
Copyright © 2013 Elsevier Ltd.

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

The content must not be changed in any way or reproduced in any format or medium without the formal permission of the copyright holder(s)

When referring to this work, full bibliographic details must be given

http://eprints.gla.ac.uk/78465/

Deposited on: 26 April 2013

Enlighten – Research publications by members of the University of Glasgow
http://eprints.gla.ac.uk
Role of oxidative stress in physiological albumin glycation: a neglected interaction

Vlassopoulos A, Lean MEJ* and Combet E

Human Nutrition, School of Medicine, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow, G3 8SJ, UK

*Corresponding Author: Tel +44 141 211 4686, Fax: +44(0)141 211 4844, E-mail: mike.lean@glasgow.ac.uk
Abstract

Background: Protein glycation is a key mechanism involved in chronic diseases development in both diabetic and non-diabetic individuals. About 12-18% of circulating proteins are glycated in vivo in normoglycaemic blood, but in-vitro studies have hitherto failed to demonstrate glucose-driven glycation below concentration of 30mM.

Methods: Bovine Serum Albumin (BSA), reduced BSA (mercaptalbumin), (both 40g/L) and human plasma were incubated with glucose concentrations 0-30 mM for 4 weeks at 37°C. All were tested pre-oxidized for 8 hours prior to glycation with 10nM H₂O₂, or continuously exposed to 10nM H₂O₂ throughout the incubation period. Fructosamine was measured (nitroblue tetrazolium method) at two and four weeks.

Results: Oxidized BSA (both pre-oxidised and continuously exposed to H₂O₂) was more readily glycated than native BSA at all glucose concentrations (p=0.03). Moreover, only oxidized BSA was glycated at physiological glucose concentration (5 mM) compared to glucose-free control (glycation increased by 35% compared to native albumin p<0.05). Both 5 and 10 mM glucose led to higher glycation when mercaptalbumin was oxidised than un-oxidised (p<0.05). Fructosamine concentration in human plasma was also significantly higher when oxidized and exposed to 5 mM glucose, compared to non-oxidised plasma (p=0.03). The interaction between glucose concentration and oxidation was found to be significant in all protein models (p<0.05).

Conclusion The current study has for the first time demonstrated albumin glycation in-vitro, using physiological concentrations of albumin, glucose and hydrogen peroxide, identifying low-grade oxidative stress as a key element early in the glycation process.

Key-Words: oxidative stress, hydrogen peroxide, albumin, plasma, glucose, mercaptalbumin, glycation, glucose
**Introduction**

Protein glycation is the non-enzymatic glycosylation reaction between reducing sugars and amine residues in proteins[1]. It is usually considered to be driven by elevated glucose concentrations. Products of this reaction include Advanced Glycation End-products (AGEs) which are stable and accumulate in the body where they may exert antigenic effects, and contribute to tissue damage such as atherogenesis[2-3]. One early-glycation product is glycated hemoglobin (HbA1c), used as a biomarker for the diagnosis of diabetes and monitoring of glucose control in diabetic individuals[4]. Monitoring, and minimizing, glycative damage in diabetic care is of high importance, as glycated proteins and AGEs are implicated in cataract, neuropathy, nephropathy as well as macrovascular diseases[5].

However, glycation also occurs in non-diabetic people, in whom up to 6 % of hemoglobin and 12-16% of serum albumin is glycated[6], without exposure to high glucose concentrations (fasting plasma glucose <6 mmol/L). Protein glycation heralds tissue damage and function loss, in the normal aging process and as part of the pathogenesis of various chronic diseases. Receptors for Advanced Glycation End-products (RAGE), found in most tissues, have potent immunomodulatory actions, promoting reactive oxygen species (ROS) production and inflammation. Elevated HbA1c can serve as a proxy for both pre-diabetes and metabolic syndrome, as shown in large longitudinal studies[7]. In both non-diabetic and diabetic subjects, HbA1c correlates with coronary heart disease (CHD) risk factors and predicts future CHD and strokes[8]. A recent study showed that among non-diabetic individuals who did not develop diabetes in the next 3.5 years, those with a higher yet physiological level of HbA1c had higher risk for CVD, in both men and women and after controlling for traditional risk factors[9].

The mechanisms leading to protein glycation in the non-diabetic state are not established. The very few *in vitro* studies which have used physiological concentrations are inconclusive as to whether glucose, alone, can successfully promote glycation[10-11]. In diabetic subjects, protein glycation is assumed to be mainly a mass action effect driven by high glucose concentration. However the full process of glycation in diabetes is in fact driven by two separate factors - the concentration of sugars in the initiation phase (mainly glucose, due to its high concentration in blood), and later the pro-oxidant status during Maillard reactions to generate stable AGEs[12]. We hypothesize that at physiological concentrations of glucose,
oxidation may have another role in initiating glycation, supporting earlier speculations from a cross-sectional study in non-diabetic individuals which showed inverse associations between protein glycation and dietary fruit and vegetable consumption, plasma vitamin C and plasma tocopherol[13]. Defining an early, preventable oxidative component to the overall glycation mechanism could be of importance in the management of pre-diabetes, when glucose metabolism is only mildly disrupted and glucose-centered clinical approaches might have little effect.

The current study investigates the effect of introducing a mildly pro-oxidative state (hydrogen peroxide at a low physiological concentration of 10nM[14]) on the susceptibility of protein (albumin) to glycation, particularly at physiological and near-physiological glucose concentrations. Another common glycation driver, methylglyoxal, which causes glycative damage in a more oxidative fashion than glucose, was used in physiological concentration both alone and in combination with glucose, to explore possible synergistic effects. This oxidation-driven glycation hypothesis was also tested on reduced albumin (mercaptalbumin) on the assumption that commercially source native albumin would be already partly oxidized. This work was also extended to proteins in human plasma to extend the physiological relevance of our findings.

Material and methods
Impact of constant oxidation & pre-oxidation on BSA glycation
Chemicals
Bovine serum albumin (BSA), sodium azide, nitroblue tetrazolium, d-glucose, methylglyoxal, PBS, 1-deoxy-1-morpholinofructose (DMF), hydrogen peroxide, sulphuric acid, dithiothreitol and quinine were purchased from Sigma-Aldrich (Dorset, UK). SnakeSkin Dialysis Tubing, 3.5K MWCO was purchased from Thermo Fisher Scientific (Nottinghamshire, UK).

Glycation of pre-oxidized BSA
BSA (80g/L) was incubated with H₂O₂ (10nM) for 8 hours at 37°C in PBS and was then dialyzed against PBS (8:1) for another 8 hours. The dialysate was discarded and replaced with fresh PBS three times during dialysis.
To measure the effect of protein pre-oxidation on the susceptibility of BSA to glycation, both native and pre-oxidized albumin (40g/L) were incubated in the presence of glucose (0, 5, 10, 20 and 30mM) for 4 weeks. The combination of methylglyoxal (150nM) and glucose (0, 10 or 20mM) was also studied in order to replicate glyoxidative conditions of albumin in the circulation. All incubations were repeated in 6 replicates.

**Albumin glyoxidation**

All incubations took place in PBS with sodium azide (0.2g/L) and a final volume of 1.5ml. To investigate the effect of continuous exposure to oxidative damage in the progress of the glycation reaction (glycoxidation) native BSA was incubated with glucose (0, 5, 10, 20, or 30mM) and combinations of methylglyoxal (150nM) plus glucose (0, 10, 20mM) for 4 weeks in the presence of H₂O₂ (10nM). Native BSA was also incubated under the same conditions without H₂O₂ (10nM) to serve as a reference. All incubations were repeated in 6 replicates.

**Comparison of the effect of constant glycation among BSA, mercaptalbumin & human plasma**

To enhance a concern over the oxidation status of the bovine serum albumin sold by Sigma-Aldrich, the BSA used in the experiments was i) pre-treated with 1.5mM dithiothreitol (DTT) at 37°C for 15min and then DTT was removed by extensive dialysis against PBS for 10 hours to create mercapralbumin and ii) BSA as bought from Sigma-Aldrich.

In order to investigate the effect of hydrogen peroxide exposure in plasma proteins glycation, pooled plasma from 8 healthy, normal weight volunteers was collected in heparin tubes after an overnight fast.

**Protein glyoxidation**

All incubations took place in PBS with sodium azide (0.2g/L) and a final volume of 1.5ml. To investigate the effect of constant exposure to oxidative damage in the progress of the glycation reaction (glycoxidation) native BSA and native mercaptalbumin was incubated with glucose (0, 5, 10 or 20mM) for 4 weeks in the presence of H₂O₂ (10nM). Proteins were also incubated under the same conditions without H₂O₂ (10nM) to serve as a reference.

The glucose concentration of 30mM was not employed as it is a highly supra-physiological concentration and the combinations of methylglyoxal plus glucose were also not employed.
All incubations were repeated in 5 replicates.

**Fructosamine measurement**

The NBT assay was modified in this experiment using a larger amount of sample (25 vs 10 µL) which resulted in a smaller Coefficient of Variation.

Fructosamine levels were measured at week 2 and 4 with the NBT assay, performed in microplates as described previously [15]. Briefly, samples (25µL) were added to of sodium carbonate buffer (100µL, 100mM, and pH 10.8) with Nitroblue Tetrazolium (0.25mM).

Microplates were incubated for 15 min at 37°C and measured spectrophometrically against controls at 550nm after 10 and 15 min of incubation. The difference between the two readings was used to calculate concentrations. The fructosamine analog 1-deoxy-1-morpholinofructose (DMF) was used as a standard. All fructosamine measurements were performed in duplicate. Standards and NBT reagent were made fresh every week and stored at -20°C and 4°C respectively. All samples were stored at -20°C.

**Statistical analysis**

All combinations of oxidative damage and glycation drivers were tested as five or six true replicates, according to the experiment. The independent sample t-test was used to assess the differences in glycation between native BSA and either of the oxidation set-ups. Difference in fructosamine production between glucose levels and glycation drivers were tested using a one-way ANOVA and Tukey’s post-hoc test. Differences between exposure to oxidation and no oxidation were studied in each protein system separately using one-way ANOVA and the interaction between glucose levels and oxidation was studied using two-way ANOVA. Statistical analysis was performed using PASW 18.

**Results**

**Effect of constant oxidation on BSA glycation**

Incubation of native BSA (40g/L) with glucose concentrations below 20mM did not lead to measurable levels of glycated BSA measured after 2 weeks, with or without 150nM methylglyoxal, compared to glucose-free control (Table 1). After 4 weeks, incubation with 10mM glucose (with or without methylglyoxal) significantly promoted glycation in native BSA compared to glucose-free control. Exposure to a physiological concentration of
hydrogen peroxide (10nM), however, led to significantly higher glycated BSA (measured as fructosamine) at the lower glucose concentrations of 10mM after 2 weeks, and 5 mM after 4 weeks incubation (both p<0.05 vs. glucose free control).

Using constantly-oxidized BSA generated significantly more fructosamine than native BSA after 2 weeks of incubation with 5, 10 and 20mM glucose (increased by 23%, 36% and 35% respectively). Similar results were observed with methylglyoxal (150 nM) alone (19% increase in fructosamine), and with a combination of methylglyoxal (150 nM) plus glucose at concentrations of 10 and 20mM, with 35% and 26% increases in fructosamine respectively (Figure 1). Significantly greater glycation of constantly-oxidized BSA compared to native BSA was also observed after 4 weeks at all glucose concentrations, and with combinations of glucose (10 & 20mM) and methylglyoxal (150nM) (Figure 2). In particular, incubation of BSA with 5mM glucose and 10mM H$_2$O$_2$ led to a 35% higher fructosamine concentration compared to the non-oxidised control (p=0.04). Although incubating BSA in presence of methylglyoxal (150nM) alone did not lead to significantly increased glycation after neither 2 nor 4 weeks (Table 1), combining methylglyoxal (150nM) and glucose (10mM) had a synergistic effect on glycation of constantly-oxidised BSA after 4 weeks (p=0.02 vs. glucose alone), as well as some suggestion of an effect on native BSA (p=0.08) (data not shown).

The individual impacts of glucose concentration and oxidation, as well as their interaction, on glycation over periods of 2 and 4 weeks were investigated using a two-way ANOVA. Oxidation had a significant effect on glycation at both two and four weeks (p<0.001) There was strong evidence for an interaction between continuous-oxidation and glucose concentration in driving glycation after 2 weeks of incubation (p<0.001) with a non-significant indication of an effect of this interaction on glycation after 4 weeks (p=0.058). While removing data relating to methylglyoxal and glucose incubations from the analysis did not change impact on the significance of the effect of oxidation on protein glycation (p <0.001 at both two and four weeks), the interaction between oxidation and glucose concentration significantly affected glycation after both two and four weeks (p=0.001 and p=0.01, respectively).

**Effect of pre-oxidation on BSA glycation**

Incubation of native and pre-oxidised BSA with glucose concentrations lower than 20mM for two weeks did not lead to significantly more fructosamine being produced than the glucose-
free control. Nonetheless the pre-oxidation step led to significantly higher glycation, compared to native BSA, after two weeks at the lowest glucose concentration (5mM) (p=0.016).

A 4-week incubation with 5mM glucose alone was sufficient to drive glycation of pre-oxidized BSA (p=0.03 vs. glucose-free control), but not native (un-oxidized) BSA which required at least 10mM glucose (p=0.001, Table 1).

Comparison of the effect of constant-oxidation on BSA and mercaptalbumin glycation

Reduction of BSA to mercaptalbumin was employed in order to investigate whether commercially available BSA, possibly being oxidised to some extent, would be more or less prone to subtle oxidation driven glycation. For this reason, DTT-treated BSA was incubated with glucose (0-20mM) in presence or absence of 10nM H$_2$O$_2$.

When mercaptalbumin was incubated with 5 and 10mM glucose under constant oxidation for two weeks, significantly higher fructosamine levels were observed compared to non-oxidised mercaptalbumin (p=0.03 & p=0.006; respectively). While incubation for two weeks with 5mM glucose was sufficient to drive higher glycation in constantly-oxidized mercaptalbumin than glucose-free control (p<0.001), non-oxidised mercaptalbumin required incubation with 10mM glucose to lead to higher glycation than the glucose-free control (p<0.001). No differences between oxidized and non-oxidised mercaptalbumin were observed at week 4 (Figure 3).

Two-way ANOVA, analysing the effect of oxidation at all glucose levels, showed that oxidized mercaptalbumin was subject to higher glycation than non-oxidised, at both weeks two and four (p=0.003 & p=0.035 respectively). The interaction between glucose and oxidation was not significantly affecting glycation in both weeks (p for interaction glucose × oxidation = 0.48 & 0.78 for week 2 and 4 respectively).

When mercaptalbumin glycation was compared to BSA glycation. Mercaptalbumin was more prone to glycation than BSA in both the presence and absence of H$_2$O$_2$. In the absence of H$_2$O$_2$ mercaptalbumin had higher fructosamine concentration than BSA at 5 and 10mM glucose at two weeks (p=0.004 & p=0.002 respectively) and that was significant at week 4 for 5mM glucose (p=0.005) and nearly significant for 10mM glucose (p=0.06). In the
presence of H$_2$O$_2$ mercaptalbumin was again more successfully glycated than BSA at 5&10 mM glucose at week 2 (p<0.001, for both) and nearly significantly more at 20mM (p=0.057). At week 4 mercaptalbumin was significantly more glycated than BSA only at 5mM glucose (p=0.04) (data not shown).

Employing two-way ANOVA showed that mercaptalbumin was more prone to glycation than BSA (p<0.001) and there was a significant positive interaction between oxidation and the type of protein employed, in favour of mercaptalbumin (p interaction protein-type x oxidation= 0.047) at week 2. Although the interaction between the protein type and oxidation was not documented at week 4 (p=0.33), glycation was still positively affected by using mercaptalbumin rather than BSA (p<0.001) (data not shown).

**Effect of constant-oxidation on human plasma glycation**

Protein glycation in human plasma was studied to explore the reactions studied previously in a more complex protein system with antioxidant mechanisms in place and closer to human physiology. Plasma exposure to constant hydrogen peroxide (10nM) promoted glycation when incubated with 5mM glucose for 2 weeks, compared to non-oxidised plasma (p=0.03). Surprisingly, this effect of oxidation was no present after 4 weeks, and actually led to significant lower fructosamine concentration for incubations with 10mM glucose compared to non-oxidised plasma (p=0.001) (Figure 5).

Two-way ANOVA suggested that oxidation had no impact on glycation in human plasma at week two but it had a significant negative impact at week four (p=0.01). The interaction glucose x oxidation was also significant (p<0.001) at week four, only.

**Discussion**

Protein glycation was first described as part of the Maillard reactions cascade, as a cause of food-spoiling. Although the same reactions have been identified *in-vivo*, and glycated proteins are probably causally associated with the tissue damage that occurs during aging, in diabetes and other chronic diseases[5], there are still gaps in the understanding of the exact mechanisms involved. It is striking that 12-18% of circulating albumin, and up to 6% of
haemoglobin is glycated in apparently healthy non-diabetic people, amongst whom most heart disease occurs[6].

Perhaps surprisingly, this study is the first to demonstrate in vitro protein glycation at physiological glucose concentrations. The clear effect of including an oxidative agent (hydrogen peroxide at a low, physiologically relevant concentration [14]), supports the concept of oxidative stress as a key mechanism behind in-vivo glycation of albumin in normoglycaemic individuals. It is worth stressing that the concentration of hydrogen peroxide used was very low. Although the literature is still controversial over the exact concentration of hydrogen peroxide in plasma with values up to 35 μM being documented[16], even the supporters of the theory that hydrogen peroxide concentration is not important in plasma documented values of 250nM[17], which 25 times higher than the concentration equipped in the current study. Several factors might explain why previous studies have failed to achieve glycation in vitro under physiological conditions. We used a physiological concentration of albumin, while previous studies have used lower (sub-physiological) concentrations (0.01-7 g/L)[18-19], and/or high (supra-physiological) glucose concentrations (30mM-0.5M)[20-21]. Albumin glycation had previously been achieved in the presence of 15mM glucose alone at 5 weeks or 30mM glucose alone at 4 weeks [22]. With physiological glucose concentrations, results have varied according to sample treatment and methods used to measure glycation. Bourdon et al. [10] reported that incubation of albumin for 4 weeks with 5mM glucose did not promote glycation, while later reporting contradictory findings (with, however, glycation only implied from qualitative results[11]).

Similarly, while methylglyoxal is a potent glycative molecule in supra-physiological conditions[19], it did not show significant glycative activity in physiological concentrations. Our data suggest it might act synergistically with glucose to promote glycation at lower glucose concentrations (10mM), but we demonstrated that both glucose and methylglyoxal at physiological concentrations will glycate albumin if it is oxidised.

In both the BSA and mercaptalbumin models, glycation of the protein was significantly higher than the glucose-free control when exposed to a physiological concentration of hydrogen peroxide for two weeks. Although the effect of pre-oxidation and continuous oxidation were very similar with a favour towards continuous oxidation, in so low hydrogen peroxide concentrations the continuous oxidation model is more likely to be of physiological
Continuous exposure to hydrogen peroxide led to higher fructosamine concentrations at all glucose levels and oxidation was also shown to act synergistically with glucose, as the interaction between the two was found to be significant in our experiments. Hydrogen peroxide positively interacts with glucose in promoting glycation reactions. One has to keep in mind that the hydrogen peroxide exposure was weak in term of concentration in order to resemble physiological condition and hence it is likely to induce important but subtle effects. When human plasma was exposed to hydrogen peroxide, glycation was significantly higher with 5mM glucose after two weeks, compared to the non-oxidized control. The opposite was found when oxidised plasma was incubated with 10mM glucose for 4 weeks, but the lower fructosamine concentration of the oxidised plasma in that case could be attributed both to increased protein instability and/or glycation being driven to the production of AGEs (not detectable by the NBT method used) rather than early-glycation products as fructosamine.

Constant oxidative stress is clearly damaging and relevant to diabetic and obese chronic pro-oxidant states, however a dynamic balance between pro- and anti-oxidant factors is usually present in plasma and other body fluids. The level of oxidative stress fluctuates during the day (e.g. higher post-prandially) and a variety of events can trigger short-term production of Reactive Oxygen Species. Our results suggest that episodes of relatively unopposed oxidation, e.g. from infection or inflammation, or smoking, could damage proteins to promote subsequent glycation, as we have demonstrated with the increased susceptibility of pre-oxidized albumin to glycation at a physiological glucose concentration. This mechanism could apply in vivo.

Recognizing that, in physiological systems, protein glycation depends on oxidative damage as well as glucose concentration has implications for scientific understanding and potentially for clinical practice. The term ‘glyoxidation’, currently restricted to the latter stages of Maillard reactions, seems more appropriate than simply ‘glycation’ to describe the overall in vivo protein glycation process, and similar protocols to ours would be appropriate to study the phenomenon in vitro. The quest for normoglycaemia in diabetes management is important to delay vascular and other complications, but potentially hazardous interventions are entirely directed at glucose-lowering: using insulin or anti-diabetic drugs intensively to reduce glycation has been associated with increased risk of hypoglycemia, and of mortality [23]. If oxidative stress is also involved as a trigger for protein glycation and tissue damage, then
approaches aimed solely at glucose handling are insufficient, and reducing oxidative stress might be less hazardous. This is not arguing for indiscriminate or high-dose antioxidant treatments. Several trials have suggested worse outcomes from antioxidant vitamin supplementation[24], leading to understandable prejudice against their effectiveness and safety, but a recent meta-analysis of 66 randomised controlled trials indicates benefit from vitamin E supplementation for primary prevention (where there is some baseline insufficiency)[25], and vitamin E also contributes to secondary prevention[26], renewing interest in antioxidant interventions. Our results support findings from the cross-sectional study of Bates et al.[13], which led to the hypothesis that dietary antioxidants may reduce tissue glycation. Also evidence from in-vitro studies suggest that antioxidants are having a protective role in protein glycation[20, 27]. Exposure to oxygen radicals such as TBH and H$_2$O$_2$ significantly increased haemoglobin glycation in-vitro and pre-treatment with vitamin E blocked that effect[28]. Replenishment of the antioxidant defences of GSH-deficient red blood cells, on the other hand, protected them against increased haemoglobin glycation[29], both supporting the hypothesis of oxidative stress being involved in protein glycation.

The present studies suggest some important avenues for future research, as well as changes to commonly-used experimental models. It is important to question accepted pathological mechanisms if they cannot be demonstrated in vivo at physiological concentrations. Our evidence that mild oxidation plays an early role in AGE production is novel and explains a gap in the literature. Prior reduction of BSA might indeed increase the oxidation potential of the protein and hence strengthen the effect of oxidation on glycation, as shown by the two-way ANOVA analysis. While being designed to replicate physiological conditions and employing a large number of replicates (6 instead of the usual 3) to reduce random errors under physiological conditions, the current study does have limitations. Albumin, although the major circulating protein, may not be representative of other glycation-prone proteins, and the results cannot provide an exact mechanism linking oxidative damage to glycation. Using human plasma led to slightly different results than BSA and mercaptalbumin. No effect on glycation was seen from oxidation at glucose levels above 10mM after two weeks of incubation; that could be attributed to the fact that plasma from healthy volunteers involves different proteins with different degrees of pro-oxidation and glycation and also a much more competent antioxidant system which would be expected to rapidly scavenge ROS. Glycated proteins already present in plasma could also affect the speed and general kinetics of the reaction. Possible mechanisms involve protein damage by
hydrogen peroxide and/or increased glucose autoxidation in the presence of hydrogen peroxide, both likely to increase the affinity of the molecules for the non-enzymatic sugar linkage. There may be selective oxidation of amino acids: for example, tryptophan, a main site for protein glycation, is an oxidation site for human albumin [30-31], suggesting that oxidized amino acids maybe more susceptible to further glycative damage.
**Conclusion**

Oxidative damage, although known to be important for the late stages of protein glycation, has not previously been linked with the early stage of the Maillard reaction. Our data suggest that oxidative damage, induced by a very low (physiological) concentration of hydrogen peroxide, plays a critical early role in fructosamine production. Importantly, the effect is seen at physiological glucose concentrations, potentially opening an avenue for new preventive treatments. Our experiments highlight the importance of oxidative stress on protein glycation, as a promoter and even a necessary condition to achieve glycation in physiological glucose concentrations.

**Acknowledgements**

AV is in receipt of a scholarship from Yorkhill Children’s Foundation. The authors have no conflicts of interest to declare.
Figure 1. Fructosamine concentration (mM DMF equivalent) after two weeks incubation with glucose and constant exposure to oxidation from hydrogen peroxide (10 nM). Two-way ANOVA analysis showed a significant effect of oxidation to promote glycation.

*\(p<0.05\) native vs. constant oxidation for each given glucose concentration
Figure 2. Fructosamine concentration (mM DMF equivalent) after four weeks incubation with glucose and constant exposure to oxidation from hydrogen peroxide (10 nM). Two-way ANOVA analysis showed a significant effect of oxidation to promote glycation.

- *p<0.05 native vs. constant oxidation for each given glucose concentration
### Table 1: Fructosamine concentration after two weeks incubation of albumin with different glucose concentrations, between and within treatments (glucose / MGO exposure and oxidation)

<table>
<thead>
<tr>
<th>Glucose levels (mM)</th>
<th>Oxidation status</th>
<th>Week 2</th>
<th></th>
<th></th>
<th>Week 4</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native Mean (SD)</td>
<td>H₂O₂ 10nM Mean (SD)</td>
<td>p-value</td>
<td>Pre-Oxidized Mean (SD)</td>
<td>p-value</td>
<td>Oxidised vs native*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.26(0.04)</td>
<td>0.27(0.02)</td>
<td>0.669</td>
<td>0.26(0.01)</td>
<td>0.26(0.03)</td>
<td>0.807</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.31(0.04)</td>
<td>0.38(0.02)</td>
<td>0.017</td>
<td>0.23(0.02)</td>
<td>0.29(0.05)</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.35(0.02)</td>
<td>0.53(0.02)*</td>
<td>0.001</td>
<td>0.26(0.04)</td>
<td>0.28(0.03)</td>
<td>0.177</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.51(0.05)*</td>
<td>0.69(0.07)*</td>
<td>0.002</td>
<td>0.57(0.06)*</td>
<td>0.57(0.04)*</td>
<td>0.940</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.82(0.06)*</td>
<td>0.82(0.03)*</td>
<td>0.987</td>
<td>0.79(0.10)*</td>
<td>0.64(0.10)*</td>
<td>0.041</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0+MGO</td>
<td>0.26(0.02)</td>
<td>0.31(0.01)</td>
<td>0.015</td>
<td>0.19(0.02)</td>
<td>0.19(0.03)</td>
<td>0.510</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10+MGO</td>
<td>0.34(0.05)</td>
<td>0.46(0.03)*</td>
<td>0.001</td>
<td>0.62(0.07)*</td>
<td>0.48(0.05)*</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20+MGO</td>
<td>0.53(0.08)*</td>
<td>0.67(0.01)*</td>
<td>0.008</td>
<td>0.46(0.05)*</td>
<td>0.39(0.07)*</td>
<td>0.048</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidised vs native*</td>
<td>0.43(0.19)</td>
<td>0.51(0.20)</td>
<td>&lt;0.001</td>
<td>0.42(0.21)</td>
<td>0.40(0.16)</td>
<td>0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.37(0.03)</td>
<td>0.39(0.09)</td>
<td>0.767</td>
<td>0.20(0.02)</td>
<td>0.17(0.02)</td>
<td>0.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.40(0.09)</td>
<td>0.54(0.06)*</td>
<td>0.015</td>
<td>0.26(0.02)</td>
<td>0.24(0.02)*</td>
<td>0.063</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.55(0.04)*</td>
<td>0.68(0.06)*</td>
<td>0.003</td>
<td>0.31(0.02)*</td>
<td>0.31(0.02)*</td>
<td>0.825</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.78(0.04)*</td>
<td>0.92(0.04)*</td>
<td>&lt;0.001</td>
<td>0.56(0.05)*</td>
<td>0.52(0.07)*</td>
<td>0.292</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.95(0.08)*</td>
<td>1.17(0.10)*</td>
<td>0.002</td>
<td>0.62(0.05)*</td>
<td>0.64(0.03)*</td>
<td>0.844</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0+MGO</td>
<td>0.43(0.02)</td>
<td>0.40(0.04)</td>
<td>0.590</td>
<td>0.22(0.02)</td>
<td>0.17(0.01)</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10+MGO</td>
<td>0.70(0.03)*</td>
<td>0.84(0.09)*</td>
<td>0.010</td>
<td>0.52(0.01)*</td>
<td>0.46(0.03)*</td>
<td>0.028</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20+MGO</td>
<td>0.86(0.07)*</td>
<td>1.05(0.12)*</td>
<td>0.015</td>
<td>0.40(0.02)*</td>
<td>0.33(0.01)*</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidised vs native*</td>
<td>0.64(0.22)</td>
<td>0.75(0.29)</td>
<td>&lt;0.001</td>
<td>0.38(0.16)</td>
<td>0.36(0.17)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05 vs. glucose 0mM, *two-way ANOVA analysis
Figure 3. Fructosamine concentration (mM DMF equivalent) after two and four weeks incubation with glucose and constant exposure to oxidation from hydrogen peroxide (10 nM) in mercaptalbumin. Two-way ANOVA analysis showed a significant effect of oxidation to promote glycation.

Mercaptalbumin

Week 2

![Graph showing fructosamine concentration (mM DMF equivalent) for mercaptalbumin in week 2 with glucose levels ranging from 0 to 20 mM. The graph compares fructosamine levels without and with hydrogen peroxide (H2O2). *p<0.05 native vs. constant oxidation.]

Week 4

![Graph showing fructosamine concentration (mM DMF equivalent) for mercaptalbumin in week 4 with glucose levels ranging from 0 to 20 mM. The graph compares fructosamine levels without and with hydrogen peroxide (H2O2). *p<0.05 native vs. constant oxidation.]

*p<0.05 native vs. constant oxidation
Figure 4. Fructosamine concentration (mM DMF equivalent) after two and four weeks incubation with glucose and constant exposure to oxidation from hydrogen peroxide (10 nM) in human plasma. Two-way ANOVA analysis showed a significant effect of oxidation to promote glycation.

**Human Plasma**

**Week 2**

![Graph showing fructosamine concentration (mM DMF equivalent) at different glucose levels (mM) with and without H2O2 for Week 2.](image)

**Week 4**

![Graph showing fructosamine concentration (mM DMF equivalent) at different glucose levels (mM) with and without H2O2 for Week 4.](image)

*p*<0.05 native vs. constant oxidation
REFERENCES


