-$ (peroxynitrite) with lysine residues (Scheme 1B). However, allysine formation by this pathway is unlikely based upon the results of Tien et al. [21]. In this study [21], although allysine was not directly measured, negligible amounts of carbonyl groups were formed when proteins were reacted with $ONOO^-$ under physiological conditions. Interestingly, lysine appeared to be preferentially targeted by this modification at high pH $\sim$ 10, although no conclusions could be made from this observation due to the small number of lysine residues modified relative to the minute amounts of total carbonyl groups formed during these experiments.

Alternatively, and more importantly for collagen, lysine residues may be converted into allysine by LOX (Scheme 1B) similarly to its mechanism of formation as the primary precursor for cross-link development in collagen during growth and maturation [3]. In this, aminoadipic acid has previously been detected in acid hydrolysates from several proteins including peroxidase-treated casein and β-lactoglobulin oxidized with performic acid [9], non-collagenous preparation of phosphoprotein purified from unerupted bovine teeth [10], bovine skin collagen and elastin [12]. Bailey et al. [12] proposed a mechanism involving oxidation of the Schiff base between LOX-derived allysine with an adjacent lysine or hydroxylysine residue resulting in the hypothetical non-reducible cross-link, ε-[δ-adipyl]-(hydroxy)-lysine, which upon acid hydrolysis would yield 2-aminoadipic acid. However, neither level of this cross-link nor 2-aminoadipic acid was reported.

Of further importance is that the activity of LOX decreases with age in skin [22] as well as in most other tissues [3]. Thus the importance of 2-aminoadipic acid originating from allysine based upon a LOX mechanism in explaining the age-related increase observed in Figure 1B is questionable. Interestingly, however, Buckingham and Reiser [23] reported a significant elevation in two important LOX-derived cross-links in human skin during diabetes, suggesting increased LOX activity as a marker of collagen synthesis. However, most studies conclude that collagen synthesis is decreased in skin during diabetes [24] although it is increased in diabetic nephropathy and may actually be enhanced in skin [25]. In spite of these controversies, LOX is not thought to act on mature triple helical collagen [26] making it unlikely that 2-aminoadipic acid stems from LOX activity.

A more plausible hypothesis for lysine oxidation especially in the diabetic milieu is oxidative degradation of lysine residues by α-dicarbonyl sugars, such as methylglyoxal, or their fragments by MCO-mediated SKD (Strecker degradation; Scheme 1B) as proposed by Suyama and colleagues [27]. In this reaction, the autoxidation of glucose or the decomposition of the Schiff base or the Amadori product itself, in the presence of M^{n+} results in α-dicarbonyl fragments which, in turn, undergo a reaction with the ε-amino groups of lysine residues resulting in intermediates involved in allysine formation as depicted in Scheme 1(B). *In vitro* evidence supports such a mechanism for allysine formation in proteins incubated with reducing sugars in the presence of transitional metals M^{n+} [27]. Indeed, elevated levels of allysine

were detected in plasma proteins of diabetic rats compared with controls [27]. In the present study, 2-aminoadipic acid was found significantly ($P < 0.0001$) elevated in diabetic skin collagen whereas allysine was not ($P > 0.05$) (Figures 2A, 2B and 3). Although SKD may explain the elevation in the diabetic state where excessive levels of sugars are present, its role in the aging phenomenon as well as other disease states such as sepsis is far from clear and needs further investigation. Interestingly, we found that sepsis correlated significantly ($P < 0.05$), using Spearman's correlation analysis, with levels of the methylglyoxal-derived AGEs, CEL, argpyrimidine and MODIC in non-diabetic subjects (Table 1). Since evidence suggests that elevated levels of CEL in diabetes most likely originates from aldehydic lipid and not from methylglyoxal [28], the Suyama pathway (Scheme 1B) may well explain the increased 2-aminoadipic acid levels in collagen.

The role of septicaemia in 2-aminoadipic acid formation is intriguing. Ample evidence suggests that sepsis induces oxidant stress and carbonyl formation in tissue proteins as shown in clinical studies with humans [29] and experimental sepsis using rodents [30,31]. Interestingly, evidence suggests that the virulence of the invading microorganism depends on the availability of redox-active iron [32] whereas levels increase during sepsis [32] and can catalyse protein carbonyl formation [7].

A fifth mechanistic hypothesis is that allysine and 2-aminoadipic acid originate from inflammation reactions involving SSAO (semicarbazide-sensitive amine oxidase) [33]. SSAO is identical with VAP-1 (vascular adhesion protein-1) which is located on endothelial surfaces and participates in the adhesive events between leucocytes and the vascular wall. Of significance is that VAP-1/SSAO is highly expressed in skin where it is up-regulated by various inflammatory reactions [34]. SSAO catalyses the deamination of low molecular mass, volatile, short-chain aliphatic amines such as methylamine and aminoacetone (Scheme 1B) resulting in the formation of formaldehyde and methylglyoxal respectively, as well as hydrogen peroxide and ammonia [33]. In short, the SSAO hypothesis is attractive for several reasons. First, the resultant hydrogen peroxide formed can easily oxidize the aldehyde group of allysine to form the carboxylic acid group of 2-aminoadipic acid. Secondly, SSAO activity increases in diabetes [35] as well as increasing plasma levels of aminoacetone and methylglyoxal [36]. However, the relationship between SSAO and sepsis has not been established. Furthermore, SSAO activity does not increase with age [37] and thus cannot explain the noted age-related increase of 2-aminoadipic acid shown in Figure 1(B).

Finally and most pertinent to our data, neutrophil MPO (myeloperoxidase) can oxidize lysine residues into lysine mono- and di-chloramine derivatives by chlorination reactions involving hypochlorous acid (HOCl) and hypochlorite (OCl^-). These derivatives, in turn, can decompose into allysine and 2-aminoadipic acid (Scheme 1B) [11]. Strong evidence implicates MPO in sepsis [38] as well as in acute [39,40] and chronic [41,42] renal failure. MPO activity increases with age in rat kidney, levels of which can be attenuated by caloric restriction [43]. In diabetes where neutrophil activity is impaired [44], patients are more prone to infections [44]. These findings may explain the observation made in Figure 3 where sepsis significantly increased 2-aminoadipic acid levels in non-diabetic individuals (0.61 versus 1.21, $P = 0.001$), but not diabetic individuals (1.08 versus 1.1, $P > 0.05$).

In conclusion, 2-aminoadipic acid, a pan-marker for all forms of lysine oxidation, significantly increased in aging human skin. Levels of its precursor, allysine, are in steady-state suggesting ongoing oxidation of allysine to form the stable end-product, 2-aminoadipic acid. Diabetes in the absence of renal complications significantly increased levels of 2-aminoadipic acid as well as renal failure in the absence of diabetes. Septicaemia dramatically elevated 2-aminoadipic acid levels in non-diabetic individuals and was found to correlate with other glycoxidative markers such as CML, and the methylglyoxal-derived AGEs, CEL, argpyrimidine and MODIC, which provides support for the methylglyoxal/copper (Suyama) pathway for lysine oxidation. Thus 2-aminoadipic acid is a more reliable marker for protein oxidation than its precursor, allysine. The mechanism of formation of 2-aminoadipic acid in diabetes, renal disease and sepsis needs to be elucidated.

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