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2 **Role of oxidative stress in physiological**

3 **albumin glycation: a neglected interaction**

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31 **Abstract**

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

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

41 **Results:** Oxidized BSA (both pre-oxidised and continuously exposed to H₂O₂) was more
42 readily glycated than native BSA at all glucose concentrations (p=0.03). Moreover, only
43 oxidized BSA was glycated at physiological glucose concentration (5 mM) compared to
44 glucose-free control (glycation increased by 35% compared to native albumin p<0.05). Both
45 5 and 10 mM glucose led to higher glycation when mercaptalbumin was oxidised than un-
46 oxidised (p<0.05). Fructosamine concentration in human plasma was also significantly higher
47 when oxidized and exposed to 5 mM glucose, compared to non-oxidised plasma (p=0.03).
48 The interaction between glucose concentration and oxidation was found to be significant in
49 all protein models (p<0.05).

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

53

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

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59 **Introduction**

60
61 Protein glycation is the non-enzymatic glycosylation reaction between reducing sugars and
62 amine residues in proteins[1]. It is usually considered to be driven by elevated glucose
63 concentrations. Products of this reaction include Advanced Glycation End-products (AGEs)
64 which are stable and accumulate in the body where they may exert antigenic effects, and
65 contribute to tissue damage such as atherogenesis[2-3]. One early-glycation product is
66 glycated hemoglobin (HbA1c), used as a biomarker for the diagnosis of diabetes and
67 monitoring of glucose control in diabetic individuals[4]. Monitoring, and minimizing,
68 glycative damage in diabetic care is of high importance, as glycated proteins and AGEs are
69 implicated in cataract, neuropathy, nephropathy as well as macrovascular diseases[5].

70
71 However, glycation also occurs in non-diabetic people, in whom up to 6 % of hemoglobin
72 and 12-16% of serum albumin is glycated[6], without exposure to high glucose
73 concentrations (fasting plasma glucose <6 mmol/L). Protein glycation heralds tissue damage
74 and function loss, in the normal aging process and as part of the pathogenesis of various
75 chronic diseases. Receptors for Advanced Glycation End-products (RAGE), found in most
76 tissues, have potent immunomodulatory actions, promoting reactive oxygen species (ROS)
77 production and inflammation. Elevated HbA1c can serve as a proxy for both pre-diabetes and
78 metabolic syndrome, as shown in large longitudinal studies[7]. In both non-diabetic and
79 diabetic subjects, HbA1c correlates with coronary heart disease (CHD) risk factors and
80 predicts future CHD and strokes[8]. A recent study showed that among non-diabetic
81 individuals who did not develop diabetes in the next 3.5 years, those with a higher yet
82 physiological level of HbA1c had higher risk for CVD, in both men and women and after
83 controlling for traditional risk factors[9].

84
85 The mechanisms leading to protein glycation in the non-diabetic state are not established. The
86 very few *in vitro* studies which have used physiological concentrations are inconclusive as to
87 whether glucose, alone, can successfully promote glycation[10-11]. In diabetic subjects,
88 protein glycation is assumed to be mainly a mass action effect driven by high glucose
89 concentration. However the full process of glycation in diabetes is in fact driven by two
90 separate factors - the concentration of sugars in the initiation phase (mainly glucose, due to its
91 high concentration in blood), and later the pro-oxidant status during Maillard reactions to
92 generate stable AGEs[12]. We hypothesize that at physiological concentrations of glucose,

93 oxidation may have another role in initiating glycation, supporting earlier speculations from a
94 cross-sectional study in non-diabetic individuals which showed inverse associations between
95 protein glycation and dietary fruit and vegetable consumption, plasma vitamin C and plasma
96 tocopherol[13]. Defining an early, preventable oxidative component to the overall glycation
97 mechanism could be of importance in the management of pre-diabetes, when glucose
98 metabolism is only mildly disrupted and glucose-centered clinical approaches might have
99 little effect.

100

101 The current study investigates the effect of introducing a mildly pro-oxidative state (hydrogen
102 peroxide at a low physiological concentration of 10nM[14]) on the susceptibility of protein
103 (albumin) to glycation, particularly at physiological and near-physiological glucose
104 concentrations. Another common glycation driver, methylglyoxal, which causes glycative
105 damage in a more oxidative fashion than glucose, was used in physiological concentration
106 both alone and in combination with glucose, to explore possible synergistic effects.

107 This oxidation-driven glycation hypothesis was also tested on reduced albumin
108 (mercaptalbumin) on the assumption that commercially source native albumin would be
109 already partly oxidized. This work was also extended to proteins in human plasma to extend
110 the physiological relevance of our findings.

111

112

113 **Material and methods**

114 **Impact of constant oxidation & pre-oxidation on BSA glycation**

115 **Chemicals**

116 Bovine serum albumin (BSA), sodium azide, nitroblue tetrazolium, d-glucose, methylglyoxal,
117 PBS, 1-deoxy-1-morpholinofructose (DMF), hydrogen peroxide, sulphuric acid, dithiothreitol
118 and quinine were purchased from Sigma-Aldrich (Dorset, UK). SnakeSkin Dialysis Tubing,
119 3.5K MWCO was purchased from Thermo Fisher Scientific (Nottinghamshire, UK).

120

121 **Glycation of pre-oxidized BSA**

122 BSA (80g/L) was incubated with H₂O₂ (10nM) for 8 hours at 37°C in PBS and was then
123 dialyzed against PBS (8:1) for another 8 hours. The dialysate was discarded and replaced
124 with fresh PBS three times during dialysis.

125

126 To measure the effect of protein pre-oxidation on the susceptibility of BSA to glycation, both
127 native and pre-oxidized albumin (40g/L) were incubated in the presence of glucose (0, 5, 10,
128 20 and 30mM) for 4 weeks. The combination of methylglyoxal (150nM) and glucose (0, 10
129 or 20mM) was also studied in order to replicate glycoxidative conditions of albumin in the
130 circulation. All incubations were repeated in 6 replicates.

131

132 **Albumin glycooxidation**

133 All incubations took place in PBS with sodium azide (0.2g/L) and a final volume of 1.5ml.
134 To investigate the effect of continuous exposure to oxidative damage in the progress of the
135 glycation reaction (glycooxidation) native BSA was incubated with glucose (0,5, 10, 20, or
136 30mM) and combinations of methylglyoxal (150nM) plus glucose (0,10,20mM) for 4 weeks
137 in the presence of H₂O₂ (10nM). Native BSA was also incubated under the same conditions
138 without H₂O₂ (10nM) to serve as a reference. All incubations were repeated in 6 replicates.

139

140 **Comparison of the effect of constant glycation among BSA, mercaptalbumin & human 141 plasma**

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

146

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

150

151 **Protein glycooxidation**

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

157

158 The glucose concentration of 30mM was not employed as it is a highly supra-physiological
159 concentration and the combinations of methylglyoxal plus glucose were also not employed.

160 All incubations were repeated in 5 replicates.

161

162 **Fructosamine measurement**

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

165 Fructosamine levels were measured at week 2 and 4 with the NBT assay, performed in
166 microplates as described previously [15]. Briefly, samples (25 μL) were added to of sodium
167 carbonate buffer (100 μL , 100mM, and pH 10.8) with Nitroblue Tetrazolium (0.25mM).
168 Microplates were incubated for 15 min at 37°C and measured spectrophotometrically against
169 controls at 550nm after 10 and 15 min of incubation. The difference between the two
170 readings was used to calculate concentrations. The fructosamine analog 1-deoxy-1-
171 morpholinofructose (DMF) was used as a standard. All fructosamine measurements were
172 performed in duplicate. Standards and NBT reagent were made fresh every week and stored
173 at -20°C and 4°C respectively. All samples were stored at -20°C.

174

175 **Statistical analysis**

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

184

185

186 **Results**

187

188 **Effect of constant oxidation on BSA glycation**

189 Incubation of native BSA (40g/L) with glucose concentrations below 20mM did not lead to
190 measurable levels of glycated BSA measured after 2 weeks, with or without 150nM
191 methylglyoxal, compared to glucose-free control (Table 1). After 4 weeks, incubation with
192 10mM glucose (with or without methylglyoxal) significantly promoted glycation in native
193 BSA compared to glucose-free control. Exposure to a physiological concentration of

194 hydrogen peroxide (10nM), however, led to significantly higher glycated BSA (measured as
195 fructosamine) at the lower glucose concentrations of 10mM after 2 weeks, and 5 mM after 4
196 weeks incubation (both $p < 0.05$ vs. glucose free control).

197

198 Using constantly-oxidized BSA generated significantly more fructosamine than native BSA
199 after 2 weeks of incubation with 5, 10 and 20mM glucose (increased by 23%, 36% and 35%
200 respectively). Similar results were observed with methylglyoxal (150 nM) alone (19%
201 increase in fructosamine), and with a combination of methylglyoxal (150 nM) plus glucose at
202 concentrations of 10 and 20mM, with 35% and 26% increases in fructosamine respectively
203 (Figure 1). Significantly greater glycation of constantly-oxidized BSA compared to native
204 BSA was also observed after 4 weeks at all glucose concentrations, and with combinations of
205 glucose (10 & 20mM) and methylglyoxal (150nM) (Figure 2). In particular, incubation of
206 BSA with 5mM glucose and 10mM H_2O_2 led to a 35% higher fructosamine concentration
207 compared to the non-oxidised control ($p=0.04$). Although incubating BSA in presence of
208 methylglyoxal (150nM) alone did not lead to significantly increased glycation after neither 2
209 nor 4 weeks (Table 1), combining methylglyoxal (150nM) and glucose (10mM) had a
210 synergistic effect on glycation of constantly-oxidised BSA after 4 weeks ($p=0.02$ vs. glucose
211 alone), as well as some suggestion of an effect on native BSA ($p=0.08$) (data not shown).

212

213 The individual impacts of glucose concentration and oxidation, as well as their interaction, on
214 glycation over periods of 2 and 4 weeks were investigated using a two-way ANOVA.
215 Oxidation had a significant effect on glycation at both two and four weeks ($p < 0.001$) There
216 was strong evidence for an interaction between continuous-oxidation and glucose
217 concentration in driving glycation after 2 weeks of incubation ($p < 0.001$) with a non-
218 significant indication of an effect of this interaction on glycation after 4 weeks ($p=0.058$).
219 While removing data relating to methylglyoxal and glucose incubations from the analysis did
220 not change impact on the significance of the effect of oxidation on protein glycation (p
221 < 0.001 at both two and four weeks), the interaction between oxidation and glucose
222 concentration significantly affected glycation after both two and four weeks ($p=0.001$ and
223 $p=0.01$, respectively).

224

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

226 Incubation of native and pre-oxidised BSA with glucose concentrations lower than 20mM for
227 two weeks did not lead to significantly more fructosamine being produced than the glucose-

228 free control. Nonetheless the pre-oxidation step led to significantly higher glycation,
229 compared to native BSA, after two weeks at the lowest glucose concentration (5mM)
230 ($p=0.016$).

231

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

235

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

237 Reduction of BSA to mercaptalbumin was employed in order to investigate whether
238 commercially available BSA, possibly being oxidised to some extent, would be more or less
239 prone to subtle oxidation driven glycation. For this reason, DTT-treated BSA was incubated
240 with glucose (0-20mM) in presence or absence of 10nM H_2O_2 .

241

242 When mercaptalbumin was incubated with 5 and 10mM glucose under constant oxidation for
243 two weeks, significantly higher fructosamine levels were observed compared to non-oxidised
244 mercaptalbumin ($p=0.03$ & $p=0.006$; respectively). While incubation for two weeks with
245 5mM glucose was sufficient to drive higher glycation in constantly-oxidized mercaptalbumin
246 than glucose-free control ($p<0.001$), non-oxidised mercaptalbumin required incubation with
247 10mM glucose to lead to higher glycation than the glucose-free control ($p<0.001$). No
248 differences between oxidized and non-oxidised mercaptalbumin were observed at week 4
249 (Figure 3).

250

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

256

257 When mercaptalbumin glycation was compared to BSA glycation. Mercaptalbumin was more
258 prone to glycation than BSA in both the presence and absence of H_2O_2 . In the absence of
259 H_2O_2 mercaptalbumin had higher fructosamine concentration than BSA at 5 and 10mM
260 glucose at two weeks ($p=0.004$ & $p=0.002$ respectively) and that was significant at week 4
261 for 5mM glucose ($p=0.005$) and nearly significant for 10mM glucose ($p=0.06$). In the

262 presence of H₂O₂ mercaptalbumin was again more successfully glycated than BSA at 5&10
263 mM glucose at week 2 (p<0.001, for both) and nearly significantly more at 20mM (p=0.057).
264 At week 4 mercaptalbumin was significantly more glycated than BSA only at 5mM glucose
265 (p=0.04) (data not shown).

266

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

273

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

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

282

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

286

287

288 **Discussion**

289

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

295 haemoglobin is glycated in apparently healthy non-diabetic people, amongst whom most
296 heart disease occurs[6].

297

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

317

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

323

324 In both the BSA and mercaptalbumin models, glycation of the protein was significantly
325 higher than the glucose-free control when exposed to a physiological concentration of
326 hydrogen peroxide for two weeks. Although the effect of pre-oxidation and continuous
327 oxidation were very similar with a favour towards continuous oxidation, in so low hydrogen
328 peroxide concentrations the continuous oxidation model is more likely to be of physiological

329 relevance Continuous exposure to hydrogen peroxide led to higher fructosamine
330 concentrations at all glucose levels and oxidation was also shown to act synergistically with
331 glucose, as the interaction between the two was found to be significant in our experiments.
332 Hydrogen peroxide positively interacts with glucose in promoting glycation reactions. One
333 has to keep in mind that the hydrogen peroxide exposure was weak in term of concentration
334 in order to resemble physiological condition and hence it is likely to induce important but
335 subtle effects. When human plasma was exposed to hydrogen peroxide, glycation was
336 significantly higher with 5mM glucose after two weeks, compared to the non-oxidized
337 control. The opposite was found when oxidised plasma was incubated with 10mM glucose
338 for 4 weeks, but the lower fructosamine concentration of the oxidised plasma in that case
339 could be attributed both to increased protein instability and/or glycation being driven to the
340 production of AGEs (not detectable by the NBT method used) rather than early-glycation
341 products as fructosamine.

342

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

352

353 Recognizing that, in physiological systems, protein glycation depends on oxidative damage
354 as well as glucose concentration has implications for scientific understanding and potentially
355 for clinical practice. The term ‘glycooxidation’, currently restricted to the latter stages of
356 Maillard reactions, seems more appropriate than simply ‘glycation’ to describe the overall *in*
357 *vivo* protein glycation process, and similar protocols to ours would be appropriate to study the
358 phenomenon *in vitro*. The quest for normoglycaemia in diabetes management is important to
359 delay vascular and other complications, but potentially hazardous interventions are entirely
360 directed at glucose-lowering: using insulin or anti-diabetic drugs intensively to reduce
361 glycation has been associated with increased risk of hypoglycemia, and of mortality [23]. If
362 oxidative stress is also involved as a trigger for protein glycation and tissue damage, then

363 approaches aimed solely at glucose handling are insufficient, and reducing oxidative stress
364 might be less hazardous. This is not arguing for indiscriminate or high-dose antioxidant
365 treatments. Several trials have suggested worse outcomes from antioxidant vitamin
366 supplementation[24], leading to understandable prejudice against their effectiveness and
367 safety, but a recent meta-analysis of 66 randomised controlled trials indicates benefit from
368 vitamin E supplementation for primary prevention (where there is some baseline
369 insufficiency)[25], and vitamin E also contributes to secondary prevention[26], renewing
370 interest in antioxidant interventions. Our results support findings from the cross-sectional
371 study of Bates et al.[13], which led to the hypothesis that dietary antioxidants may reduce
372 tissue glycation. Also evidence from *in-vitro* studies suggest that antioxidants are having a
373 protective role in protein glycation[20, 27]. Exposure to oxygen radicals such as TBH and
374 H₂O₂ significantly increased haemoglobin glycation *in-vitro* and pre-treatment with vitamin E
375 blocked that effect[28]. Replenishment of the antioxidant defences of GSH-deficient red
376 blood cells, on the other hand, protected them against increased haemoglobin glycation[29],
377 both supporting the hypothesis of oxidative stress being involved in protein glycation.

378

379 The present studies suggest some important avenues for future research, as well as changes to
380 commonly-used experimental models. It is important to question accepted patho-
381 physiological mechanisms if they cannot be demonstrated *in vivo* at physiological
382 concentrations. Our evidence that mild oxidation plays an early role in AGE production is
383 novel and explains a gap in the literature. Prior reduction of BSA might indeed increase the
384 oxidation potential of the protein and hence strengthen the effect of oxidation on glycation, as
385 shown by the two-way ANOVA analysis. While being designed to replicate physiological
386 conditions and employing a large number of replicates (6 instead of the usual 3) to reduce
387 random errors under physiological conditions, the current study does have limitations.
388 Albumin, although the major circulating protein, may not be representative of other
389 glycation-prone proteins, and the results cannot provide an exact mechanism linking
390 oxidative damage to glycation. Using human plasma led to slightly different results than BSA
391 and mercaptalbumin. No effect on glycation was seen from oxidation at glucose levels above
392 10mM after two weeks of incubation; that could be attributed to the fact that plasma from
393 healthy volunteers involves different proteins with different degrees of pro-oxidation and
394 glycation and also a much more competent antioxidant system which would be expected to
395 rapidly scavenge ROS. Glycated proteins already present in plasma could also affect the
396 speed and general kinetics of the reaction. Possible mechanisms involve protein damage by

397 hydrogen peroxide and/or increased glucose autoxidation in the presence of hydrogen
398 peroxide, both likely to increase the affinity of the molecules for the non-enzymatic sugar
399 linkage. There may be selective oxidation of amino acids: for example, tryptophan, a main
400 site for protein glycation, is an oxidation site for human albumin [30-31], suggesting that
401 oxidized amino acids maybe more susceptible to further glycative damage.

402

403

404

405 **Conclusion**

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

414

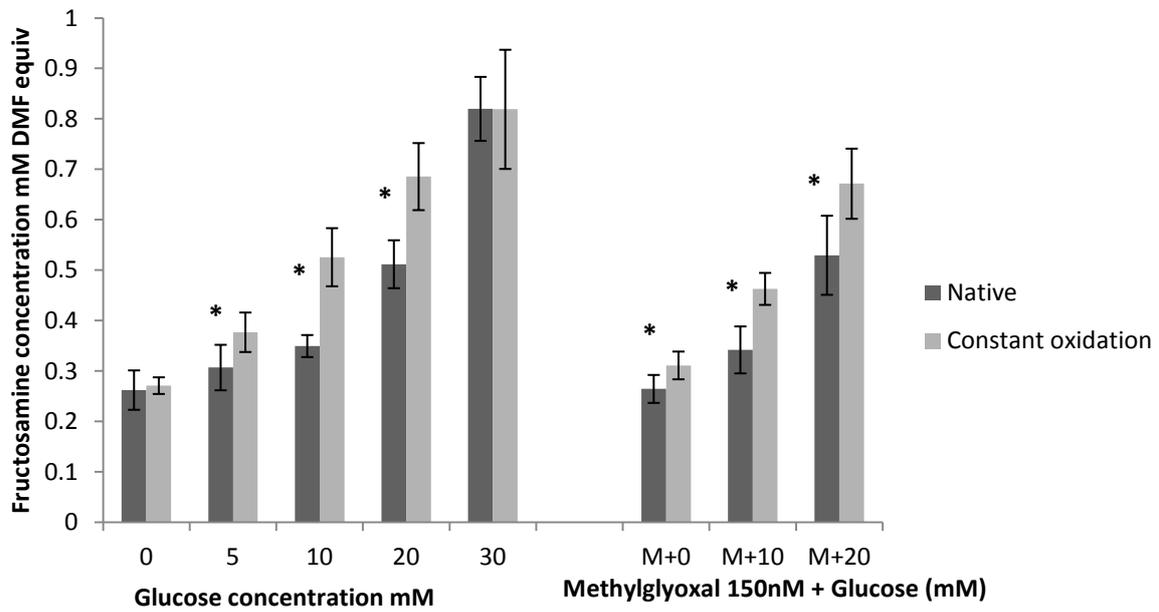
415

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419

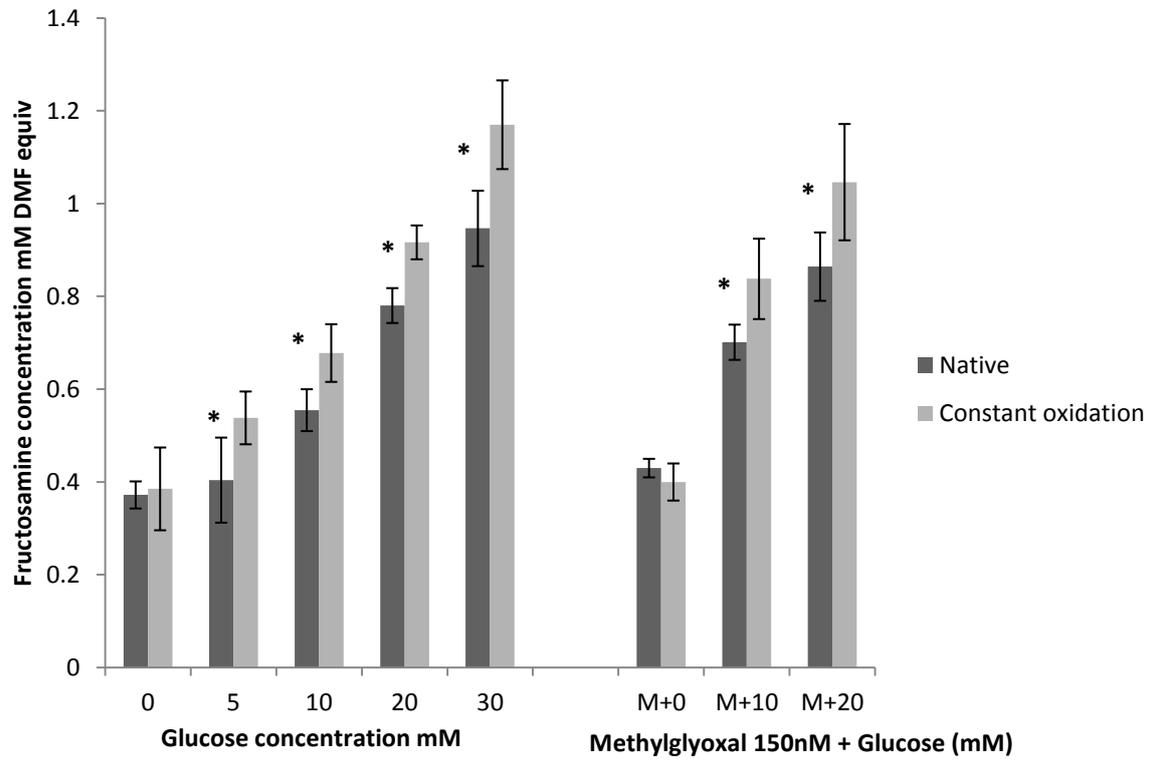
420 **Figure 1.** Fructosamine concentration (mM DMF equivalent) after two weeks incubation with glucose and
421 constant exposure to oxidation from hydrogen peroxide (10 nM). Two-way ANOVA analysis showed a
422 significant effect of oxidation to promote glycation.



423
424
425
426
427

*p<0.05 native vs. constant oxidation for each given glucose concentration

428 **Figure 2.** Fructosamine concentration (mM DMF equivalent) after four weeks incubation with glucose and
 429 constant exposure to oxidation from hydrogen peroxide (10 nM). Two-way ANOVA analysis showed a
 430 significant effect of oxidation to promote glycation.



431 *p<0.05 native vs. constant oxidation for each given glucose concentration

432
 433
 434
 435

436 **Table 1.** Fructosamine concentration after two weeks incubation of albumin with different glucose
 437 concentrations, between and within treatments (glucose / MGO exposure and oxidation)
 438

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

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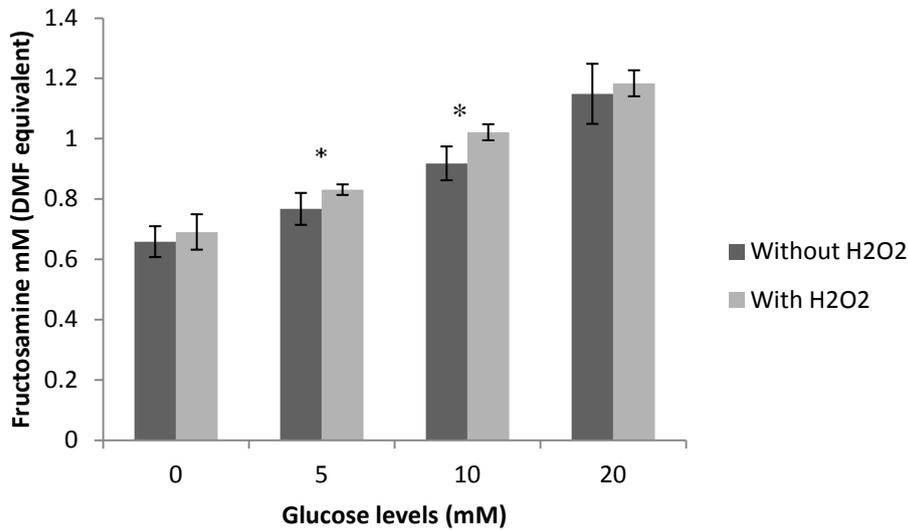
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Figure 3. Fructosamine concentration (mM DMF equivalent) after two and four weeks incubation with glucose and constant exposure to oxidation from hydrogen peroxide (10 nM) in mercaptalbumin. Two-way ANOVA analysis showed a significant effect of oxidation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

Mercaptalbumin

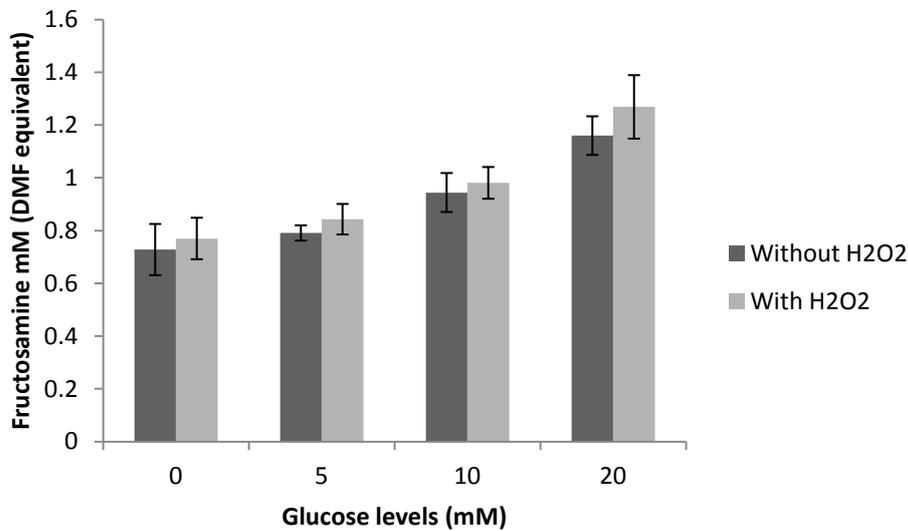
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Week 2



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Week 4

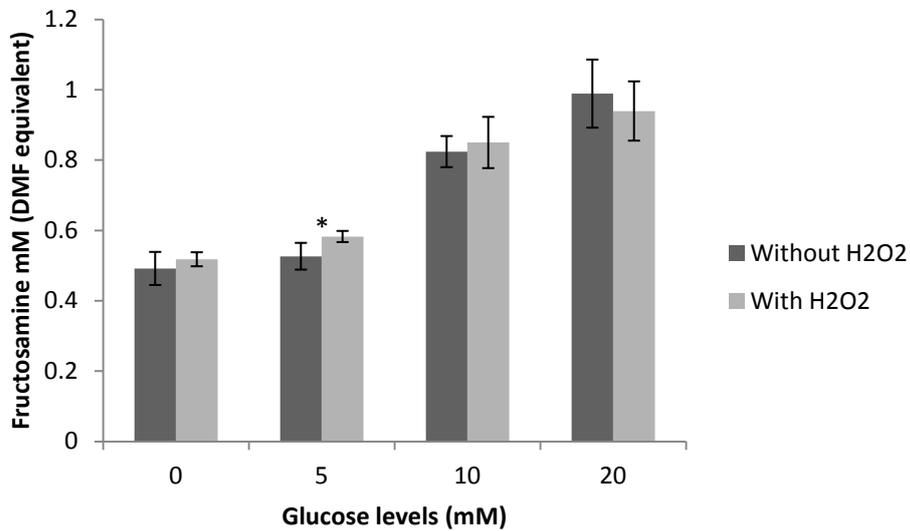


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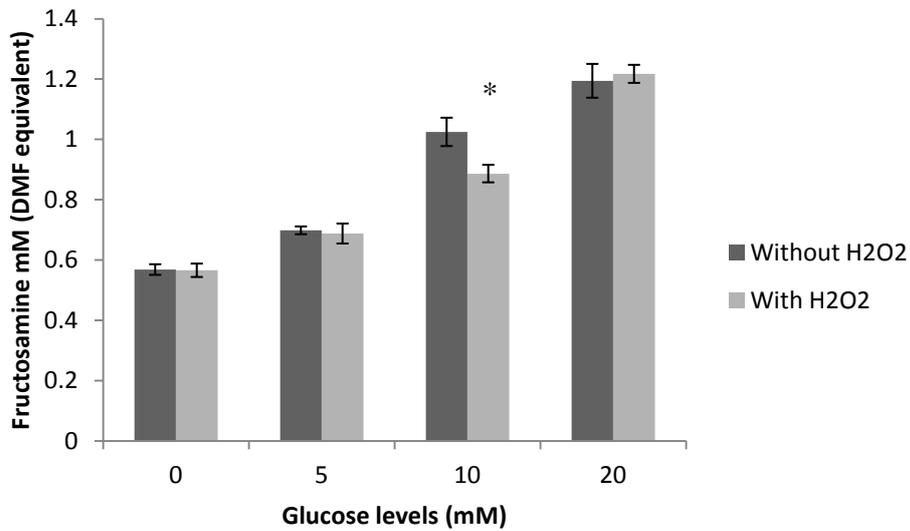
*p<0.05 native vs. constant oxidation

456 **Figure 4.** Fructosamine concentration (mM DMF equivalent) after two and four weeks incubation with
457 glucose and constant exposure to oxidation from hydrogen peroxide (10 nM) in human plasma. Two-
458 way ANOVA analysis showed a significant effect of oxidation to promote glycation.
459

460
461 **Human Plasma**
462 **Week 2**



463
464 **Week 4**



465
466 *p<0.05 native vs. constant oxidation
467
468

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