Anti-glycative effect of palladium and platinum nanoparticle solution

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Abstract

Aim: Glycation, the non-enzymatic reaction between proteins and reducing sugars, generates advanced glycation end products (AGEs), and accumulation of AGEs is exceeded in diabetic patients. Oxidation is one of the critical steps in the process of glycation. In the present study, we investigated the effect of PAPLAL (palladium and platinum nanoparticles), which is known to be a potent antioxidant on glycation.

Methods: AGEs were formed by using three different combinations of proteins and sugars. Human serum albumin (HSA) and glucose were incubated at 60 °C for 40 hours. Both type I collagen and type II collagen were mixed with fructose and were incubated at 60 °C for 24 hours. Fluorescent AGEs were measured by their typical fluorescence of 370/440 nm. Non-fluorescent AGEs, \(N_\varepsilon\)-(carboxymethyl)lysine (CML) and intermediates of AGEs: 3-deoxyglucosone (3-DG), glyoxal (GO) and methylglyoxal (MGO) were determined using ELISA and HPLC-UV analyses, respectively.

Results: Palladium and platinum nanoparticles inhibited the formation of fluorescent AGEs in all three protein-sugar models. In the HSA-glucose model, PAPLAL inhibited CML, 3-DG, GO and MGO formation in a dose dependent manner.

Conclusions: These results suggest that the palladium and platinum nanoparticle eliminated the formation of several different kinds of AGEs and their intermediates in vitro.

KEY WORDS: Glycation, palladium and platinum nanoparticle, fluorescent AGEs, \(N_\varepsilon\)-(carboxymethyl)lysine (CML), intermediates of AGEs

Introduction

Advanced glycation end products (AGEs) are generated and accumulated in the body due to non-enzymatic reactions between proteins and excess substances which have reduction abilities, such as reducing sugar and aldehydes. This phenomenon is named glycation and several lines of evidence indicated that accumulation of AGEs is associated with age-related diseases such as cancer, osteoporosis, Alzheimer’s disease, and cardiovascular disease. If not a deadly disease, when AGEs are formed in a long-lived protein such as collagen and elastin, modified protein may cause dysfunction in the body.

\(N_\varepsilon\)-(carboxymethyl)lysine (CML) is one of the well studied AGEs. CML is accumulated in cardiovascular tissue in diabetic patients. Hwang et al. also reported that serum levels of CML associated with diabetic neuropathy. In human skin, CML is predominantly accumulated in epidermis in aged skin, and cytokeratin 10 in stratum corneum is one of the proteins modified by CML. CML also accumulated in elastic fibers in dermis, especially sun-exposed areas. Iwai et al. reported that the amount of protein carboxylation in stratum corneum correlates to loss of skin clarity. Thus, prevention of AGEs formation is a beneficial for not only lifestyle-disease prevention, but also anti-aging and health promotion.

In the process of AGE formation, proteins and sugars firstly form a Schiff base, they then rearrange amadori products and intermediates of AGEs. Once their structures were cleaved by oxidation, various kinds of AGEs are generated, such as pentosidine, CML, \(N_\varepsilon\)-(carboxymethyl) arginine (CMA), \(N_\varepsilon\)-(carboxyethyl)lysine (CEL), pyrralin, crossline and so on. CML is also directly formed through autoxidation of glucose. Therefore, oxidation is one of the critical steps in glycation.

Platinum (Pt) nanoparticles are recently reported as strong and broad antioxidants which scavenge superoxides and peroxides. However, the surface of Pt nanoparticles is easy to oxidize, even in the air, at room temperature.
and that causes loss of anti-oxidative effects. Okamoto et al. demonstrated that co-treatment of palladium (Pd) nanoparticles suppressed oxidative stress-induced deterioration of Pt nanoparticles. PAPLAL, the mixture of Pt (0.2 mg/mL) and Pd (0.3 mg/mL) nanoparticles, has been used for various diseases associated with inflammation for many years in Japan. However, to our knowledge, there is no report about its anti-glycative effect.

In the present study, we investigated the effect of PAPLAL, which is known to be a potent antioxidant on glycation.

Materials and Methods

Materials

Human serum albumin (HSA) was purchased from Sigma-Aldrich (St. Louis, MO). Type I collagen was obtained from Nippi (Tokyo, Japan) and type II collagen was provided from Elastin Products Company (Owensville, MO). CML ELISA kit was purchased from MBL (Nagoya, Japan). Other chemicals were obtained from Wako (Osaka, Japan) or Dojindo (Kumamoto, Japan) for analytical grade.

Nanoparticles

Palladium and platinum nanoparticle solution, namely PAPLAL, was supplied from Musashino Pharmaceutical Co. (Tokyo Japan). PAPLAL is contained with 0.3 mg/mL (2.82 mM) of Palladium nanoparticles and 0.2 mg/mL (1.03 mM) of Platinum nanoparticles.

Preparation of glycated proteins

Three glycation models, i) HSA with glucose, ii) type I collagen with fructose and iii) type II collagen with fructose were used. Briefly, i) 8 mg/mL HSA was mixed with 0.2 M glucose in 50 mM phosphate buffer (PB, pH 7.4) and incubated at 60 °C for 40 hours, ii) and iii) 0.6 mg/mL collagen with 0.4 M fructose in 50 mM PB (pH 7.4) were incubated at 60 °C for 24 hours (named “solution A”). As a background, heated proteins without sugars were also prepared (solution B). To determine the effects of PAPLAL on glycation, indicated amounts of PAPLAL were also prepared (solution B). To determine the effects of PAPLAL on glycation, indicated amounts of PAPLAL were also prepared (solution B). To determine the effects of PAPLAL on glycation, indicated amounts of PAPLAL were also prepared (solution B).

Measurement of AGEs-derived fluorescence

AGEs-derived fluorescence was measured as previously described. Briefly, 200 μL of the reaction mixture was used to measure fluorescence at an excitation wavelength of 370 nm and an emission wavelength of 440 nm by a Varioskan Flash (Thermo scientific, Waltham, MA) microplate reader. The value was calculated using the equation below.

$$\text{Ratios of AGEs-derived fluorescence [\%]} = \frac{\text{fluorescence of (solution C – solution D)}}{\text{fluorescence of (solution A-solution B) x 100}}$$

CML measurement

CircuLex CML/Nε-(Carboxymethyl)lysine ELISA kits were used according to manufacturer protocol to determine the amount of CML in glycated proteins.

Measurement of intermediates of AGEs

Three kinds of intermediates of AGEs, 3-deoxyglucosone (3-DG), glyoxal (GO) and methylglyoxal (MGO), were measured using a Shimadzu high-performance liquid chromatography ultraviolet (HPLC-UV) system (Shimadzu Corporation, Kyoto, Japan). Samples were prepared as previously described with slight modification. Briefly, reaction mixture were deproteinized using 6% perchloric acid. After centrifugation, supernatant was immediately neutralized by excess amounts of sodium bicarbonate. Then, 3-DG, GO and MGO were labeled with 2,3-diaminonaphthalene (Dojindo) for 24 hours at 4 °C. The HPLC conditions were as follows; Column, UnisonUK - Phenyl, 75 mm x 3 mm I.D. column (Imtakt Corp, Kyoto, Japan); eluent, 50 mM phosphoric acid and acetonitrile = 89 : 11. The flow rate and detection wavelengths were 1.0 mL/min and 268 nm.

Statistics

Data were expressed as mean ± standard deviation (SD) of at least three independent experiments. The statistical analyses were performed by the Student’s t-test and an analysis of variance (ANOVA) was taken using Tukey-Kramer test for multiple comparisons. Differences were considered significant at p values less than 0.05.

Results

PAPLAL inhibited fluorescent AGEs formation in various glycation models.

First, we investigated that whether or not PAPLAL affected glycation. Ten percent volume content of PAPLAL was introduced into three different kinds of protein-sugar models: HSA-glucose model, type I collagen-fructose model and type II collagen-fructose model as described in “Materials and Methods”. Fluorescent AGEs were measured at 370/440 nm which is the characteristic wavelength of fluorescent AGEs. As shown in Fig. 1, 10% PAPLAL inhibited fluorescent AGEs formation significantly in all three models. Especially, in the HSA-glucose model, ten % PAPLAL decreased glycation-derived fluorescent AGEs by over 70% (Fig. 1A).

PAPLAL inhibited fluorescent AGEs formation in a dose-dependent manner.

As shown in Fig. 1A, 10% PAPLAL has a drastic effect against fluorescent AGEs formation in the HSA-glucose model. We further evaluated the effects of PAPLAL at lower concentrations. PAPLAL inhibited fluorescent AGEs formation dose-dependently in the HSA-glucose model; even 0.1% PAPLAL suppressed it significantly (Fig. 2).

Effect of PAPLAL on CML formation in the HSA-glucose model.

Even though 370/440 nm is a characteristic fluorescent wavelength of AGEs, there are several AGEs which do not have fluorescence. In this experiment, we assessed the effects...
**Fig. 1.** Effect of PAPLAL on fluorescent AGEs formation in various glycation models.

To determine the inhibitory effect of PAPLAL on fluorescent AGEs formation, 10% volume content of PAPLAL was used. Fluorescent AGEs were measured at 370/440 nm. (A) HSA-glucose model, (B) type I collagen-fructose model and (C) type II collagen-fructose model. All data were shown as the mean ± SD (n = 3) of the ratios against water. **p < 0.01 vs. water.**

AGEs, advanced glycation end products; HSA, human serum albumin; SD, standard deviation.

**Fig. 2.** Dose-dependent study of PAPLAL on fluorescent AGEs formation in HSA-glucose model.

Indicated percentages of PAPLAL were used to investigate the inhibitory effect of PAPLAL on fluorescent AGEs formation in HSA-glucose model. Fluorescent AGEs were measured at 370/440 nm. All data were shown as the mean ± SD (n = 3) of the ratios against 0% PAPLAL. **p < 0.01 vs. 0% PAPLAL.** AGEs, advanced glycation end products; HSA, human serum albumin; SD, standard deviation.
of PAPLAL on the formation of CML, one of the non-fluorescent AGEs, by using ELISA. As well as fluorescent AGEs, PAPLAL decreased CML formation in a dose dependent manner, while PAPLAL did not alter basal levels of CML. (Fig. 3).

Effect of PAPLAL on formation of intermediate of AGEs in the HSA-glucose model.

In the process of AGE formation, various kinds of intermediates are produced and accumulated in the body. In this experiment, we investigated the effects of the serial dilution of PAPLAL on the formation of intermediates of AGEs in the HSA-glucose model. We performed HPLC-UV analyses to measure three different kinds of intermediates, 3-DG, GO and MGO. GO and MGO were reduced by PAPLAL in a dose-dependent manner (Figs. 4A, B), while 3-DG was slightly, but significantly increased to over a 0.5 % content in the reaction mixture (Fig. 4C). None of them were detectable when HSA was heated without glucose.

Discussion

Glycation, the non-enzymatic reaction between reducing sugars and proteins, forms various kinds of AGEs in human tissues and organs. Mostly, AGEs-modified proteins are decomposed or metabolized, thus, the body can maintain physiological homeostasis. However, when long-lived proteins are glycated, it may disturb their metabolism and affect their functions. Also accumulated AGEs induce inflammation in tissues, leading to dysfunctions. In bone collagen, glycation causes loss of bone stiffness and resilience and is involved in age-related diseases such as osteoarthritis, rheumatoid arthritis and osteoporosis 3,18-22. Gomi reported that glycation of long-lived protein in skin tissue, such as type I collagen and elastic fibers, mediates loss of skin elasticity 23. Accumulation of AGEs in skin are evaluated using a noninvasive autofluorescence reader. In diabetic patients, skin autofluorescence is strongly associated with cardiac mortality 24. It also has a strong correlation between age 25 and short sleeping hours 26 in healthy subjects. These findings suggested that AGEs in skin tissues are not only a diagnostic indicator but also may be a serious cause of skin aging.

In our previous study, we investigated over 500 kinds of food materials such as tea leaves, vegetables, fruits and spices against glycation 27-31. Also, we demonstrated that the anti-lycative effect in herbs has a strong correlation with their polyphenol content 32. Because oxidation plays a critical role in the early steps of glycation, antioxidants such as polyphenol may affect glycation. PAPLAL, the mixture of Pt and Pd nanoparticles is known to be a strong antioxidant and has been used for a many years in Japan 33. In clinical trials and experimental studies on skin disorders, topical use of PAPLAL cream improved vitiligo subjects 34 and transdermal PAPLAL treatment mitigated skin atrophy associated with increased lipid peroxidation in Sod1-/- mice 17.

In the present study, we investigated the efficacy of PAPLAL on anti-glycation by using three different proteins such as HSA, type I collagen and type II collagen. Serum albumin is the most abundant protein in blood and exposed

Fig. 3. PAPLAL inhibited CML formation in a dose-dependent manner in HSA-glucose model.

Indicated percentage of PAPLAL were introduced into HSA-glucose model and heated at 60 °C for 40 hours. CML formations were determined by ELISA. All data were shown as the mean ± SD (n = 3). * p<0.05 vs. glucose with 0 % PAPLAL. CML, Nε-(carboxymethyl)lysine; HSA, human serum albumin; SD, standard deviation.
Fig. 4. PAPLAL inhibited formation of intermediate of AGEs in HSA-glucose model.

Indicated percentages of PAPLAL were introduced into HSA-glucose model and heated at 60 °C for 40 hours. Intermediates of AGEs were detected by HPLC-UV. (A) GO, (B) MGO and (C) 3-DG. All data were shown as the mean ± SD (n = 3) of the ratios against 0% PAPLAL. ** p<0.01 vs. 0% PAPLAL. AGEs, advanced glycation end products; HSA, human serum albumin; GO, glyoxal; MGO, methylglyoxal; 3-DG, 3-deoxyglucosone; SD, standard deviation.
to blood glucose constitutively, even though its half life is within a couple of weeks. Collagen is one of the long-life proteins; type I collagen reside in bone and dermis and type II collagen exist in cartilage. We demonstrated that 10% PAPLAL inhibited fluorescent AGEs formation in all three proteins (Fig. 1). Even 0.1% PAPLAL showed significant suppression against HSA-glucose model (Fig. 2). We previously demonstrated that the pattern of formed AGEs were different in each protein). Therefore, these results suggest that PAPLAL is an extraordinary effective product against glycation-related protein disorders.

Accumulation of CML in the dermis is one of the causes of dullness and loss of skin clarity. Even though CML is a non-fluorescent AGEs, its amount has a correlation with skin autofluorescence AGEs. Also, accumulation of skin AGES has a correlation with glycated proteins in blood, such as hemoglobin Alc (HbAlc). In the present study, we demonstrated that PAPLAL inhibited CML formation in HSA-glucose model (Fig. 3). This result indicates that orally administrated PAPLAL may also be potentially effective for skin problems caused by blood glycation. CML was detectable in heated HSA in the absence of glucose, and PAPLAL had no effect on CML amount in this reaction (Fig. 3). We considered that HSA which isolate from human blood originally contained certain amount of CML, and PAPLAL could not decompose already formed CML. However, further studies are needed to discuss the effect of PAPLAL on breakdown of AGES.

Intermediates of AGES such as GO, MGO and 3-DG are highly reactive compounds to modulate proteins to form various kinds of AGES. In our data, PAPLAL inhibited GO (Fig. 4A) and MGO (Fig. 4B) formation. Because GO is an intermediate of CML, PAPLAL inhibited CML formation through inhibition of GO formation. MGO is known to be an intermediate of CEL, PAPLAL may affect CEL formation, as well. In skin collagen, Monnier et al. demonstrated that accumulation of AGEs including CML and CEL increased with age. These findings suggest that PAPLAL may have the potential to reduce AGEs formation in skin, leading to better maintenance of skin health, while 3-DG was elevated slightly, but significantly, by PAPLAL (Fig. 4C). Niwa et al. reported that imidazoleone, metabolite of 3-DG accumulated in glomeruli and in the renal artery wall in an advanced stage of diabetic nephropathy in diabetes patients. 3-DG inhibited mesangial cell adhesion, leading to the loss of critical cell-matrix interactions in the kidney. From this point of view, an increase in 3-DG is undesirable side effect. The mechanism by which PAPLAL induce 3-DG is still unknown, however, elevated levels of 3-DG by PAPLAL was very little, as while GO and MGO, other critical intermediated against diabetes were drastically inhibited. Further study is required to determine the relevant concentration of PAPLAL for treatment.

In conclusion, our study shows that PAPLAL, the colloids of Pt, and Pd nanoparticles are potentially effective in the maintenance of healthy and young tissues and organs, which are easily glycated. Even though the AGEs we investigated in this study are limited, our present results strongly indicate that the production of CML, reported to be strongly related to dullness in skin, may be inhibited in human skin by topical PAPLAL application. Further, our findings provide a novel approach for anti-aging of the skin.

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**Conflict of Interest Statement**

The authors state that the performance of this study entailed no issues representing a conflict of interest.

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**References**

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