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Advanced Glycation Endproducts form During Ovalbumin Digestion in the Presence of Fructose

Alejandro Gugliucci *, Yasmin Bains and Russell Caccavello

Glycation, Oxidation and Disease Laboratory, Dept. of Research, College of Osteopathic Medicine, Touro University-California, Vallejo, CA 94592, USA;
yasmin.bains@tu.edu (Y.B.); russell.caccavello@tu.edu (R.C.)

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Correspondence should be directed to:

Alejandro Gugliucci

Professor and Associate Dean of Research,

Touro University-California,

1310 Club Drive, 94592, Vallejo, CA, USA

Phone: 1 707 638 5237

Fax: 1 707 638 5998

Email: alejandro.gugliucci@tu.edu

Abstract

Background: One mechanism by which fructose could exert deleterious effect in metabolism and inflammation is via its potency vis-à-vis de Maillard reaction. We employed simulated stomach and duodenum digestion of ovalbumin to test the hypothesis that indeed AGEs are formed by fructose during simulated digestion of an ubiquitous food protein with intrinsic allergenic potential and under model physiological conditions. **Methods:** OVA was subjected to simulated gastric and intestinal digestion using standard models, in presence of fructose or glucose (0-100 mM). Peptide fractions were analyzed by fluorescence spectroscopy and intensity at Excitation: λ370 nm, Emission: λ 440nm. **Results:** AGE adducts form between fructose and OVA which can be found in peptide fractions (< 5 kDa) at times (30 min) and concentration ranges (10 mM) plausibly found in the intestines, whereas no reaction occurs with glucose. The reaction is inhibited by chlorogenic acid at concentrations compatible with those found in the gut. The reaction is inhibited by AG, a specific antiglycation agent. **Conclusion:** Our proof of principle study shows that fructose-AGE formation on an ubiquitous allergenic protein indeed occurs in one hour and thereby may pave the way for the study of yet another mechanism by

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which the excess fructose in our Western diets is contributing to disease: intestinal AGE formation, absorption and RAGE engagement.

Keywords: ovalbumin, allergy; advanced glycation; fructose; fructositis; receptor for advanced glycation end products; inflammation; asthma; chlorogenic acid; Ilex paraguariensis; high fructose corn syrup

Abbreviations: AGE: advanced glycation endproducts; AG: aminoguanidine; RAGE: receptor for advanced glycation end products

1. Introduction

Eggs, along with bovine milk are the most common causes of allergic reactions to food (6% of children and 3-4% of adults in the U.S.), and ovalbumin (the chief protein in egg white-58% w/w-, is considered a dominant allergen [1-3].

Ovalbumin has a characteristic resistance to proteolysis by pepsin that may, in part explain its allergenicity. It is believed that some of its peptides are absorbed an interact with antigen-presentig cells in Peyer's patches [1-3].

The association of high fructose diets with metabolic syndrome, diabetes and obesity is undeniable [4-8]. Large epidemiological studies have so demonstrated, notably for soda and fruit juices consumption. Nevertheless, correlation cannot adjudicate causation and some researchers maintain it is just due to extra calories, not to specific mechanistic action.

One mechanism by which fructose could exert deleterious effect in metabolism and inflammation is via its potency vis-à-vis de Maillard reaction. Indeed, fructose is 7-10 times more potent as a glycating agent as glucose due to the instability of the Heyns product it produces[9].

The reaction between D-glucose or D-fructose and the N-terminal amino-acid and/or ε-amino-groups of a protein forms Schiff base adducts. In the case of glucose, the Schiff base then undergoes Amadori rearrangement to yield a more stable adduct. With fructose, the reaction is similar, but the reaction is termed as Heyns rearrangement (with carbon 2 instead of carbon 1 of the hexose) products and results in the formation of two products ([10-14]). These early glycation products undergo further rearrangement, dehydration, and condensation reactions to produce irreversibly cross-linked, fluorescent derivatives, AGEs. *N*-(carboxymethyl)lysine (CML) stems from degradation of the Amadori products. Several glucose-derived AGEs (Glc-AGE) have been characterized, such as CML, pentosidine, crosslines, and glucosepane. As shown in the figure, there is overlap between glucose and fructose AGEs: CML, carboxyethyl lysine (CEL) and pentosidine). Fructose-specific AGEs, however have not been well characterized ([9-14]). Moreover, except for the liver, fructose

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concentrations in serum are low, therefore the role of endogenous fructose glycation (with the possible exception of the polyol pathway) as a significant pathogenic agent is difficult to substantiate.

Until recently, little attention has been paid to the possibility of endogenous formation of AGEs between fructose and proteins, precisely where they are more concentrated: the intestinal lumen [9, 15]. We reasoned that endogenous advanced glycation endproducts (AGEs) formation on ovalbumin may enhance or multiply its immunogenic potential by means of their interaction with the receptor for advanced glycation endproducts (RAGE).

RAGE is involved in many inflammatory conditions [16-19]. RAGE is a pattern recognition receptor that binds AGE, among several ligands related to primary immunity [18-19]. AGEs may be endogenous and exogenous [9]. Indeed, AGEs from dietary sources have been shown to play a deleterious role in many conditions. AGEs are certainly important in baked and roasted foods, the Maillard reaction being important as a flavor and color component of palatable food[9] . However, they may not be the only source of AGEs from food. One hypothesis that is getting attention is the possibility of AGEs being formed in the intestines when fructose in excess of glucose is ingested together with proteins. According to this "fructositis" hypothesis [15], fructose derived advanced glycation end-products (AGE) form in the intestines due to underlying fructose malabsorption. Notably, fructose malabsorption occurs after consumption of fructose at levels that exceed glucose, i.e. when fructose to glucose ratios exceed 1:1, but not after consumption of sucrose, or equal monomers of fructose and glucose. Importantly, foods and beverages sweetened with high fructose corn syrup (HFCS) contain elevated levels of fructose [20]. The same is true for apple juice. Recent research suggests that the ratio in US soft drinks may be as high as 1.9:1.

Using data from 2801 adults aged 20-55 years from the National Health and Nutrition Examination Survey (NHANES), DeChristopher et al found that independent of all covariates, , regular intake of sodas (≥5 times p/wk) vs never/ seldom consumption was associated with nearly twice the likelihood of having chronic bronchitis [21], but not with diet soda. In another cross sectional study with NHANES, children who regularly consumed any combination of high excess free fructose beverages, including apple juice, fruit drinks and HFCS sweetened soft drinks were five times more likely to have asthma vs seldom/ never consumers, independent of potential confounders [22]. Regular apple juice drinkers were twice as likely vs seldom/ never consumers to have asthma[22]. There was no association with orange juice a juice, with a 1:1 fructose to glucose ratio. Further, young adults who regularly consumed any combination of high excess free fructose beverages were three times as likely to have non-wear-and-tear, non-age associated arthritis vs seldom/ never consumers [23]. The authors suggest these three associations may be mediated through in situ enteral formation of fructose AGEs, which, after being absorbed, may contribute to inflammatory diseases via engagement of RAGE [15, 21-23]. Other food AGEs (preformed in cooking) have been shown to actually be absorbed and be pro-inflammatory in animal and human intervention studies [16]. They may participate in chronic inflammation enhancing insulin resistance in metabolic syndrome and diabetes as well as in renal failure [24-26]

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Given the ubiquity of ovalbumin and excess fructose in our diets, we reasoned that the possibility of an interaction between them during digestion needed exploration, especially in the light of the plausibility of this new hypothesis and our recent proof of principle study [27]. We showed that fluorescent AGE adducts form between fructose and amino acids at times and concentration ranges plausibly found in the intestines, whereas no detectable reaction occurs with glucose [27] . The reaction is inhibited by AG, a specific antiglycation agent. In this follow up study we employed simulated stomach and duodenum digestion of ovalbumin to test the hypothesis that indeed AGEs are formed by fructose during simulated digestion of an ubiquitous food protein with intrinsic allergenic potential and under model physiological conditions.

2. Material and Methods

All reagents were purchased from Sigma St Louis, MO.

2.1 OVA digestion was conducted employing classical models [1-3]

2.1.1. In Vitro Gastric Digestion. Ovalbumin (OVA, A-5503, 98% purity, Sigma, MO, USA) was dissolved in simulated gastric fluid (SGF, 150 mM NaCl, 20 mM KH_2PO4) at pH 1.2, to a final concentration of 25 mg/ml (0.42 mM) and subjected to an in vitro gastric digestion at 37° C with porcine pepsin (EC 3.4.23.1, 3440 units/mg, P7012, Sigma) at an enzyme/substrate ratio of 1:20 w/w (172 units/mg) [2]. Digestion was conducted in 50 ml cell culture tubes under rotatory movement at 100 rpm. After 2 h the reaction was stopped by adding 0.1 M NaOH to titrate pH to 7.0 At least triplicate digestions were conducted for each condition.

2.1.2. In Vitro Duodenal Digestion. Duodenal digestions were performed by using, as the starting material, the 120 min gastric digests as described above adjusted to pH 7.0, with the addition of 1 mM $CaCl_2$, 1 mM $MgCl_2$, and a 0.125 M bile salt mixture B3426 (Sigma). After preheating at 37°C for 15 min, a 10 X solution of pancreatin from porcine pancreas (containing all pancreatic enzymes) in $NaHCO_3$ buffer ph 8. The final composition of the mixture was 0. 4 mM OVA at an enzyme/substrate ratio of 1:25 w/w, 20 mM HCO_3, 1 mM $CaCl_2$, 1 mM $MgCl_2$, ph 7.0. At least triplicate digestions were conducted for each condition.

Digestions were performed in the absence or presence a range of fructose or glucose concentrations (0-100 mM), and with and without the presence of glycation inhibitors. Aminoguanidine (AG, the classic AGE inhibitor, Sigma St Louis, MO) as well as 5-caffeoylquinic acid (cholorogenic acid, Sigma St Louis, MO) were employed as inhibitors. Aliquots were taken at different times and digestion was stopped by changing pH and briefly heating at 90 °C. The mixture was chilled in dry ice and peptides were immediately separated.

2.2. Peptide separation

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Digested peptides and free aminoacids (< 10 kDa) were separated by ultrafiltration through 0.5 ml Amicon Ultra Centrifugal filters spun at 14,000 g for 70 min in a refrigerated centrifuge at 4 °C. Samples (triplicates of ultrafiltrates) were read in a Biotek Synergy H1. Top fluorescence was recorded at Excitation: λ370 nm, Emission: λ 440nm against blanks, corrected by absorbance at 280 nm and expressed in AU as previously described.

2.3.Fluorescence Spectroscopy

The interaction between fructose and ovalbumin peptides was studied by fluorescence spectroscopy. AGE fluorescence spectra between 390 and 600 nm (excitation, 370 nm) were recorded at room temperature on a Biotek Synergy.

2.4.SDS-PAGE. Samples were dissolved to a protein concentration of 1 mg/ml or 1/1 for peptide fractions in 10 mM Tris-HCl buffer, pH 8, containing 2.5% SDS, 5% 2-β-mercaptoethanol, and 10 mM EDTA and heated at 100° C for 10 min. SDS-PAGE was performed using precast Mini-PROTEAN TGX 4-20% gels (Bio-Rad, USA). Following the electrophoretic gels were stained with silver nitrate. A LMW Calibration Kit for SDS (Amersham Biosciences, USA) was used.

2.5.Statistical analysis. Significance of differences was assessed with the Student's t test.

3. Results

3.1. Figure 1 shows digestion of OVA (lane 1) during the stomach (lane 2) and intestinal phases (lanes 3 and 5) of simulated digestion. Peptic digestion of OVA (lane 1, time 0) leads to multiple peptides of decreasing molecular weights, as shown in lane 2, which is enhanced by intestinal digestion. The peptide fractions (<10 kDa, lanes 4 (control) and 6 (fructose) run off the gels for the most part, indicating that they contain peptides smaller than 5 kDa as well as free aminoacids. No significant difference in the digestion pattern was seen between fructose-incubated samples (lane 5) and controls (lane 3).

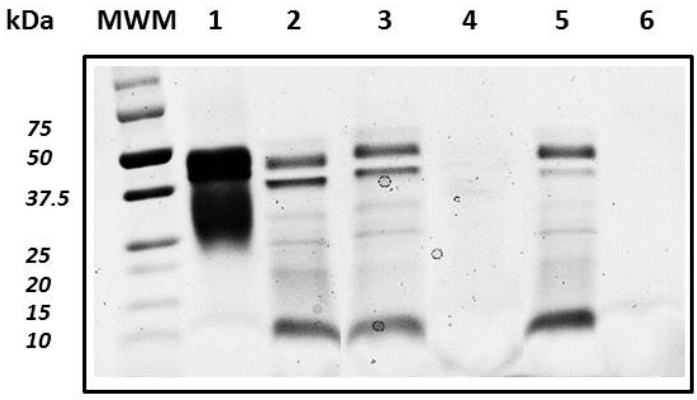

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Figure 1: SDS-PAGE analysis of OVA during in vitro simulated stomach and intestines digestion. Samples were run in 4-20% polyacrylamide gels under reducing conditions. OVA was digested with pepsin for 2 h at 37° C to simulate gastric digestion, followed by digestion with pancreatic enzymes for 2 h at 37° C to simulate enteral digestion, as described in Methods. MWM: molecular weight markers. 1: OVA at time 0; 2: OVA after 2 h pepsin;3: OVA after 2 h pancreatic enzymes; 4: peptide fraction of 4; 5:OVA + 50 mM fructose after 2 h pancreatic enzymes; 6: peptide fraction of 5.

3.2. As shown in Figure 2, simulated digestion of OVA in the presence of fructose (but not with glucose,) leads to the formation of compounds, present in the molecular weight fraction < 10 kDa that possess the characteristic fluorescence spectrum of AGEs at excitation 370 nm. Glucose incubation did not produce changes in the spectrum over the control (data not shown). Of note, fluorescent compounds were also seen at time 0, indicating the purified OVA already contains AGEs.

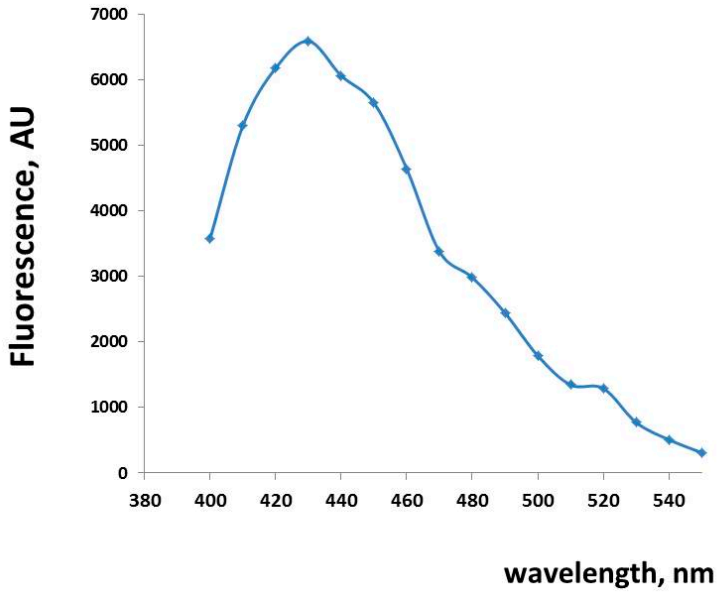

Figure 2. Emission absorption spectrum of AGEs in the peptide fraction of OVA digestion.

OVA was digested in the presence of 50 mM fructose or buffer (control) for 2 h of intestinal digestion. The peptide fraction was obtained by ultrafiltration through 10 kDa cut off Amicon filters. Spectrum was obtained after excitation at 370 nm using OVA incubated in the absence of fructose as blank.

3.3. Figure 3 shows time (3A) and dose dependent (3B) formation of AGE fluorescence in peptides during simulated digestion. Notably, at 50 mM fructose AGE fluorescence is already apparent after just 30 min duodenal digestion, a time frame well compatible with the digestive process. At 120 min of intestinal digestion AGE formation is already apparent at 10 mM fructose concentrations.

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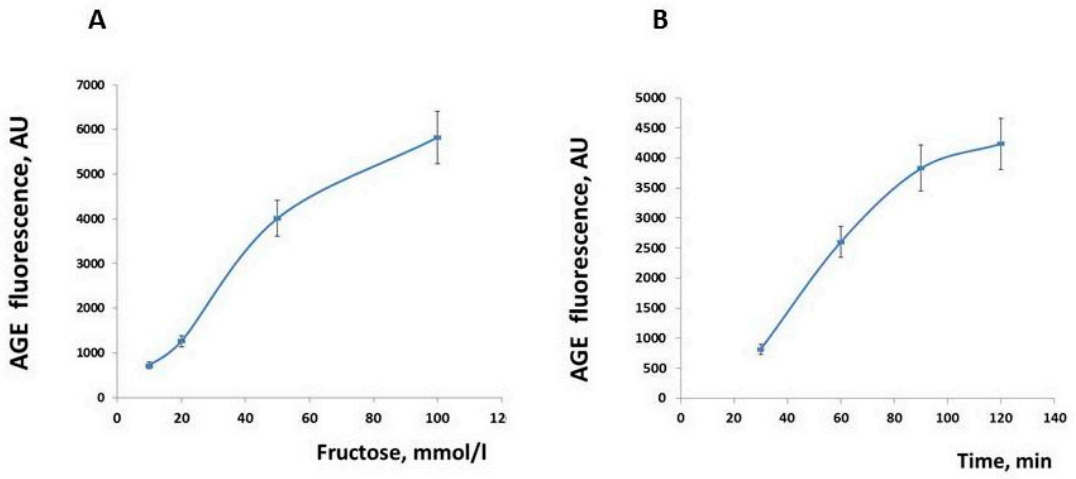

Figure 3. Time and concentration dependent formation of AGE-peptides during OVA digestion

A) Concentration dependent fructose AGE formation. OVA was incubated in the presence or absence (blanks) of fructose for 2 h of stomach conditions and 2 h of intestinal conditions as described in Methods, for 3 h.
B) Time dependent fructose AGE formation. OVA was incubated in the presence or absence (blanks) of 50 mM fructose for 2 h of stomach conditions and the indicated times of intestinal conditions as described in Methods.
C) Data are means +/- SD of triplicates. Top fluorescence (expressed in arbitrary units) was recorded at Excitation: λ350 nm, Emission: λ 440nm against blanks (OVA incubated under the same conditions in the absence of fructose). Experiments were run 3 times. Data are mean +/- SD. All differences found are significant $p < 0.05$.

AGE formation was inhibited by aminoguanidine (AG) with an IC 50 of 11 mM. Cholorogenic acid (CGA) was a potent fructose-AGE inhibitors as AG with an IC50 of 5 mM.

4. Discussion

This study was designed to test a biochemical hypothesis which was elucidated and motivated by the results of a rigorous food elimination diet with HFCS [15, 20] and tested by us in an earlier proof of principle study[27] where we showed that unlike glucose, fructose forms fluorescent advanced glycation endproducts with basic and neutral aminoacids at conditions and times compatible with those in the digestive system lumen.
In this follow-up work we have simulated the gastrointestinal proteolysis of OVA using an in vitro digestion system in two steps, which mimics the successive passage through the stomach and duodenum. We show for the first time that fructose produces AGE-peptides in OVA during simulated digestion, at times and concentrations compatible with physiological conditions. The allergenic potential of OVA may be enhanced by endogenous formation of AGEs during digestion when consumed with fructose.

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Ovalbumin was chosen as a model protein for several reasons. First, it is a classic nutritional protein in our diets. Second, its digestion has been thoroughly studied. Finally, it is particularly interesting since it is somewhat resistant to peptic digestion and its peptides are allergenic and pose a problem to some children [1-3]. If our hypothesis is correct, adding AGEs to those peptides would aggravate the problem.

The concentrations chosen were adequate to mimic a meal of an average protein consumption of 1 g/kg weight per day, in 3 meals. That is 20-25 g protein, diluted in about 1 l of gastric fluid.

Eggs, are among the most common causes of allergic reactions to food and ovalbumin is considered a key allergen [1-3]. OVA is a glycoprotein with a molecular mass of 47 kDa. It contains six cysteines with a single disulfide bond between Cys73 and Cys120 [1-3]. OVA partially resists hydrolysis by pepsin, a property common to several allergenic proteins. They keep some three-dimensional structural integrity to be able to trigger an immune reaction. Actually, stability to digestion is considered one criterion to assess the allergenic potential of new proteins; e. g: transgenic proteins. Therefore, a known pathway exists for OVA peptides to interact with antigen presenting cells in the gut. Would that be enhanced by these peptides containing fructose-derived AGEs formed in situ? If this is true, they would engage RAGE on top of their intrinsic known deleterious action and this would help explain the epidemiological evidence showing that only fructose in excess of glucose is strongly associated with asthma, arthritis and other inflammatory ailments.

In our work we demonstrate that fructose at low milimolar concentrations (compatible with those achieved by excess fructose in many beverages) forms AGEs that can be retrieved in low molecular weight peptide and free aminoacid fractions of OVA. The reaction happens in contact duodenal times as short as 30 minutes and at concentrations of fructose as low as 10 mM in longer co-incubation times. We demonstrate this reaction with a classic model nutritional protein, OVA. As OVA peptides can be immunogenic per se, their AGE content may add a “second hit” to enhance its allergenic potential.

Moreover, since digestion by trypsin involves hydrolysis at lysine and arginine residues, if peptic peptides are attacked by fructose and form Heyns products and then AGEs, these peptides would become resistant to tryptic digestion, thereby becoming yet more immunogenic.

It is important to point out that extended contacts between fructose and proteins, peptides and amino acids is greatly enhanced by fructose malabsorption [28-29]. This usually follows ingestion of food or beverages that contain a ratio of fructose to glucose greater than 1 [21, 23]. Otherwise, enhanced absorption of fructose by glucose would tend to shorten contact times.For instance, fructose malabsorption due to large intake of high fructose corn syrup (HFCS) and apple juice has been shown [28-31]

We show a time and dose dependent formation of fluorescent AGEs between fructose (but not glucose) and OVA during simulated digestion at concentrations plausibly found in the

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gastrointestinal lumen (10-50 mmol/l). Indeed, in the typical Western diet over 200 mmoles of fructose in liquid form can be ingested in two glasses of apple juice or soda usually together with a load of protein and fat that delays gastric motility [32-33]. This usually leads to 12-24 g (up to 130 mmoles) of excess fructose that is not co-absorbed with glucose and therefore has an increased luminal residence time. Even if we allowed for an exaggerated 1/5 dilution of these amounts due to fluid changes and poor absorption, these concentrations are compatible with the ones shown here to allow for formation of AGEs.

AG inhibited fructose AGE fluorescence formation in a concentration dependent manner which attests to the specificity of the reaction explored. AG is a specific AGE formation inhibitor, very efficacious and widely used in animal models to study the role of glycation in disease [27, 34-36]. We reasoned that better candidates to avoid intraluminal AGE formation by fructose would be known dietary compounds, especially those with poor absorption which get concentrated in the lumen. Chlorogenic acids are a family of esters formed between certain trans cinnamic acids and (−)-quinic acid and are major phenolics compounds in coffee, strawberries, pineapple, apple, sunflower, blueberries [37-40] . We and others have shown a potent effect of these compounds as glycation inhibitors in model systems under intracellular conditions [27, 41-42]. The formation of these compounds is readily inhibited by chlorogenic (5-caffeoylquinic acid) acid with potencies comparable to the standard antiglycating agent aminoguanidine.

In this regard these results build up on our prior report showing AGE formation between fructose and amino acids in short time and physiological conditions and give plausibility to the proposed mechanism advances to explain the epidemiological association between fructose malabsorption (due to ingestion of beverages with a ratio of fructose to glucose greater than 1 and up to 2) and inflammatory diseases (asthma, chronic bronchitis and arthritis). It has been proposed that the association is due to RAGE activation by AGE adducts formed in situ in the intestines when fructose, peptides and amino acids co-exists for abnormally long periods [15, 21, 23, 28, 31]. We chose OVA not only as a well-studied classic model protein to be employed in simulated digestion employed but also as a key allergenic per se.

5. Conclusions

Our results suggest that 1) AGE adducts form between fructose and OVA which can be found in peptide fractions (< 5 kDa) at times and concentration ranges plausibly found in the intestines, whereas no reaction occurs with glucose. Fructose ketone carbonyl is known to be 7-10 times more reactive than glucose due to the high concentration of the open ring form in fructose vis-à-vis glucose[11, 43] 2) The reaction is inhibited by chlorogenic acid at concentrations compatible with those found in the gut 3) The reaction is inhibited by AG, a specific antiglycation agent. These findings add another layer of support to our previous report that fructose-AGE adducts may be forming in situ (in the intestinal lumen) and contributing to circulating AGE load [27]. This phenomenon, in particular when OVA is involved, may be of pathogenic significance, as US average per capita HFCS consumption levels remain high vs other parts of the world, and the fructose to glucose ratio in US soft

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drinks (and more significantly in apple juice where the ratio is 2/1) has been found to exceed levels generally recognized as safe. In this regard, this study provides further in vitro evidence for the mechanism proposed by DeChristopher [15] which explains how consumption of high fructose corn syrup, but not sucrose, could result in airway mucus hypersecretion that overwhelms the airways and contributes to asthma, chronic bronchitis, and other co-morbidities including non-wear-and-tear, non-age-associated arthritis, and possibly other pro-inflammatory chronic diseases. The proposed hypothesis is supported by cross sectional epidemiological studies with nationally representative data including intake frequency of high excess free fructose beverages (HFCS sweetened soft drinks, fruit drinks, and apple juice) and the above mentioned outcomes, independent of potential confounders including smoking and exposure to in-home smoke and has recently been reviewed by us [9].

Limitations are that we did not measure non-fluorescent specific AGEs. However since many AGEs are non-fluorescent, we may be underestimating the formed AGEs. LC-MS/MS studies to characterize these adducts and animal testing of their immunogenicity are warranted to fully establish this mechanism.

Our proof of principle study shows that fructose-AGE formation on an ubiquitous allergenic protein indeed occurs in one hour and thereby may pave the way for the study of yet another mechanism by which the excess fructose in our Western diets is contributing to disease. Our study also suggests ways to decrease the damage: enteral fructose-AGE formation would be partially inhibited by co-intake of beverages, fruits and vegetables with concentrations of phenolics high enough to serve as anti-glycation agents.

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The authors declare no conflict of interest.

AG conceived and planned the work. executed some experiments and wrote the paper, YB and RC executed experiments, read and had input on the paper.

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