Structure of advanced Maillard reaction products and their pathological role

V. M. Monnier¹, R. H. Nagaraj¹, M. Portero-Otin¹, M. Glomb¹, A-H Elgawish¹, D. R. Sell¹ and M. A. Friedlander³

Departments of ¹Pathology, ²Ophthalmology and ³Medicine, Case Western Reserve University School of Medicine, and University Hospitals of Cleveland, Cleveland, OH 44106, USA

Abstract. In this article we review recent progress and controversies relating to three areas of the field of advanced glycosylation end-products (AGE). A controversy exists as to whether pyrraline, an AGE detectable by immunohistochemistry in kidneys from patients with renal failure, exists in vivo. Recent data from the authors’ laboratory revealed that pyrraline is present in alkaline or protease digests from human skin and plasma. However, the amounts are very low and pyrraline was found to undergo further reactions to form an ether with itself (dipyrraline) as well as a thioether with cysteine. This high reactivity of pyrraline may explain the difficulty of quantitating it accurately in biological material. In contrast, the glycoxidation products carboxymethyllysine (CML) and pentosidine are stable, very resistant to acid hydrolysis and easy to quantitate. They are present in elevated concentrations in the extracellular matrix in diabetes mellitus and ageing. In the diabetic human lens, CML is not elevated, in contrast to pentosidine, suggesting a different mechanism of formation. Recent data in diabetic dogs have shown that pentosidine is elevated only in lenses from poorly controlled dogs, in contrast to LM-1, a fluorophore thought to arise from ascorbate. Further studies are needed to clarify the intracellular mechanism of glycoxidation. The greatest concentrations of AGEs and glycoxidation products are found in patients with end-stage renal disease, and they are almost completely normalized by renal transplantation. Comparison of peritoneal dialysis (PD) with haemodialysis (HD) showed that PD is associated with lower plasma protein pentosidine, possibly due to selective transport of pentosidine-rich protein across the peritoneal wall. Fractionation of plasma proteins from ESRD patients by size showed that 90% of pentosidine is linked to HMW protein and 1–2% is in free form. The mechanism of accelerated glycoxidation in ESRD is still not understood.

Key words: diabetes mellitus; end-stage renal disease; glycoxidation; pentoside; peritoneal dialysis; pyrraline

Introduction

The traditional scheme of the Maillard reaction implicates Amadori products, the major products resulting from the condensation of glucose with primary amines, as the sole major precursor of advanced glycation end-products (AGEs) [1]. This view was challenged when Hayashi et al. [2] discovered that the Schiff base adduct could undergo fragmentation to form glyoxal, C-3 fragmentation products and fairly stable pyrazine radicals. More confusion arose when Wolff and Dean [3] showed that glucose could autoxidize in the presence of trace metals, thus leading to reactive compounds that were recently shown to correspond to glyoxal and arabinose [4]. One study in the authors’ laboratory showed that ~50% of the glycoxidation product, carboxymethyllysine (CML), originates from cleavage of Amadori products, ~40% from a stage intermediate of glucose autoxidation and the Amadori products, i.e. a possible Schiff base adduct, and <10% from spontaneous oxidation of glucose [5]. Similar results with more emphasis on glucose autoxidation were reported by Wells-Knecht et al. [6]. From another perspective, several studies have shown that not only CML but also pentosidine, the other glyoxidation product, can originate through pathways that involve multiple sugars, various fragmentation mechanisms of Amadori products or ascorbate [7,8]. The composition of the products obtained depends substantially on the reaction conditions (molar ratios of reactants, type of buffer, presence of chelating agents, etc.).

All in all, great progress has been achieved recently in...
our understanding of the synthetic pathways leading to the formation of the advanced Maillard reaction products that are currently assayed in vivo. However, the discovery of multiple pathways for the formation of advanced Maillard reaction products increases the difficulty of interpreting results from biological studies.

Much emphasis has been placed on the role of oxidative modifications, especially in protein cross-linking and fluorescence formation [9]. As a result, the classical, non-oxidative pathway of degradation of Amadori products, which are the major in vivo modifications of the Maillard reaction, has become neglected, in part due to the paucity of simple assays for this arm of the advanced Maillard reaction.

Below, we first discuss recent progress on the biochemistry of pyrraline, an AGE formed non-oxidatively from Amadori products. We then address the problem of the source and mechanism of formation of glycoxidation products in the lens, a model of intracellular glycation. Finally, we present very recent data elucidating mechanisms of glycation and pentosidine formation in patients with end-stage renal disease.

The pyrraline controversy

Pyrraline, also called pyrrole-lysine, is a major product resulting from the reaction between glucose and primary amines when heated under reflux [10]. At slightly acidic pH, pyrraline yield can reach 10% if the reactant is e-amino caproic acid [11]. Pyrraline was isolated from a reaction mixture of glucose and lysine at 37°C after 7 days of incubation in yields of ~1% [12].

Because of its lability to acid hydrolysis, poly- and then monoclonal antibodies were produced which allowed quantitation of pyrraline by ELISA in diabetic plasma from the rat and humans [11,13]. Immunocytochemical studies with monoclonal antibody revealed immunostaining of sclerosed glomeruli and arterioles in the extracellular matrix from end-stage renal disease kidneys, as well as from basement membranes in brain microvessels, bronchioles and skin collagen [13]. In addition, positive immunostaining was found in lesions from brains of patients with Alzheimer's disease [14]. In each of these studies the immunostaining could be blocked by preincubating the antibody with as low a concentration as 25 μM of the free hapten. Furthermore, independent investigators studying the immunoreactivity of pyrraline in purified Descemet and lens capsule basement membranes from cows confirmed the role of 3-deoxyglucosone as a pyrraline precursor, and also noted an age-related increase in pyrraline immunoreactivity [15]. Again, preincubation with hapten blocked all binding.

These data were challenged by the report of Smith et al. [16], who failed to detect pyrraline immunoreactivity in glucose-incubated protein and in plasma protein from diabetic patients. The assays, however, were performed using proteins incubated with submillimolar glucose concentrations in the incubation mixtures, and the incubation duration was too short for significant amounts of Amadori products to build up. Furthermore, a positive control with 3-deoxyglucosone-incubated protein was not performed, thus making it difficult to assess whether the antibody had sufficient affinity to recognize native protein-bound pyrraline.

A report stating that pyrraline was stable to alkaline hydrolysis [17] led us to use both this method and proteolytic digestion to detect and quantitate pyrraline in glucose-incubated protein and in biological tissues. Reverse-phase HPLC was used to determine the time course of pyrraline formation in protein incubated at 37°C with 200 mM glucose [18]. Pyrraline formed only very slowly after a lag period. Incubation with 3-deoxyglucosone dramatically catalyzed pyrraline formation. Repetitive injections and peak collections of digest from skin collagen led to identification of a pyrraline peak, the UV spectrum of which overlapped with that of authentic pyrraline. Concentrations, however, were <10 pmol/mg collagen and showed no age-related increase. Similarly, pyrraline-like material was recovered from human plasma, and its HPLC peak was elevated in plasma digest from diabetic patients, thus strengthening the previous finding of increased pyrraline immunoreactivity in plasma from diabetic patients. The latter results are not surprising since 3-deoxyglucosone, the immediate pyrraline precursor, is found present in micromolar concentrations in plasma from diabetic individuals [19,20]. Although these findings are suggestive of pyrraline formation in vivo, mass-spectral data would be required to prove unequivocally the presence of pyrraline in biological tissues.

The low concentrations of pyrraline found in vivo, and the difficulty encountered in purifying large quantities of native pyrraline from tissue for spectral analysis (unpublished observation), may relate to the high reactivity of the pyrrole group of pyrraline. A pyrraline sample that was recovered from the freezer at –80°C after 1 year of storage was found to be pink in color [21]. Reverse-phase HPLC of this material revealed several new peaks in addition to pyrraline. The major peak absorbing at the pyrraline wavelength (290 nm) was isolated and analysed by NMR and FAB-MS. The results indicate that it was a pyrraline ether, i.e. two molecules of pyrraline condensed with each other. We therefore investigated whether another pyrraline ether could form with residues of serine, threonine, hydroxlysine or hydroxyproline. We found no major modifications with these amino acids [21]. In contrast, extensive pyrraline degradation was observed after just 24 h in pyrraline incubated with N-acetyl-L-cysteine. From this reaction mixture, a thioether involving a cysteine residue linked to the pyrrole hydroxymethyl carbon 4 was isolated (Figure 1). Its structure was confirmed by NMR and MS spectroscopy [21].

From the data above it is now clear that pyrraline is not a very stable product. Considerable degradation can be expected whenever HPLC fractions are concentrated, a procedure which leads to increased
concentration of the poorly volatile acids present in the solvent. Thus, these may act as catalysts for pyrraline degradation. At the present time, data are needed on whether dipyrraline or pyrraline thioether exist in vivo. Such data would be helpful to investigate the non-oxidative advanced pathways of the Maillard reaction in vivo.

Glycoxidation and intracellular AGEs

The oxidative degradation of Amadori products leads, in vitro, to the formation of the 'glycoxidation' products pentosidine and CML [7-9]. As pointed out earlier, however, these advanced Maillard products are not specific for glucose, and most, if not all, reducing sugars tested so far were able to act as precursor compounds of glycoxidation products. Furthermore, ascorbate, which oxidizes in the presence of free metals into the highly labile dehydroascorbate, undergoes further decomposition to form pentoses and tetroses [22], which can also serve as pentosidine precursors [23].

Recent studies on the in vivo mechanism of formation of CML have shown that at least 50% form from a step prior to the Amadori product when glucose is incubated with a protein, and at most 6-7% from glucose autoxidation [5]. That step appears to be identical with the Namiki pathway. Yet another study suggests that only 20% of CML comes from glycated protein when reincubated with glucose, and that up to 80% originates from a step before the Amadori product, perhaps in part from glucose autoxidation [6]. Thus, these recent developments clearly emphasize the great complexity of the pathways leading to glycoxidation products. At the same time, however, they may facilitate our understanding of the biosynthetic mechanism of in vivo glycoxidation, while concomitantly helping to clarify the nature and impact of altered cellular and extracellular processes.

Quantitation of glycoxidation products in lenses from diabetic humans and animals, or animals exposed to high concentrations of reducing sugars, as in galactosaemia, revealed surprising findings. For example, pentosidine was found elevated in one study [24] but not in another [23]. In the former study, CML was normal in diabetic lenses in spite of highly increased amounts of glycated crystallins. Thus, obviously, pentosidine in the diabetic lens must be forming through a mechanism different from CML. As to the discrepancy between the outcome of pentosidine determination in the two studies with diabetic patients, recent data from the authors' laboratory revealed the presence of a glycaemic threshold for pentosidine formation in the lens of diabetic dogs [R. H. Nagaraj et al., submitted for publication] (Figure 2). Whereas crystallin-bound fluorescence and the ascorbate/ribose-derived fluorophore LM-1 [25] were elevated in lenses from chronic diabetic dogs in moderate control of glycaemia (HbA1 = 8.0%), pentosidine was normal in such lenses. In contrast, pentosidine was elevated only in lenses from poorly controlled dogs (HbA1 > 8.0%). Thus, crystallin-bound fluorescence and LM-1 cannot form through the same mechanism as
pentsidine. In fact, *in vitro* studies have shown that ascorbate is a precursor for both compounds, but that only LM-1 can form under de-aerated conditions from dehydroascorbate. Furthermore, glucose and fructose were not found to be precursors of LM-1. It can therefore be concluded that ascorbate is most likely the precursor of LM-1 in the lens. However, pentsidine formation in lenses from poorly controlled dogs may also be linked to membrane permeability increase and increased crystallin glycoxidation [30].

These data clearly indicate that the biosynthetic mechanisms for the formation of advanced Maillard reaction products are unclear, and that the precursor may vary according to ambient glucose concentration. In light of the renewed interest in the role of glycaemic thresholds in the pathogenesis of diabetic complications, advanced products of the Maillard reaction may become very useful as markers of metabolic pathways that are differentially influenced by glycaemic stress.

**Glycation and glycoxidation in end-stage renal disease**

The first evidence for a dramatic acceleration of the advanced Maillard reaction in end-stage renal disease came to light when it was found that pentsidine was highly elevated in diabetic patients who were suffering from end-stage renal disease [26]. Subsequent studies using immunoreactive AGEs confirmed this finding [27], and further demonstrated that renal transplantation almost completely normalized plasma AGE, including pentsidine [27,28].

A detailed study of the kinetics of glycation and pentsidine formation in patients treated with chronic ambulatory peritoneal dialysis was performed by the authors [29]. This treatment modality offers a unique opportunity for *in vivo* kinetic studies of glycation and advanced glycation, since it consists of filling the peritoneal cavity with a concentrated glucose solution (130 mM) in order to promote solute exchange through osmosis. Samples of dialysate can be retrieved at various time intervals for analysis. Over the course of a typical dialysate ‘dwell’ period, the mean peritoneal glucose concentration is estimated at 50 mM.

Using this approach, we first found that the glycation rate of peritoneal proteins (measured as furosine [29]) increases rapidly, i.e. within 2 h of infusing a fresh bag of dialysate. Surprisingly, whereas glycation values were identical with those from plasma proteins at time zero, pentsidine was already highly elevated (Figure 3), suggesting either *in situ* formation of pentsidine or selective passage of pentsidine-rich proteins from the blood into the peritoneum. In order to clarify these possibilities, rates of glycation and pentsidine formation were determined by incubating normal plasma as well as peritoneal proteins isolated from patients treated by peritoneal dialysis with 2.5% Dianeal® (a commercial peritoneal dialysate solution). Whereas protein glycation increased within hours of incubation, only a minor change was noted in pentsidine concentrations. Interestingly, glycation rate was slower in peritoneal proteins than in plasma.
proteins incubated with Dianeal, suggesting a possible protein as a basis for the accelerated glycation rate of peritoneal proteins.

Whereas the rapid increase in furosine, an acid-hydrolysed product of Amadori compounds, is readily explained by the presence of glucose in the dialysis bag, the high pentosidine levels at time zero may reflect the selective passage of pentosidine-rich proteins from the plasma into the peritoneal cavity. Alternatively, this gradient may stem from the trapping of AGE proteins by the peritoneum. In order to further clarify the increase in peritoneal protein pentosidine, we have determined furosine and pentosidine in peritoneal fluid proteins aspirated from normal women at the time of laparoscopic tubal ligation. The results showed that whereas furosine was identical in plasma and peritoneal fluid proteins, there was a 7-fold increase in pentosidine in peritoneal fluid, thereby strongly suggesting a role.

![Graphs](https://academic.oup.com/ndt/article-abstract/11/supp5/20/1856983)

Fig. 4. Bio-Gel P6 gel filtration chromatography of pooled plasma proteins from patients undergoing haemo- or peritoneal dialysis. The chromatograms in the insets show the high-molecular-weight fraction from a Bio-Gel P30 column. Full circles before dialysis, empty circles after dialysis. The major peak at fraction 24-25 corresponds to free pentosidine.
for the peritoneum in the formation of pentosidine-rich proteins in peritoneal fluid, in normal individuals as well as patients treated by peritoneal dialysis.

The distribution of pentosidine in pooled plasma fractions from patients with HD and PD was investigated by fractionation using Bio-Gel P30 and P6 chromatography. The insets in Figure 4 show that neither therapy modality had an effect on pentosidine linked to high-molecular-weight proteins of >40 kDa. In contrast, there was a marked decrease in pentosidine immediately following haemodialysis but not peritoneal dialysis. Whereas this finding could be interpreted to indicate that haemodialysis is more effective at removing pentosidine from plasma, mean steady state concentrations of total pentosidine in peritoneal dialysis patients are 30-40% lower than in haemodialysis, again suggesting that active transport or release of PD-rich proteins may occur at the peritoneal wall.

The rapid effect of HD in decreasing plasma pentosidine without affecting high-molecular-weight protein-linked pentosidine suggests that a fraction of pentosidine is present in free form. Indeed, the major peak in Figure 4 was found to coincide with free pentosidine, and a time-related increase in free pentosidine could be measured over the course of peritoneal equilibration of fresh dialysate (not shown). Similarly, free plasma pentosidine declined after a haemodialysis treatment, whereas no change was observed in peritoneal dialysis patients after a dialysis exchange because of the steady-state nature of peritoneal dialysis. Interestingly, investigation into the nature of pentosidine distribution in peritoneal fluid revealed that ~15% is in free form, 20% linked to low-molecular-weight protein and 65% linked to high-molecular-weight protein. However, in both HD and PD, >95% of total plasma pentosidine is protein-bound.

In summary, determination of kinetics of glycation and advanced glycation in peritoneal dialysis patients has shown that (i) mean plasma pentosidine concentrations are lower in peritoneal than in haemodialysis patients; (ii) active passage through or release of pentosidine-rich proteins from the peritoneal wall exists; and (iii) most plasma pentosidine is protein-bound regardless of mode of therapy.

Conclusions

Structure elucidation and kinetic studies on the formation of advanced Maillard reaction products have shown that pyrraline undergoes further modifications in vivo, especially in the presence of sulphydryl agents such as cysteine. The biological significance of this modification remains to be assessed. Intracellular glycation and glycoxidation products do not follow an expected pattern as a function of the degree of hyperglycaemia, as revealed in experimental animals. Glycation and glycoxidation (pentosidine) are enhanced only in poorly controlled diabetic dog lenses, whereas LM-1 is elevated at moderate levels of hyperglycaemia. Thus, ascorbate may lead to more rapid formation of AGEs than glucose at equivalent concentrations, e.g. as found in the lens. Finally, the dramatic acceleration of AGE formation in plasma from patients with end-stage renal disease is lower in peritoneal dialysis than in haemodialysis. This observation seems paradoxical in view of the intraperitoneal diabetic state induced by glucose-containing dialysate associated with increased intraperitoneal glycated and pentosidine-rich proteins. In both treatment groups most pentosidine is present in the high-molecular-weight protein fraction. Approximately 5% of total pentosidine is in free form, the origin of which remains to be determined.

Acknowledgements: This work was supported by grants EY 07099 (to V. M. M.), EY 09912 (to R. H. N.) from the National Eye Institute, grants 05601 (to V. M. M.) and 64242 (to D. R. S.) from the National Institute on Aging, and grant DK 45619 to M. A. F. from the NIDDK.

References


