Inhibitory Effect of 6-gingerol on Fructose-Mediated Protein Glycation *in Vitro*

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Abstract

Background: High fructose comsumption is associated with the development of diabetic complication. Fructose, like other reducing sugars, can react non-enzymatically with proteins through the Maillard reaction, leading to form irreversible advanced glycation end product (AGEs). However, it is more potent in producing AGEs than glucose. AGEs formation are also accompanied by the formation of free radicals via metal ion-catalyzed autoxidation of sugar and glycated proteins. The increase in free radicals during glycation process may cause protein modifications, leading to development of diabetic complication. Therefore, the inhibition of AGEs formation may be a promising target for therapeutic intervention in these AGEs-related disorders. The aim of this study was to evaluate the inhibitory effect of 6-gingerol on fructose-mediated protein glycation and oxidation of human serum albumin (HSA).

Methods: Antioxidant activity of 6-gingerol was assessed using ferrous iron chelating activity methods. The protein glycation inhibitory potential was evaluated using *in vitro* HSA/fructose model. HSA and fructose (0.5 M) were incubated at 37° C in the presence or absence of 6-gingerol (1-100 μM) for 14 days. The effect of 6-gingerol on AGEs formation was investigated by measuring AGE-specific fluorescence. In addition glycation induced protein oxidation was assessed by using the protein thiol oxidation and carbonyl assays.

Results : 6-gingerol exhibited the potent metal chelation properties. The formation of fructose (0.5 M) derived AGEs was significantly decreased by 6-gingerol (1 - 100 μM) in a concentration-dependent manner. Moreover, fructose caused a significant elevation of protein carbonyl content and oxidation of thiols in glycated than in native HSA. 6-gingerol (100 μM) decreased high fructose-induced oxidative damages to protein by reducing protein carbonyl formation and preserving protein thiols from oxidation.

Conclusion: These results suggest that 6-gingerol is capable of suppressing the formation of AGEs and protein oxidation in vitro, which might involve their metal chelating activity that inhibits glycoxidative-AGEs formation.

Keywords: 6-gingerol, glycation, advanced glycation end products, fructose

Introduction

Non-enzymatic reaction occurring between free amino groups of proteins and reducing sugars such as glucose and fructose results in Maillard reaction or non-enzymatic glycation of protein. The reaction is initiated by free amino groups of proteins react with reducing sugar to the reversible formation of a Schiff base, which undergoes a rearrangement to form the reversible Amadori products. Finally, the Amadori products degrade into α- dicarbonyl compounds. These compounds react with amino groups of proteins to form cross-linked, yellow-brown, fluorescent, insoluble, irreversible compounds, called AGEs (Ahmed, 2005). Many studies have demonstrated that the ability of fructose to form AGEs is greater than that of glucose (Schalkwijk, Stehouwer, & van Hinsbergh, 2004; Tappy, Le, Tran, & Paquot, 2010). Fructose is sweeter than glucose or sucrose and is commonly used in many food products and soft drinks. Therefore, the high dietary fructose consumption might result in the accumulation of fructose and its metabolites in tissues and fluids, leading to rapidly accelerate the Millard

reaction and AGEs formation (Tappy, et al., 2010). The accumulation of AGEs in living organisms can inactivate tissue proteins or modify their biological activities, leading to the development and progression of pathological conditions found in diabetic patients and in the aging process (Negre-Salvayre, Salvayre, Auge, Pamplona, & Portero-Otin, 2009; Simm, 2013).

The formation of AGEs is accelerated under oxidative conditions. (Baynes, & Thorpe, 1999; Pennathur, & Heinecke, 2007). In the presence of transition metals, monosaccharides, such as glucose or fructose, undergo auto-oxidation reactions to form radicals and other reactive intermediates (e.g. H_2O_2 and other peroxides) which can also contribute to AGEs formation. (Ahmed, 2005; Hayase, Shibuya, Sato, & Yamamoto, 1996). In addition the generation of free radicals from these process cause protein fragmentation and oxidation of protein and lipids, resulting in their biological dysfunctions. It is also known that AGEs are formed by sequential glycation and oxidation reactions termed glycoxidation. Therefore, agents with antioxidant or metal-chelating activity may suppress the process of AGEs formation. In this regard, several natural compounds which have antioxidant activity, is considered to be of the potential benefit for preventing diabetic complication (Ahmed, 2005; Ardestani, & Yazdanparast, 2007).

6-gingerol, was a major pharmacologically active compenent of ginger. Many studies have shown the pharmacological properties of 6-gingerol, including antioxidant, anti-inflammatory and anticarcer activities (Kim, Kim, Na, Surh, & Kim, 2007; Lee, Seo, Kang, & Kim, 2008; Park, Wen, Bang, Park, & Song, 2006; Wang, Chen, Lee, & Yang, 2003; Young, et al., 2005). Recent studies have demonstrated that 6-gingerol suppressed AGEs-induced reactive oxygen species in RIN-5F pancreatic β-cells and decreased the blood glucose levels in diabetic db/db mice (Son, Miura, & Yagasaki, 2014). However, there were no studies supporting the ability of 6-gingerol in the prevention of diabetic complication by inhibiting AGEs formation and protein oxidation. Therefore, the aim of the present study was to investigate the inhibitory effect of 6-gingerol on fructose-mediated glycation and oxidation dependent damages to albumin.

Materials and Methods

Chemicals

Human serum albumin (HSA), 6-gingerol, aminoguanidine hydrochloride (AG), nitroblue tetrazolium (NBT), D-fructose, ferrozine, 5,5'-dithiobisnitrobenzoic acid (DTNB) and L-cysteine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA) was purchased from Merck (Darmstadt, FR, Germany). Protein carbonyl assay kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). All chemicals used in this study were of analytical grade.

Metal chelating activity

The chelation of ferrous ions by 6-gingerol was estimated by a previous method with slight modifications (Ardestani, & Yazdanparast, 2007). Different concentrations of 6-gingerol (1 -100 μM) or aminoguinidine (1 mM) were added to a solution of 1 mM $FeCl_2$. The reaction was initiated by the addition of 1 mM ferrozine. After incubation at room temperature for 10 min, the absorbance of the Fe^{2+}-Ferrozine complex was measured spectrophotometrically at 562 nm. Percentage of inhibition was calculated in respect of the values of that of control.

In Vitro Glycation of Human Serum Albumin (HSA)

Glycation of HSA was done according to a previous method with minor modifications (Ardestani, & Yazdanparast, 2007). HSA (10 mg/mL, fatty acid free) was incubated with fructose (0.5 M) in 0.1 M phosphate buffer (PBS), pH 7.4 containing 0.02% sodium azide (NaN_3) at 37 °C for 14 days in the absence or presence of 6-gingerol (1 -100 μM) or aminoguanidine (1mM, the AGEs inhibitor). After 14 days of incubation, the formation of AGE-linked fluorescence was determined spectrofluorometrically at the excitation wavelength of 330 nm and emission wavelength of 410 nm. The inhibitory effect of 6-gingerol and aminoguanidine was evaluated by the calculation of percentage inhibition compared with maximum glycation elicited by fructose.

Determination of protein carbonyl content

The effects of 6-gingerol (1 -100 μM) on oxidative modification of HSA during glycoxidation process were carried out according to method described previously (Levine, et al., 1990). For determination of protein carbonyl content in the samples, the reaction mixture was mixed with 1ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH). Samples were incubated in the dark at room temperature with vortexing every 15 min for 1 hour. Then, 1 ml of 10% (w/v) TCA was added to the mixture and centrifuged at 3000*g* for 10 min. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1 v/v) and dissolved in 1 ml of 6 M guanidine hydrochloride. The absorbance of the sample was measured at 370 nm. The carbonyl content of each sample was calculated based of the extinction coefficient for DNPH (ε=22,000 $M^{-1}cm^{-1}$). The results were expressed as nmol carbonyl/mg protein).

Thiol Group Estimation

The free thiols in glycated samples were measured by Ellman's assay with minor modifications (Ellman, 1959). Briefly, glycated samples were incubated with 5 mM DTNB in 0.1 M PBS, pH 7.4 at 25° C for 15 min. The absorbance of samples was measured at 410 nm. The free thiol concentration of samples was calculated based on the standard curve prepared by using various concentration of L-cysteine and expressed as nmol/mg protein.

Statistical analyses

Results are expressed as the mean ± SEM. for N = 8. Data were analyzed using one-way analysis of variance (ANOVA) with post hoc multiple comparison using Newman-Keuls test (Prism version 4.0, GraphPad Software Inc, San Diago, CA, USA). In all cases, statistical differences were accepted when $p < 0.05$.

Results

Metal chelating

In the metal chelating assay, ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted with consequent decrease in the intensity of the red color of the complex. In addition 6-gingerol was assayed for Fe^{2} chelating power, and this activity was compared with the chelating activity of aminoguinidine. Aminoguanidine is a known inhibitor of glycation reaction through carbonyl trapping and chelating activity. Our results (Figure 1) showed that 6-gingerol (100 μM) was able chelating ferrous ions by 86 ± 6%, whereas the chelating activity of aminoguinidine (1 mM) was 57± 4 %. The results indicated that 6-gingerol (10- 100 μM) exhibited chelating properties.

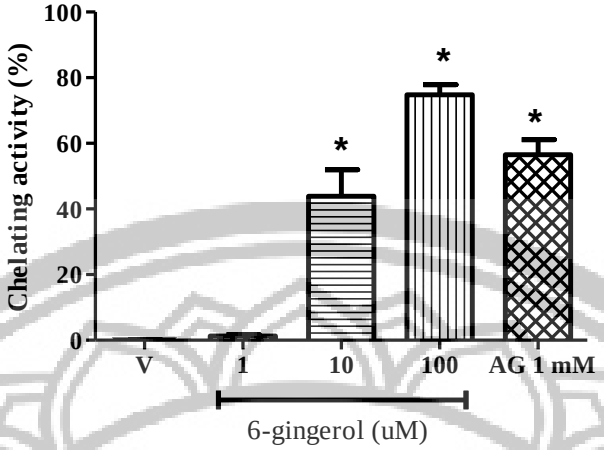

Figure 1 Chelating activity of 6-gingerol (1-100 μM), aminoguinidine (AG, 1 mM) or vehicle (methanol). Each value represents the mean ± SEM (n=8). *P<0.05 compared to vehicle group.

The effects of 6-gingerol on AGEs formation

As shown in Figure 2, the addition of fructose to the HSA solution for 14 days significantly increased the fluorescence intensity of AGEs, compared to HSA in the absence of fructose. 6-Gingerol (100 μM) suppressed the fluorescence intensity of AGEs by approximately 28 ± 2 % when compared to HSA incubated with fructose. In addition, aminoguinidine (1 mM) reduced fluorescent AGEs in HSA incubated with fructose by 59 ± 4%.

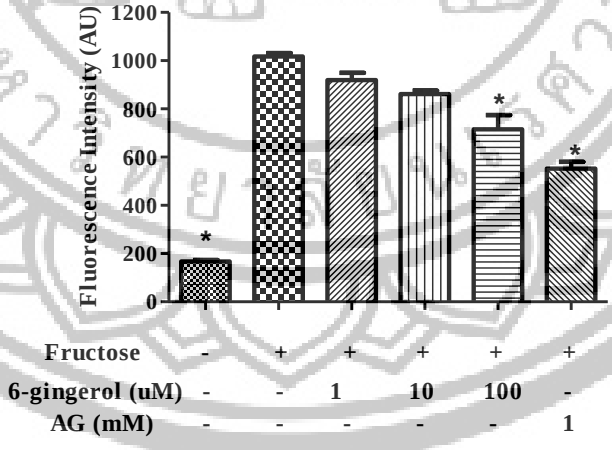

Figure 2 Effect of 6-gingerol (1- 100 μM) or aminoguanidine (AG, 1 mM) on fluorescent AGEs formation in the HSA/fructose system. Each value represents the mean± SEM (n = 8). *P<0.05 compared to HSA / fructose.

The effects of 6-gingerol on protein oxidation

The determination of carbonyl content and thiol groups was used in order to assess the protein oxidation during the glycation process. As shown in Figure 3, the carbonyl content of glycated HSA was significantly higher than non-glycated HAS. However, there was a significant decrease in the carbonyl content after addition of 6-gingerol (100 μM). At the end of study, compared to HSA/fructose, the percentage reduction of carbonyl content by 6-gingerol (100 μM) was 27 ± 5 %. In addition aminoguanidine (1mM) decreased protin carbonyl formation in HSA incubated with fructose by 35± 7 %. This result indicated that 6-gingerol exhibited anti-protein oxidation activities.

The effects of 6-gingerol on the oxidation of protein thiols are shown in Figure 4. A significant decrease in free thiol groups was observed in HSA incubated with fructose, indicating that protein glycation modified thiol groups to form disulfide in HSA. 6-gingerol (100 μM) and aminoguanidine (1mM) significantly reduced the oxidation of thiol groups.

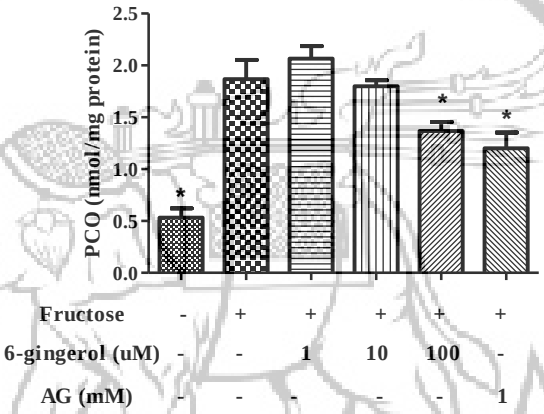

Figure 3 Effect of 6-gingerol (1- 100 μM) or aminoguanidine (AG, 1 mM) on protein carbonyl (PCO) formation in the HSA/fructose system. Each value represents the mean± SEM (n = 8). *P<0.05 compared to HSA / fructose.

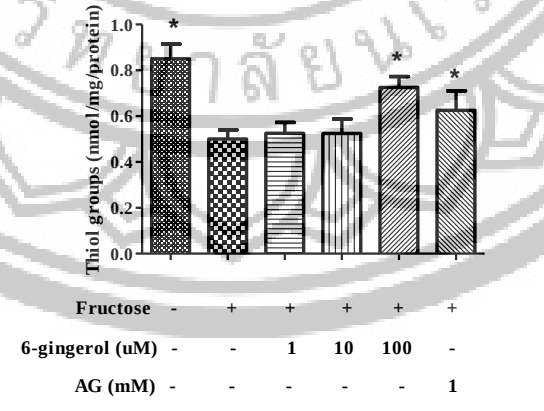

Figure 4 Effect of 6-gingerol (1- 100 μM) or aminoguanidine (AG, 1 mM) on thiol group oxidation in the HSA/fructose system. Each value represents the mean± SEM (n = 8). *P<0.05 compared to HSA /fructose.

Discussion

This study has demonstrated that 6-gingerol possessed chelating activity. Furthermore, the addition of 6-gingerol to HSA/fructose solution for 14 days reduced AGEs formation and the oxidation of albumin.

The glycation process causes various types of protein and chemical modifications, resulting in the generation of irreversible heterogeneous byproducts termed advanced glycation end products (AGEs). The accumulation of AGEs plays a primary role in the aging process and the pathogenesis of diabetic complications (Ahmed, 2005; Negre-Salvayre, et al., 2009; Prasad, Bekker, & Tsimikas, 2012; Simm, 2013). Although an increased glucose level had been thought to play a primary role in the Maillard reaction, the formation of AGEs is now known to result from the action of various metabolites other than glucose such as fructose (Ahmed, 2005). There has been research into the role of dietary fructose in the development of diabetes complications. It is well documented that an increased fructose consumtion from the diet leads to glycoxidation and generation of ROS causing oxidative damage and cellular dysfunction (Tappy, et al., 2010). Under hyperglycemic conditions, fructose can be synthesized from glucose in the polyol pathway (Narayanan, 1993; Schalkwijk, et al., 2004). Fructose and fructose metabolites participate in glycation of proteins at a much faster rate than glucose. Thus, fructose is a more potent protein glycating agent than glucose (Ahmed, 2005; Suarez, Rajaram, Oronsky, & Gawinowicz, 1989). Recent studies have demonstrated that fructose undergoes a more rapid oxidative degradation in comparison to glucose. (Meeprom, Sompong, Chan, & Adisakwattana, 2013; Schalkwijk, et al., 2004). Therefore, accumulation of fructose may cause AGEs formation and protein oxidation, leading to the development of diabetic complications.

In this study, AGEs were measured using the intensity of fluorescence, which is one of their characteristics, as a parameter. Our results demonstrated that fluorescence intensity increases during the process of glycation by fructose, similar to some other studies (Ardestani, & Yazdanparast, 2007; Meeprom, et al., 2013). However, AGE-related fluorescence was inhibited by 6-gingerol, indicating that 6-gingerol exhibited antiglycation activity.

Protein carbonyl content and thiol group formation are a markers of oxidative injury to proteins (Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003; Murphy, & Kehrer, 1989). The probable mechanism of protein damage by glycation reactions is based on the generation of free radicals. It has been reported that reducing sugars, such as glucose or fructose, can non enzymatically react with amino groups of proteins and produced stable ketoamine products. The ketoamine produces highly reactive hydroxyl radicals, by a process known as the Fenton reaction. The reactive oxygen species which generated during glycation and glycoxidation are able to oxidize amino acid residues in protein to form carbonyl derivatives and also reduce an oxidative defense of protein by decreasing protein thiol groups. Thus, protein oxidative damage and AGEs formation are direct reflection of excess of free radical generation.

We also examined whether fructose induced protein oxidation might be improved by 6-gingerol. Our study demonstrated that a significant elevation of protein carbonyl content and oxidation of thiols in HSA were observed when the protein was glycated by fructose. However, 6-gingerol suppressed high fructose-induced oxidative damages to protein by decreasing protein carbonyl formation and preserving

protein thiols from oxidation. Therefore, the present data indicated that 6-gingerol may be effective in preventing oxidative protein damages which are believed to occur under the glycoxidation processes. However, to our knowledge, this is the first study demonstrating the ability of 6-gingerol to inhibit protein glycoxidation during glycation process *in vitro*.

Regarding the presence of oxidative steps in the glycoxidation process, agents with antioxidant activities have been studies for reducing glycoxidation. Previous studies reported that 6-gingerol reduced free radical *in vitro*, including scavenging of free radical diphenylpicrylhydrazyl (DPPH) and inhibiting superoxide production. (Dugasani, et al., 2010). Moreover, 6-gingeral showed a significant ferrous ion chelating activity in our study. It has been reported that chelation of metal ions is one of the mechanisms for the inhibition in AGEs formation (Ardestani, & Yazdanparast, 2007; Meeprom, et al., 2013). There are two mechanisms of glycation processes which catalyzed by free metals such as iron and copper : the first involves the auto-oxidation of free sugar, leading to more reactive dicarbonyl compounds that react with proteins to form highly reactive ketoamines; the second mechanism involves the oxidation of the Amadori products, leading to the formation of intermediate carbonyl compounds which can generate AGEs (Piarulli, Sartore, & Lapolla, 2013). The generation of ROS during these mechanisms causes protein modifications including losses of protein functions such as the activity of enzymes, receptors, and membrane transporters, resulting in biological dysfunctions. (Ahmed, 2005; Hayase, et al., 1996). Therefore, chelation, by inhibition of sugar autoxidation and glycoxidation, would decrease the formation of AGEs and free radical derived glycation process. Our results suggested that an inhibitory effect of 6-gingerol on AGEs formation and oxidation of albumin may involve the ability of 6-gingerol to scavenge free radical and chelation of transition metal ions.

In conclusion, this is the first study to report that 6-gingerol is capable of suppressing the formation of AGEs and protein oxidation in a fructose/HSA system under *in vitro* conditions. It was proposed that chelating activity of 6-gingerol is, at least in part, involved in the inhibitory effect on glycoxidative -AGEs formation. The benifitcal effect of 6-gingerol found in this study may lead to the possibility of using 6-gingerol for preventing AGE-mediated diabetic complications.

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