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MYOGLOBIN EXHIBITS INCREASED IRON RELEASE AND FREE RADICAL MEDIATED OXIDATION REACTION ON ALPHA-OXOALDEHYDE MODIFICATION: HEME PROTEIN AS A POTENTIAL SOURCE OF OXIDATIVE STRESS

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ABSTRACT
Post-translational modification of proteins by Maillard reaction, known as glycation, is thought to be the root cause of different complications, including oxidative stress, particularly in diabetes mellitus and age-related disorders. The reactive α-oxoaldehydes, glyoxal and methylglyoxal, increases in diabetic condition and reacts with proteins to form advanced glycation end products (AGEs) following Maillard-like reaction. In this study, we report that H_2O_2-induced iron release from glyoxal or methylglyoxal-incubated myoglobin is significantly higher than that from unmodified myoglobin. Further, in presence of H_2O_2, modified myoglobin degrades deoxyribose more efficiently than the unmodified protein. Considering the increased level of α-dicarbonyls in diabetic condition, glyoxal or methylglyoxal-induced modification of the heme protein may enhance oxidative damage via Fenton reaction and associated complications. The findings thus appear physiologically relevant with clinical implications.

KEYWORDS: Myoglobin; Advanced glycation end products; Glyoxal; Methylglyoxal; Deoxyribose; Fenton reaction.

1. INTRODUCTION

Protein glycation is an irreversible, non-enzymatic post-translational modification of protein amino groups (N-terminal and arginine/lysine side chains) by carbonyl compounds leading to the formation of advanced glycation end-products (AGEs). The reaction initiates via Schiff base formation, followed by Amadori rearrangement and AGE formation, collectively known as Maillard reaction.[1, 2] Non-enzymatic modification of proteins may be significant with increased level of blood glucose over prolonged periods of time in diabetes mellitus. AGEs are thought to be the root cause of different complications in diabetes mellitus[3], which may be of quite significance due to increased levels of several active carbonyl compounds.

The reactive α-oxoaldehydes namely, glyoxal, methylglyoxal and 3-deoxyglucosone are known to initiate Maillard-like reactions and are more reactive than the parent hexose sugars with respect to their ability of protein modification and AGE formation.[4,5] Methylglyoxal has been reported to react with several proteins namely, hemoglobin, cytochrome c, myoglobin, etc., resulting in either protein cross-linking and aggregation or formation of non-crosslinking AGE adducts (namely, hydroimidazolones, argpyrimidine, carboxyethyllysine, etc.).[6-10] Like methylglyoxal, glyoxal is another reactive oxoaldehyde and its concentration increases from 215 to 230 nM in normal individuals to 350–470 nM in diabetic subjects.[11] It is a major product of glucose degradation under oxidative conditions[12] and has been reported to interact with several proteins, namely, α-crystallin[13], bovine serum albumin[14], myoglobin[15] and hemoglobin.[16, 17]

Several reports have been published on glycation-induced structural and functional modifications of haemoglobin.[18–23] Previous findings indicate that glucose-induced glycation (both in vitro and in vivo) promotes more iron release from hemoglobin and myoglobin than that from the non-glycated heme proteins and this free iron has been shown to enhance free-radical mediated oxidative stress and damage of different cell constituents.[24–27]

In a previous study, it has been shown that glycation of hemoglobin by fructose (fructation) also causes iron release from the heme protein and associated free radical reactions.[28] These findings are significant, because free radicals and oxidative stress have been implicated in

eliciting diabetes as well as causing its complications.[29, 30]

In recent studies, it has been demonstrated that glyoxal or methylglyoxal-modified hemoglobin exhibits lower iron release and subsequently lesser free-iron mediated oxidation reactions in comparison to control hemoglobin. However, no reports on the effect of compounds on the heme protein, myoglobin in terms of iron release and oxidative reactions have been published till date. The present study demonstrates the effect of the α-oxoaldehydes, glyoxal and methylglyoxal, on myoglobin with respect to the release of free iron and subsequent iron-mediated oxidative reactions. We report that modification of myoglobin induces more iron release and subsequently higher oxidation reaction in comparison to unmodified myoglobin.

2. MATERIALS AND METHODS

2.1. Materials

Horse heart myoglobin (Mb), Methylglyoxal (MG), Glyoxal, Deoxyribose, Ferrozine, Hydroxylamine Hydrochloride, Thiobarbituric acid (TBA) were purchased from Sigma Chemical Co, USA.

2.2. Methods

2.2.1. In vitro reaction of Mb with glyoxal and MG

Purchased Mb, which is mostly in met form, was dissolved in 50 mM potassium phosphate buffer (PB), pH 6.0 and its concentration was determined using $€_{408nm}$= 116 mM^{-1} cm^{-1}.[31] Mb (100 µM) was incubated with different concentrations of glyoxal (50, 100 and 200 µM) under sterile conditions for 7 days at 25°C. Mb solution incubated in the absence of glyoxal under identical conditions was used as control. In separate reaction study, Mb (100 µM) was incubated with MG (200 µM) under sterile conditions for different days (7, 14 and 18) at 25°C. Mb solution incubated without MG was used as control.

2.2.2. Estimation of H_2O_2-induced iron release

Iron release from control Mb and glyoxal or MG-treated Mb was estimated in presence of H_2O_2 according to the method of Panter.[32] Protein samples (50 µM each) were incubated with H_2O_2 (250 µM) at 37°C for 1 hr. 250 µl cold TCA (20%) was added to 250 µl sample. 250 µl protein-free supernatant was treated with 2.5 ml iron buffer reagent (1.5% hydroxylamine hydrochloride in 0.2 M acetate buffer, pH 4.5) and 50 µl iron color reagent (0.85% ferrozine in iron buffer reagent), incubated at 37°C for 30 min and absorbance was measured at 560 nm.

2.2.3. H_2O_2-mediated deoxyribose degradation

Deoxyribose degradation was assayed according to the method of Gutteridge.[33] Deoxyribose (0.67 mM) was incubated with Mb or glyoxal or MG-treated Mb (10 µM) in presence of H_2O_2 (0.67 mM) for 1 hr at 37°C. TBA reactivity was developed by adding 500 µl each of TBA (1%) and TCA (2.8%) followed by heating for 10 min in a boiling water bath. The resulting chromogen was extracted with n-butanol and fluorescence intensity was measured at 553 nm by exciting at 523 nm.

3. RESULTS AND DISCUSSION

3.1. Estimation of iron release and deoxyribose degradation

As shown in **Fig. 1A**, H_2O_2-induced iron release was found to be higher from glyoxal-treated Mb (b-d) than control Mb (a), more or less with increasing concentration of glyoxal. Degradation of deoxyribose was higher in presence of glyoxal-treated samples (b-d) than in presence of control sample (a) **(Fig. 1B)**.

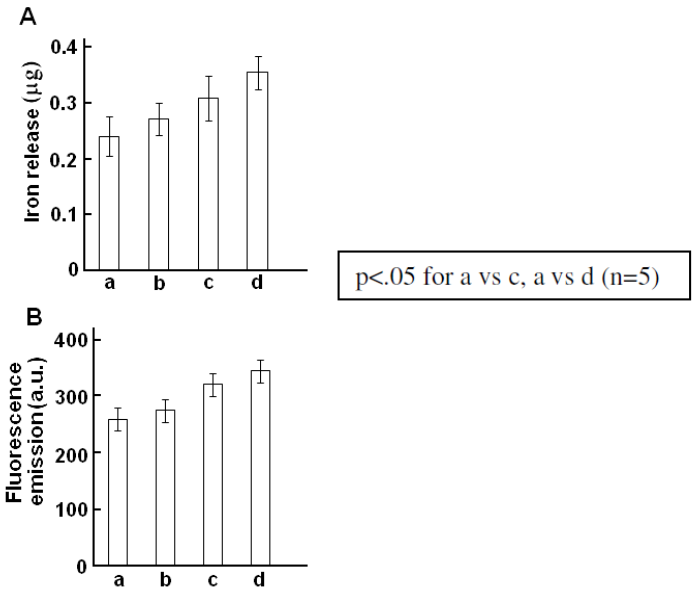

Fig. 1. Effect of glyoxal modification on H_2O_2-mediated iron release and deoxyribose degradation. (A) H_2O_2-induced iron release from Mb (a), 50 µM glyoxal-treated Mb (b), 100 µM glyoxal-treated Mb (c) and 200 µM glyoxal-treated Mb (d) estimated by ferrozine reaction. The results are mean ± SEM of four experiments. (B) Deoxyribose

degradation by Mb (a), 50 µM glyoxal-treated Mb (b),100 µM glyoxal-treated Mb (c) and 200 µM glyoxal-treated Mb (d) in presence of H_2O_2, estimated from fluorescence emission of TBA reactive substances at 553 nm with excitation at 523 nm. The results are mean ± SEM of four experiments.

As shown in **Fig. 2A**, compared to control Mb (a), H_2O_2-induced iron release was found to be higher from MG-treated Mb (b-d). Deoxyribose degradation in presence of MG-treated Mb samples (b-d) was also higher than in presence of control Mb (a) **(Fig. 2B)**. Both iron release and deoxyribose degradation were found to increase more or less with increasing period of MG incubation.

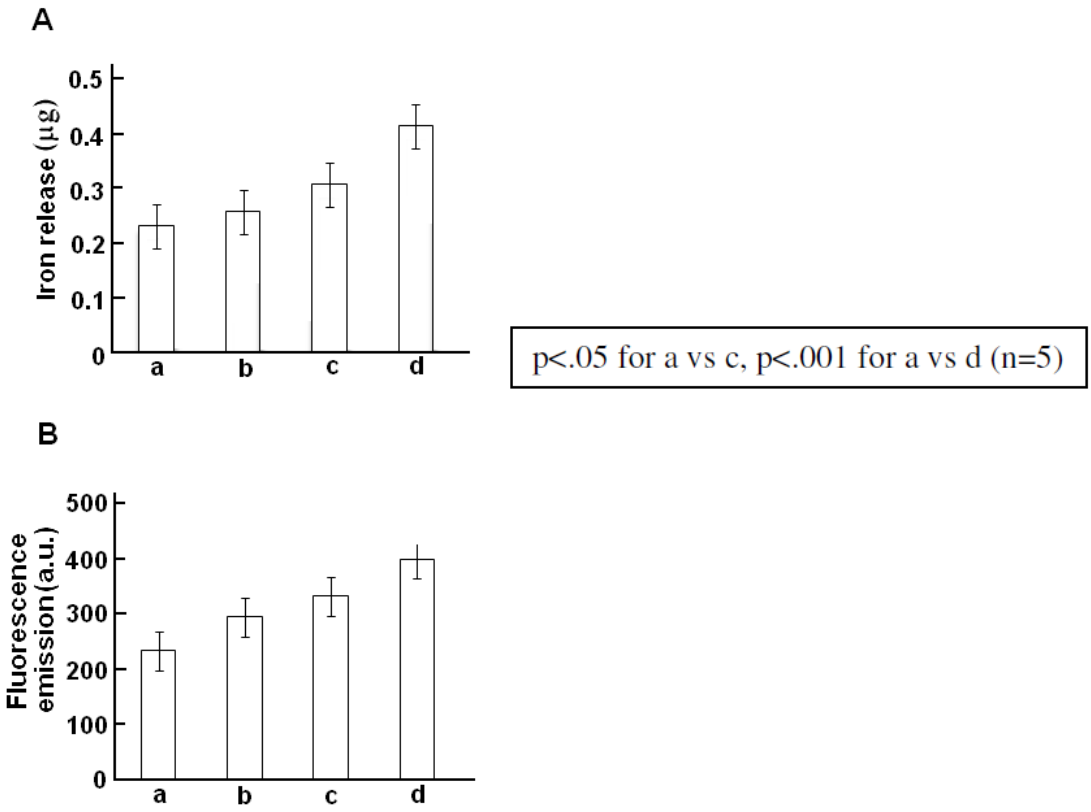

Fig. 2. Effect of MG modification on H_2O_2-mediated iron release and deoxyribose degradation. (A) H_2O_2-induced iron release from control Mb (a), Mb incubated with MG for 7 days (b), 14 days (c) and 18 days (d) estimated by ferrozine reaction. The results are mean ± SEM of five experiments. (B) Deoxyribose degradation by control Mb (a), Mb incubated with MG for 7 days (b), 14 days (c) and 18 days (d) in presence of H_2O_2, estimated from fluorescence emission of TBA reactive substances at 553 nm with excitation at 523 nm. The results are mean ± SEM of four experiments.

H_2O_2 is known to induce iron release from haemoglobin.[33, 34] Takasu et al.[35] reported stimulation of H_2O_2 generation in streptozotocin or alloxan-induced diabetic rats. H_2O_2 has been reported to promote more iron release from HbA_{1c} than that from non-glycated hemoglobin, HbA_0, and iron-mediated free radical reactions namely, lipid peroxidation, deoxyribose degradation, DNA breakdown, and carbonyl formation are more pronounced with HbA_{1c} in comparison with HbA_0.[25, 26] Cussimanio et al.[36] reported that heme degradation in in vitro-glycated hemoglobin and myoglobin might be initiated by H_2O_2 formation, followed by reaction with superoxides generated or hydroxyl radicals or with both. Release of trace metals such as iron and copper in biological systems may be significant, because they may be a source of reactive oxygen species (ROS). The accumulation of ROS in cells leads to various forms of oxidative modifications of proteins (carbonylation or nitro-modifications), lipids (hydroperoxide lipid derivatives) and DNA (adducts and breaks) leading to loss of their molecular functions. $OH^{\bullet}$ radicals are produced by Fenton reaction: $Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + OH^{\bullet}$[33] **(Fig. 3)**. $OH^{\bullet}$ radicals specifically attack the pentose sugar 2-deoxy-D-ribose to yield a mixture of products. On heating with TBA at low pH, some or all of these products react to form a pink chromogen, which is measured spectrophotometrically or spectrofluorimetrically.

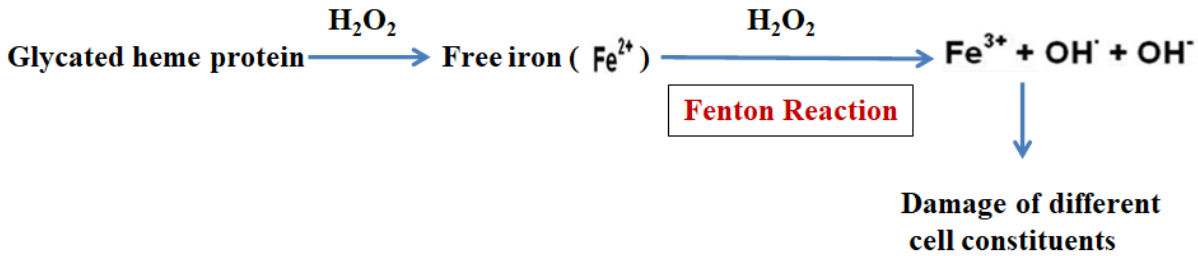

Fig. 3. Glycated heme protein as a source of oxidative stress. Glycation of heme protein generates free iron (Fe^{2+}) which reacts with H_2O_2 to generate $OH^{\bullet}$ radicals via Fenton reaction ($Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + OH^{\bullet}$). The reactive $OH^{\bullet}$ radicals induces oxidative damage to different cell constituents leading to oxidative stress.

Glycation changes the steric pattern of the protein to weaken the stability of the heme-globin linkage. Heme binding causes the globin to form a more compact structure with 20% increase in helicity.[37] Weak heme-globin linkage may be a preferential target of attack by H_2O_2 leading to heme degradation and iron release. Glyoxal or MG-induced structural modification of myoglobin may weaken heme-globin linkage resulting in more iron release. Heme-globin linkage in hemoglobin has been reported to be weakened by glycation[26], and glycation resulted in iron release from myoglobin[27] and hemoglobin.[25] Free iron then catalyses oxidative reactions like lipid peroxidation, deoxyribose degradation, and DNA breakdown.[25- 27]

MG-induced modifications in several proteins have been associated with physiological abnormalities like oxidative stress, dyslipidemia, mitochondrial dysfunction, cell detachment, and apoptosis.[38, 39] Our current finding suggests that α-dicarbonyl induced iron release from myoglobin is another source of ROS generation in the development of glyoxal or MG toxicity, and is in agreement with our earlier reports on glucose and fructose toxicities of heme proteins.[25-28] Hemoglobin samples isolated from streptozotocin-induced diabetic rats also exhibit enhanced iron-dependent oxidative reactions of the cell constituents.[40] Glyoxal or MG, being much stronger reactive agents than its parent hexoses, may have significant contribution to the observed pathological effect in diabetic condition.

4. CONCLUSION

In the present study, we find that glyoxal or MG-induced modification of myoglobin enhances its iron release and subsequent free iron-mediated oxidative reactions. Considering the increased level of α-oxoaldehydes in diabetes mellitus as well as its high reactivity, glyoxal or MG-induced modifications may be associated with the oxidative stress and pathological complications of the metabolic disorder.

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