Glyceraldehyde-derived advanced glycation end-products having pyrrolopyridinium-based crosslinks

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**ABSTRACT**

Reducing sugars and reactive aldehydes, such as glyceraldehyde, non-enzymatically react with amino or guanidino groups of proteins to form advanced glycation end-products (AGEs) by the Maillard reaction that involves Schiff base formation followed by Amadori rearrangement. AGEs are found relatively in abundance in the human eye and to accumulate at a higher rate in diseases that impair vision such as cataract, diabetic retinopathy or age-related macular degeneration. We identified two novel AGEs of pyrrolopyridinium lysine dimer derived from glyceraldehyde, PPG1 and PPG2, in the Maillard reaction of N\(^\alpha\)-acetyl-L-lysine with glyceraldehyde under physiological conditions. Having fluorophores similar to that of vesperlysine A, which was isolated from the human lens, PPGs were found to act as photosensitizers producing singlet oxygen in response to blue light irradiation. Moreover, PPG2 interacts with receptor for AGE (RAGE) in vitro with a higher binding affinity than GLAP, a well-known ligand of the receptor. We also proposed a pathway to form PPGs and discussed how they would be formed in vitro. As glyceraldehyde-derived AGEs have been studied extensively in connection with various hyperglycemia-related diseases, further studies will be required to find PPGs in vivo such as in the lens or other tissues.

1. Introduction

Advanced glycation end-products (AGEs) are produced non-enzymatically by the Maillard reaction typically between amino or guanidino groups of proteins and reducing sugars or sugar-derived reactive aldehydes. Although AGEs are well known to cause food browning, they are also found to accumulate in vivo and therefore drawing attention largely from pathogenic points of view. For instance, proteins of the human eye are highly susceptible to the formation of AGEs that accumulate with age in the lens and retina particularly at higher rates in diseases that impair vision such as cataract, diabetic retinopathy and age-related macular degeneration [1,2]. In the lens, AGEs induce irreversible changes in structural proteins to form high-molecular-weight aggregates that scatter light and impede vision. A milestone in the AGE research is the discovery of pentosidine by Sell and Monnier [3], which is a fluorescent protein- crosslink that accumulates in the lens with age [4]. Another fluorescent crosslink with lysine residues, vesperlysine A, was also identified as a specific marker for a diabetic process in the human lens [5,6]. These discoveries established that AGEs were integral to the lens aging and subsequent cataract formation. Indeed, there are more than 15 AGEs that have been identified to date in the human lens [2].

Fluorescent AGEs tend to generate reactive oxygen species (ROS) when irradiated by ultraviolet A (UVA) light. Previous studies described that pentosidine generated ROS and singlet oxygen in response to UVA irradiation [7,8]. In addition, a human lens fluorophore LM-1, which was found to be identical to vesperlysine A [6], has also been reported to produce singlet oxygen under the same conditions [7]. These reports indicate that UVA-photosensitized AGEs induce ROS-mediated damages to tropohtan and histidine residues in lens proteins. Therefore, photosensitive AGEs, if accumulated in the lens, possibly play a role in the pathogenesis of the diseases of the eye by photosensitization-induced oxidative stress.

Among many AGEs reported thus far, we have focused on glyceraldehyde-derived AGEs (Glycer-AGEs) in this study mainly because pentosidine, one of the most common fluorescent AGEs, was...
reported to be produced in the Maillard reaction of N\textsuperscript{\alpha}-acetyl-lysine and N\textsuperscript{\alpha}-acetyl-arginine with glyceraldehyde under physiological conditions [9]. In addition, Nakamura et al. identified fluorescent AGEs having a unique pyrrolopyridinium structure, vesperlysines, also in a similar reaction with glyceraldehyde and n-pentylamine (Fig. S1) [5]. Glyceraldehyde can be produced in vivo most likely from aldolase-catalyzed conversion of fructose 1-phosphate in polyol metabolism, which is facilitated under high glucose conditions [10]. Glyc-AGEs have been suggested to be associated with a variety of diseases related to hyperglycemia [11]. Furthermore, recent evidence suggests that the interaction of Glyc-AGEs with the receptor for AGEs (RAGE) elicits oxidative stress in numerous types of cells, which might contribute to the pathological changes observed in diabetic complications such as diabetic retinopathy [1,12–14]. We therefore suppose that glyceraldehyde can be a key precursor aldehyde to produce AGEs that enhance oxidative stress, particularly in the eye, both in the presence or absence of light. Although glyceraldehyde-derived pyridinium compound (GLAP) and lys-hydroxy-triosidine (L-H-T), which is a pyridinium-type crosslinked AGE, were identified as Glyc-AGEs [15,16], pathogenic roles of Glyc-AGEs still remain elusive.

In this work through extensive mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses, we have identified two new vesperlysine-like fluorescent AGEs, pyrrolopyridinium lysine dimer derived from glyceraldehyde (PPG1 and PPG2), in which two lysine molecules are crosslinked by pyrrolopyridinium ring structures (Fig. 1A), as the major products in the Maillard reaction between N\textsuperscript{\alpha}-acetyl-l-lysine and glyceraldehyde. Of these two AGEs, PPG2 was found to be photosensitive to generate singlet oxygen and to have a binding affinity to RAGE. We described herein the structural determination of these AGEs and discussed how they can be formed in vitro. In trying to find a new AGE in vivo and to explore its pathological roles, in vitro approach seems essential to elucidate the chemical structure as well as to further develop an antibody for immunological studies. Although PPGs need to be identified in vivo, we think that they might be useful to elucidate pathogenic roles of Glyc-AGEs and to study oxidative stress induced by photosensitization as well as RAGE-mediated signal transduction in the eye.

2. Materials and methods

2.1. Maillard reaction of N\textsuperscript{\alpha}-acetyl-l-lysine with glyceraldehyde

N\textsuperscript{\alpha}-acetyl-l-lysine (Ac-Lys, Tokyo Chemical Industry, Tokyo, Japan) and DL-glyceraldehyde (Nacalai Tesque, Kyoto, Japan) were dissolved at once in 0.5 M phosphate buffer (pH 7.4) at a concentration of 1.0 M each. The resulting solution was sterilized by filtration through a 0.2-μm filter and then incubated at 37 °C for one week.

![Fig. 1. Detection of GLAP, L-H-T and PPGs in the Maillard reaction mixture of Ac-Lys with glyceraldehyde. (A) Chemical structures of four Glycer-AGEs. R indicates the Ac-Lys moiety. (B) UV chromatograms of the reaction mixture. (C) UV absorbance and (D) single MS spectra of four peaks detected in the Maillard reaction mixture. Detailed analytical conditions are summarized in Table S1.](image-url)
2.2. Chromatographic purification of the maillard reaction products

The Maillard reaction mixture containing Ac-Lys and glyceraldehyde was subjected to high-performance liquid chromatography (HPLC) equipped with a diode array detector (DAD) and a mass selective detector (MSD) using an Agilent 1260 Infinity II LC/MSD system (Agilent Technologies, Santa Clara, CA, USA). The instrumentation is detailed in our previous report [22]. Chromatographic conditions are summarized in Table S1. Isolation of GLAP, L-H-T and PPGs was performed by a semi-preparative high-performance liquid chromatography (HPLC) with UV signal-triggered fraction collection; the conditions are described in Table S2. Collected fractions were evaporated in vacuo and lyophilized to give the product as formate salt.

2.3. Structural determination of PPGs

The chemical composition of PPGs was analyzed using an Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system (Agilent Technologies). The instrumentation is detailed in our previous report [22]. Analytical conditions are summarized in Tables S3 and S4. Chromatographic conditions are summarized in Table S1. Isolation of GLAP, L-H-T and PPGs was performed by a reversed-phase HPLC equipped with a DAD and a MSD. When detected at 290 nm, the most intense peak appeared at the retention time (Rt) of 3.3 min, whose absorption maximum was 290 nm (Fig. 1B and C). The MS spectrum of this peak corresponded to that of GLAP as a positive ion with mass-to-charge (m/z) of 297 (Fig. 1D). After semi-preparative purification of this peak by HPLC with UV detection as shown in Fig. S2, the chemical structure was confirmed to be GLAP using several NMR techniques, the data of which were identical to those reported previously [15] (Figs. S3-S5). In a similar manner, the peak detected at 269 nm and Rt of 9.0 min (Fig. 1B) was next isolated and its chemical structure was determined to be L-H-T based on the UV-visible spectrum (Fig. 1C), MS spectrum (Fig. 1D) and NMR spectra (Figs. S6-S8). Surprisingly, when detected with a DAD at 254 nm, two intense peaks appeared at Rt of 9.9 min and 10.9 min corresponding to PPG1 and PPG2, respectively (Fig. 1B). The UV–visible spectra indicated the absorption maxima at 252 nm, 280 nm and 380 nm for PPG1 and 254 nm, 304 nm and 388 nm for PPG2, respectively (Fig. 1C). The absorption maxima of PPGs at longer wavelengths compared to those of GLAP and L-H-T suggest that PPGs have an aromatic moiety different from pyridinium structures of GLAP or L-H-T. The single quadrupole MS spectra of PPG1 and PPG2 showed a positive ion at m/z 507 and 521, respectively (Fig. 1D), indicating that PPGs are crosslinked dimer analogs of lysine.

3. Results and discussion

3.1. HPLC analysis of the maillard reaction mixture of Ac-Lys and glyceraldehyde

Ac-Lys was incubated with an equimolar amount of glyceraldehyde under physiological conditions, and the reaction mixture was analyzed by a reversed-phase HPLC equipped with a DAD and a MSD. When detected by a reversed-phase HPLC equipped with a DAD and a MSD. When
compared to PPG1, which was assigned to the methyl group conjugated to the pyrrole ring (Figs. S14 and S15). The $^1$H-NMR spectrum measured in D$_2$O showed a peak of the methylene protons almost overlapped with that of residual water at 4.8 ppm (Fig. S15), but when measured in DMSO-d$_6$, the methylene protons were observed as a singlet with improved resolution between 4.6–5 ppm (Fig. S19). The NMR data of PPG2 in DMSO-d$_6$ are summarized in Fig. S18; $^1$H- and $^{13}$C-NMR spectra in DMSO-d$_6$ are shown in Figs. S19 and S20, respectively. The spectrum of $^1$H-$^{15}$N HMBC demonstrated that the aromatic protons of PPG2 correlated with the two nitrogens of different chemical properties (at position 1 and 5 in Fig. S17). Like PPG1, the aromatic ring of PPG2 was thought to contain both quaternary and tertiary nitrogens from the $^1$H-$^{15}$N HMBC as well as MS/MS spectral data (Figs. S9 and S17). These NMR data characterized PPG2 to have the following structure: 1,4-disubstituted 3-hydroxy-8-(hydroxymethyl)-4-methyl-$^1$H-pyrrolo[3,4-b]pyridin-1-ium, as shown in Fig. S14. To further confirm this aromatic core structure, $^{13}$C-$^{13}$C INADEQUATE experiment was carried out to observe the carbon-carbon correlation (Fig. S21). The spectral data also supported the interesting carbon skeleton in which the positional relationship of the two nitrogens in the pyrrolopyridinium ring is different from that of PPG1.

Similar pyrrolopyridinium structures were first reported as vesperlysines in the Maillard reaction of lysine with glucose [5]. However, as discussed by Tessier et al. who isolated vesperlysine A from the human lens proteins [6], the precursor aldehyde and the mechanism to produce vesperlysine A have remained unclear. As Nakamura et al. pointed out that vesperlysines were produced from one of glycoxidation products of glucose [5], we suppose that glycéraldehyde is an important precursor aldehyde to form the AGEs having pyrrolopyridinium structures.

3.3. Putative pathway to form PPGs in the maillard reaction of Ac-Lys with glycéraldehyde

Scheme 1 illustrates our proposed pathway to produce both PPG1 and PPG2. The initial Maillard reaction to expand the skeleton involves Schiff base formation between the $\varepsilon$-amino group of lysine and glycéraldehyde followed by Amadori rearrangement, resulting in the formation of adduct 2. By being dehydrated, adduct 2 cyclizes to give intermediate 3, a common precursor to GLAP as well. Although less likely, intermediate 3 can be further crosslinked to another lysine molecule via its keto functionality to give intermediate 4, which is further coupled with glycéraldehyde in two different mechanisms, as shown in path a and path b in Scheme 1. In path a which involves Schiff base formation, product 5 loses formaldehyde to give aldehyde 6, instead of Amadori rearrangements. The loss of formaldehyde is more likely in this case since it is also supposed in the formation mechanism of pentosidine [9]. Further spontaneous cyclization of aldehyde 6, followed by two-electron oxidation, finally affords PPG1. In contrast to path a, path b involves an aldol-type coupling reaction with glycéraldehyde to form the C-C bond, instead of C-N bond formation in Schiff base formation, yielding adduct 7. It seems unique that the direct C-C bond formation takes place to expand the skeleton in these steps. Adduct 7 is dehydrated to give ketone 8, which facilitates the intramolecular pyrrole ring formation yielding 9. Further dehydration of 9 finally affords PPG2.

As described above, Nakamura et al. reported the formation of AGEs of the same pyrrolopyridinium structures but with different substitution.
patterns (vesperlysines, Fig. S1) in the Maillard reaction with n-pentyl-
amine and glyceraldehyde, but they did not discuss how vesperlysines 
were produced in terms of reaction mechanism [5]. Although they 
performed the instrumental analysis of vesperlysines using fully acetyl-
ated derivatives, which differs slightly from our method, the reason that 
their similar reaction produced different molecules from PPGs is 
currently unclear.

Similarly, GLAP was also reported by Usui et al. to be the major 
product in the Maillard reaction with the same substrates as those that 
we used in our work, Ac-Lys and glyceraldehyde [15]. They carried out 
the reaction, however, under conditions with much lower substrate 
concentrations (0.1 M and 0.2 M for Ac-Lys and glyceraldehyde, 
respectively). Under the identical conditions to those used by Usui et al., 
the reaction, however, under conditions with much lower substrate 
we used in our work, Ac-Lys and glyceraldehyde [15]. They carried out 
product in the Maillard reaction with the same substrates as those that 
used Ac-Lys, which is N\(^{\alpha}\)-acylated, as a model of such modification to 
block the reactions of the \(\alpha\)-amino group. In fact, the same reaction with 
non-acylated lysine gave very complex reaction mixture that seemed 
almost impossible to isolate any single molecules. Although we think it 
unlikely that PPGs in the form of amino acids (not proteins) exist in vivo, 
further studies will be needed to consider the effect of the \(N^{\alpha}\)-acyl 
group as well as to prove that non-acylated PPG2 is also a ligand for 
RAGE. Deprotecting acetylated PPGs using a similar approach employed 
for \(N^{\alpha}\)-acylated GLAP and L-H-T, in which protection was performed by 
acid hydrolysis, might be an option [16,19].

As GLAP was also suggested by Matsui et al. to be a main structure of 
Glycer-AGEs that increased ROS production and upregulated inflamma-
tory and thrombogenic gene expression in human umbilical vein 
endothelial cells via its binding to RAGE [23], it is interesting to know 
whether PPGs, particularly PPG2 if it exists in vivo, have similar bio-
logical activities to GLAP such as stimulating RAGE-regulated oxidative 
and inflammatory reactions.

3.4. In vitro interaction of PPGs with RAGE

To investigate whether PPGs bind to RAGE, we evaluated in vitro 
inhibitory capacity of PPGs in the binding of a recombinant soluble 
RAGE with Glycer-AGEs-BSA, a well-known RAGE-binding AGE. The 
inhibitory capacity in the assay system means the binding capacity of 
PPGs to RAGE. Since quartz crystal microbalance analyses revealed that 
\(N^{\alpha}\)-acylated form of GLAP bound to RAGE in a dose-dependent manner 
[23], we used \(N^{\alpha}\)-acyt GLAP as a positive control in the RAGE-AGE 
interaction and evaluated the binding capacity of \(N^{\alpha}\)-acyt PPGs and 
L-H-T to RAGE. As shown in Fig. 2, GLAP inhibited the binding between 
RAGE and Glycer-AGEs-BSA more than 40 \% at a concentration of 6 mM 
compared to the vehicle sample. PPG2 similarly inhibited the binding 
more than 40 \% at a concentration of 4 mM, suggesting that PPG2 is a 
ligand for RAGE with a higher binding affinity than GLAP. PPG1 and 
L-H-T, in contrast, were found to show a weak binding inhibition toward 
RAGE. The 3-hydroxypyridinium structure of GLAP was previously 
described by Murakami et al. to be essential as the binding moiety in the 
interaction with RAGE [19]. Our results, however, suggest that a pyr-
ridinium ring substituted at its \(\varepsilon\) position with a hydroxymethyl 
group, which both GLAP and PPG2 have in common, would be import-
ant in binding to RAGE rather than a 3-hydroxypyridinium structure. It 
is also interesting that, despite being a crosslinked type of AGE, PPG2 
can interact with RAGE.

As mature AGEs such as pentosidine and vesperlysines are formed 
over the years as chemical modifications on the \(\varepsilon\)-amino or guanidino 
group of lysine or arginine residue, respectively, of long-life proteins, we 
used Ac-Lys, which is \(N^{\alpha}\)-acylated, as a model of such modification to 
block the reactions of the \(\alpha\)-amino group. In fact, the same reaction with 
non-acylated lysine gave very complex reaction mixture that seemed 
almost impossible to isolate any single molecules. Although we think it 
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and inflammatory reactions.

3.5. Photosensitization of PPGs

Both PPG1 and PPG2 are fluorescent with their emission maxima at 
450 nm when excited at their excitation maxima at 350 nm and 380 nm 
for PPG1 and PPG2, respectively, at pH 7.5 (Fig. S22). Since the fluo-
rescence properties of PPGs are similar to those of vesperlysin A that 
fluoresces at 442 nm (excited at its excitation maximum at 366–383 nm) 
[5], PPGs were also expected to exhibit photosensitization effects due to 
their pyrrolopyridinium structures. We tried to detect singlet oxygen 
generated from PPGs by light irradiation using an ESR spectrometer. A 
short-wavelength visible blue light (wavelength at 470 nm) was used to 
photosensitize PPGs because blue light can be more phototoxic to the 
eye. For example, blue light was reported to induce apoptosis of rat 
retinal cells [20]. In the ESR experiment, singlet oxygen was measured 
quantitatively as an amount of 4-OH-TEMP radical, a stable nitrooxide 
radical, that is produced by the reaction of 4-OH-TEMP with singlet 
oxogen, as shown in Fig. 3, in which black columns indicate that the 
samples were not irradiated (placed in dark). All AGEs were observed to 
generate singlet oxygen compared to Ac-Lys(Z), a fully protected lysine 
analogue as a negative control. The ESR signals of these AGEs decreased by 
the presence of astaxanthin, a specific quencher of singlet oxygen [21],

Fig. 2. Binding inhibition of RAGE and Glycer-
AGEs-BSA by \(N^{\alpha}\)-acylated Glycer-AGEs in vitro. 
Purified four Glycer-AGEs were used as competitors in 
the binding assay of His-tagged soluble RAGE with 
Glycer-AGEs-BSA. Relative RAGE and Glycer-AGEs- 
BSA binding rate is calculated as relative absorb-
ance at 450 nm in the presence of each competitor 
against its vehicle control and represented as an 
average of two independent experiments with stan-
dard derivatives. The concentration of competitors 
used (0.5, 1, 2, 4, 6, 8 and 10 mM) is represented as 
logarithm. Dose response curve is represented as a 
third order polynomial curve.
which further supports the generation of singlet oxygen induced by photosensitization. All crosslinked-type AGEs (PPGs and L-H-T) showed substantial photosensitization effects, whereas non-crosslinked type AGE (GLAP) was found to generate a much smaller amount of singlet oxygen under identical conditions probably because of lack of long-wavelength light absorptions (Fig. 1C).

In summary, AGEs tend to accumulate particularly in the eye which is constantly exposed to light [2]. Photosensitive AGEs, if accumulated in the eye, could enhance oxidative stress and causes various damages of the eye. Glyceraldehyde has been suggested to be a precursor aldehyde to form fluorescent AGEs, such as pentosidine and vespérysin, both of which are photosensitive as well [5–9]. Also, Glyceraldehyde-5-phosphate was suggested to play roles in various hyperglycemia-related diseases [11]. We have identified in this work novel Glycer-AGEs, PPG1 and PPG2, in which two lysine molecules are crosslinked via the pyrrolopyridinium ring structures, in the Maillard reaction between Ac-Lys and glyceraldehyde under physiological conditions. Both PPGs are photosensitive to generate singlet oxygen by light irradiation, and PPG2 shows a binding affinity toward RAGE. We have also proposed a mechanism, by which both PPGs are formed in vitro, and which involves a unique skeleton expansion reaction in addition to the typical Schiff base formation.

In order to find PPGs in vivo, it is important to know the acid stability of PPGs because it requires an acid hydrolysis of tissue samples and also helps to find conditions to deprotect acetylated PPGs. Since Nakamura et al. reported that vespérysin was stable to acid hydrolysis [5], we expect that PPGs are also stable to these acidic conditions. As an alternative approach to identify PPGs in cells and tissues, we also plan to perform immunohistochemical studies by developing monoclonal antibodies against PPGs. Although further study is required to detect PPGs in vivo, we expect future applications of PPGs as useful tools in the AGE research, in particular, to investigate pathogenic aspects of Glyceraldehyde-5-phosphate that induce oxidative stress by photosensitization as well as RAGE-mediated signal transduction in the eye.

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Credit author statement

Tomoaki Shigeta: Conceptualization, Methodology, Validation, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Project administration. Kazumi Sasamoto: Conceptualization, Writing - Original Draft, Writing - Review & Editing, Project administration, Supervision. Tetsuro Yamamoto: Writing - Review & Editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.100963.

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