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High glucose-induced membrane lipid peroxidation on intact erythrocytes and on isolated erythrocyte membrane (ghosts)

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Glucose autoxidation has been proposed to play a role in diabetes complications via free radicals damage to cells, but the mechanisms that should link high glucose to oxidative stress is still partially unknown. In this study we report that high glucose induces lipid peroxidation, membrane tocopherol consumption, and lysis in intact erythrocytes (RBC), whereas antioxidant enzyme activity was not affected. On the contrary, no lipid peroxidation or free thiols oxidation was detectable in isolated erythrocyte membranes (ghosts) incubated either with glucose or methyl-glyoxal, a carbonyl molecule, non-enzymatic byproduct of high glucose metabolism. Our study indicates that the presence of transition metals is the necessary condition to induce glucose-driven lipid peroxiation and thiols oxidation. This study also suggests that the incubation of RBC with glucose concentration similar to non-controlled hyperglycaemia may led to the release of iron in redox active form. (J. Nutr. Biochem. 7: 156–161, 1996.)

Keywords: glucose autoxidation; erythrocyte; erythrocyte membrane oxidation

Introduction

Various cytotoxic mechanisms have been proposed to explain the role of glucose in the outcome of most diabetic complications. Diabetics have an increased prevalence of atherosclerosis and an increased prevalence of many other diseases that have been claimed to have a "free radical" aetiology. Hyperglycaemia appears to be involved in many diabetic alterations through many different mechanisms such as abnormalities of polyol pathway, glycation of cellular proteins, lipid peroxidation, and protein damage during monosaccharide autoxidation.[1] Little attention has been given to this latter mechanism up to now. Like other hydroxyaldehydes, glucose can enolise and, in presence of transition metals, it reduces molecular oxygen leading to the formation of different reactive intermediates.[1] Diabetics have increased levels of NADH and decreased levels of NADPH.[2,3] This causes alterations in the concentration of monosaccharides during glycolysis and an accumulation of glyceraldehyde-3-phosphate, dihydroxyacetone phosphate and acetol and methyl-glyoxal. All these molecules are theoretically able to act as pro-oxidant through different mechanisms, which involve the trace levels of transition metal and subsequent attack to both lipid and protein substrates.[1]

Previous studies have shown that "in vitro", diabetic-like concentrations of glucose induces lipid peroxidation[4,5] and inactivation of membrane-associated enzymes[5] in erythrocytes. Increased levels of thiobarbituric acid-reactive substances (TBA-RS) were found in diabetic humans[6] and rats,[7] suggesting that prolonged hyperglycaemia is associated with increased lipid peroxidation in vivo. Thus, the true mechanism by which glucose acts as lipid pro-oxidant "in vivo" is still poorly understood.

In this study we report the effect of elevated concentration of glucose in the incubation medium on lipid peroxi-

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dation of intact erythrocytes and on isolated erythrocytes membrane.

Methods and materials

All chemicals were purchased by Sigma and reagents for HPLC analysis were of the appropriate grade of purity.

Human blood from healthy volunteers was kindly provided by the "Centro Trasfusionale Sangue" (Blood Transfusion Centre) of Rome. Erythrocytes were separated by centrifugation, washed three times with phosphate-buffered saline (PBS), and further purified by filtration through cellulose.

Experiments on intact erythrocytes

Erythrocytes were suspended at about 50% hematocrit in PBS containing 10 μl/ml of pen-strep (300 mg penicillin G and 500 mg streptomycin in 10 ml redistilled water). Erythrocytes were separated in aliquots containing no glucose or 5, 15, and 35 mM glucose and 35 mM mannitol utilised as osmotic control. These aliquots were incubated at 37°C in shaking water bath for 24 h.

At the end of the incubation time, the extent of haemolysis was evaluated by measuring by Drabkin's solution the ratio between total haemoglobin and the haemoglobin in the supernatant after centrifugation.

At the beginning and at the end of the incubation, Thiobarbituric reactive substances (TBA-RS) were assayed as described by Jain[4] after washing with PBS to remove the interference of glucose with TBA-RS test. TBA-RS is expressed as percent increase with respect to the baseline, for each experiment. Aliquots of erythrocytes were lysed in hypotonic phosphate buffer (5 mM, pH 8.2) and haemoglobin-free membranes (ghosts) were separated by centrifugation at 20.000 × g for 20 min as described by Dodge et al.[8] and furtherly washed twice in the same buffer in presence of phenyl methyl-sulphonyl fluoride (PMSF) as inhibitor of proteases.

The activity of superoxide dismutase (SOD) and Glutathione peroxidase (GSHPx) enzymes was assayed on the lysate according to Oberley & Spitz[9] e Gunzler & Flohe,[10] respectively.

Lipids from ghosts were extracted as described by Burton[11] and membrane tocopherol concentration was measured in the exane extract by HPLC associated with fluorimetric (295 Ex-345 Em) as described by[12]. Tocopherol was standardized per μg of extracted lipids.[13]

Experiment on isolated erythrocyte membranes (ghosts)

Ghosts were obtained from fresh erythrocytes as previously described and suspended in PBS at 37°C for 30 min for resealing.

Resealed ghosts were diluted in PBS plus PMSF and pen-strep solution, at a protein concentration of 1 μg/μl and incubated at 37°C for 24 h in a shaking water bath with different treatments as described under "Results."

At selected times (time 0, 3, 6, 24 hr) of incubation, lipid peroxidation was assayed by the TBA-RS test as described by Calzada and Rice-Evans.[14] Free thiols (-SH) concentration in membrane was assayed according to Haest et al.[15] Both TBA-RS and -SH are expressed as percent increase or decrease with respect to the beginning of incubation (baseline) for each experiment.

Experiments with added iron were carried out by using ADP-$FeSO_4$ complex in a molar ratio 50:1 and with a final Fe^{2+} concentration equal to 50 μM.[16]

Statistical analysis

The significativity between differences was assessed by means of ANOVA coupled to the LSD test. The minimum level of significativity was set at $P \leq 0.05$.

Table 1 TBA-RS (% increase with respect to the baseline), % Hb release in the incubation media, and tocopherol membrane concentration (ng/μg lipids) in RBC at the beginning and at the end of the incubation at 37°C with different amount of glucose. All values are the mean ± S.D. of six experiments. Figures not sharing the same superscript are significantly different ($P < 0.05$ by ANOVA)

	TBA-RS	% Hb released	Tocopherol
Baseline	100	—	1.40 ± 0.03
No glucose	130.0 ± 3.6a	1.26 ± 0.11	1.34 ± 0.02a
5 mM glucose	181.3 ± 4.2b	1.11 ± 0.05a	1.27 ± 0.03b
15 mM glucose	196.7 ± 5.5bc	1.20 ± 0.08	—
35 mM glucose	204.0 ± 6.6c	1.30 ± 0.11b	1.14 ± 0.02c
35 mM mannitol	120.0 ± 3.4d	0.88 ± 0.06c	1.50 ± 0.03d

Results

Experiments on intact RBC were performed to corroborate previous studies reporting that high glucose induces lipid peroxidation in a concentration-dependent fashion.

Human red blood cells incubated at 37°C in the presence of high glucose concentration, similar to that observed in diabetic patients, showed an increased lipid oxidation, which is proportional to the glucose concentration as determined by an increase TBA-RS. A low, though significant, lipid peroxidation was detectable at the end of the incubation time also in absence of glucose added to the medium, possibly due both to the expected autoxidation of membrane lipids and to the physical stress imposed by the continuous shaking during incubation. Concurrently RBC lysis, expressed as the release of Hb in the medium, was also significantly increased at the end of incubation time. RBC lysis in presence of low-glucose concentration was significantly lower than that observed with no glucose added to the incubation medium. Membrane tocopherol concentration also decreased during incubation, the decrease being proportional to the concentration of glucose added to the medium.

The presence of 35 mM mannitol in the incubation medium, utilized as osmotic control, induced formation of TBA-RS and lysis significantly lower than glucose at the same concentration, and no loss of membrane tocopherol with respect to the baseline value.

In the assumption that increased glucose concentration, either through direct glycation or by inducing oxidative damage, could affect enzymatic function and drain on the cellular antioxidant defenses, SOD and GSHPx activity were assayed under the same conditions at 5 and 35 mM glucose.

As summarized in *Table 2*, no significant differences

Table 2 SOD activity (Units/g Hb) and GSHPx activity (mUnits/mg protein) at the beginning and at the end of the incubation at 37°C with 5 or 35 mM glucose. All values are the mean ± S.D. of six experiments

	SOD activity	GSHPx activity
Baseline	2806 ± 129	7.84 ± 0.19
5 mM glucose	2799 ± 132	7.90 ± 1.40
35 mM glucose	2843 ± 21	8.00 ± 0.96

were observed at the end of incubation time, the activity of both enzymes remaining stable independently from glucose concentration. To understand the mechanism underlying glucose driven oxidation, we have simplified the model system and performed further experiments using ghosts instead of RBCs.

Haemoglobin-free membranes were incubated for 24 hr at 37°C in PBS containing either 35 mM glucose or t-Butyl hydroperoxide (tBOOH), employed as a reference pro-oxidant agent.[17] *Figure 1* shows lipid peroxide formation, assessed as TBA-RS, and the loss of free sulphydryl group during the incubation.

High concentration of glucose did not lead to the formation of TBA-RS eventually protecting the loss of free sulphydryl groups in comparison with control ghosts. The possibility of a glucose-dependent pro-oxidation catalysed by trace metal was investigated by repeating the incubation experiments in the presence of added chelated iron.

The addition of Fe-ADP to ghosts induced a significant, time-dependent lipid oxidation, whereas free-sulphydryl groups were not affected (*Figure 2*). The presence in the incubation mixture of both ADP-Fe and glucose 35 mM produced a distinct additive effect, strongly stimulating both lipid peroxidation and thiols depletion.

It is known that subsequent to high glucose, glyceraldehyde phosphate, a product of the glycolytic pathway, may give rise to methyl-glyoxal, as a non-enzymatic byproduct. In this context the experiments described were repeated using methyl-glyoxal instead of glucose (*Figure 3*).

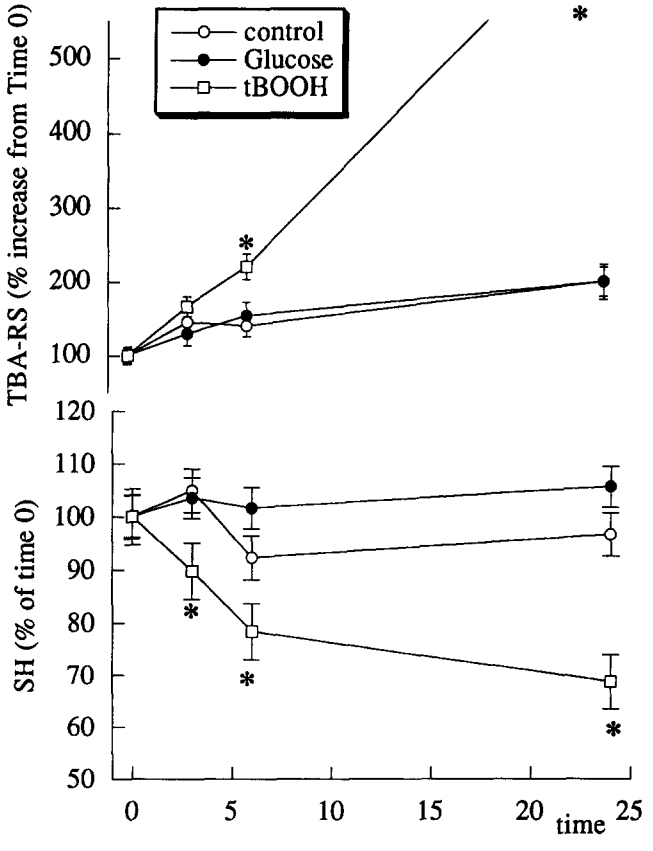

Figure 1 TBA-RS and -SH in ghosts incubated in PBS with glucose and tBOOH 25 μM. The asterisk (*) indicates a significant difference (<0.05 by ANOVA) from the control at the same incubation time.

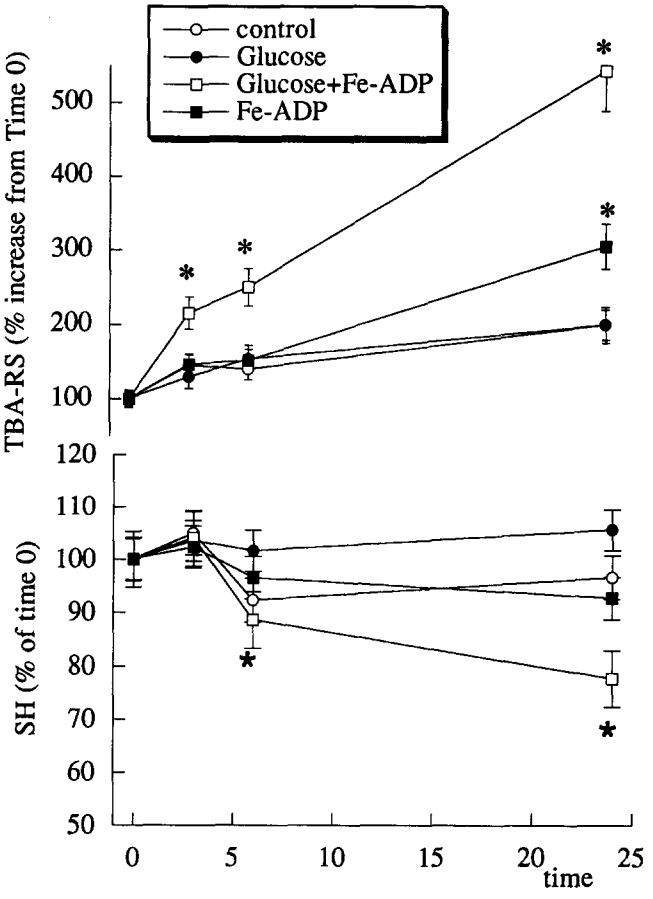

Figure 2 TBA-RS and -SH in ghosts incubated in PBS with glucose, ADP-Fe, and glucose plus ADP-Fe. The asterisk (*) indicates a significant difference (<0.05 by ANOVA) from the control at the same incubation time.

As in the case of high glucose, methyl-glyoxal potentiated the lipid peroxidation induced by Fe-ADP complex. However, methyl glyoxal plus Fe-ADP had no significant effect on membrane free suphydril groups.

On the basis of previous studies suggesting that oxidative stress in erythrocytes results in a release of iron in diffusible and redox-active form, we have tried to assess if the lipid peroxidation observed in intact erythrocytes was due to iron-containing molecules possibly released in the course of high-glucose incubation. To test this possibility, we first incubated intact RBC with both high glucose (35 mM) or with no glucose for 24 hr at 37°C. At the end of the incubation, RBC were lysed in hypotonic buffer, centrifuged, and the lysate was filtered through a molecular membrane with a cut-off limit of 30.000. The low molecular weight (LMW) filtrate, obtained from both glucose and no-glucose incubation, was then added to a suspension of freshly prepared ghost with added glucose and incubated at 37°C for 24 hr.

Figure 4 shows the time course of the production of TBA-RS and the sulphydryl levels of ghosts incubated with glucose plus either the LMW fraction obtained from RBC incubation with glucose (LMWG) or without glucose (LMWnG).

LMWG induced a light, though significative, oxidative

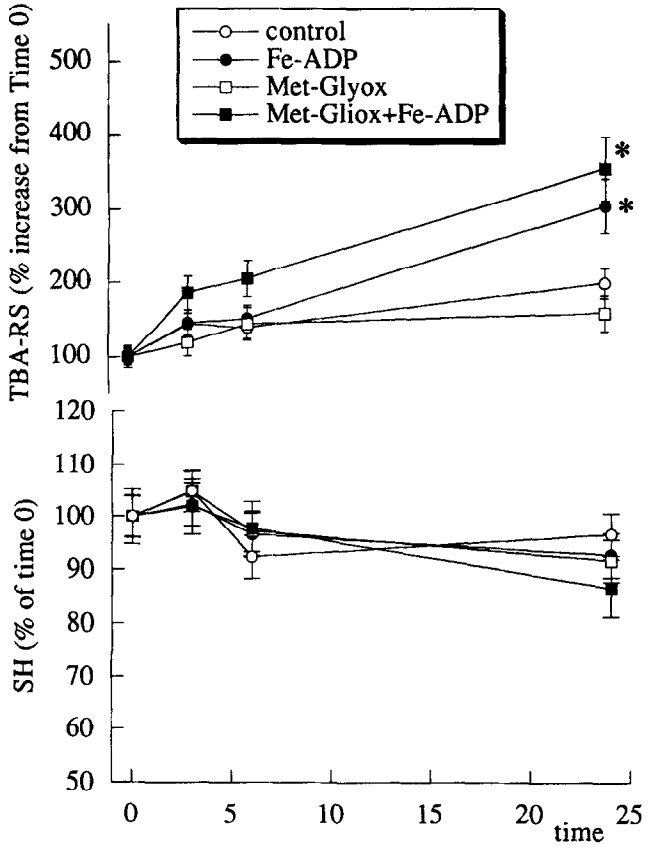

Figure 3 TBA-RS and -SH in ghosts incubated in PBS with methylglyoxal and methyl glyoxal plus ADP-Fe. The asterisk (*) indicates a significant difference (<0.05 by ANOVA) from the control at the same incubation time.

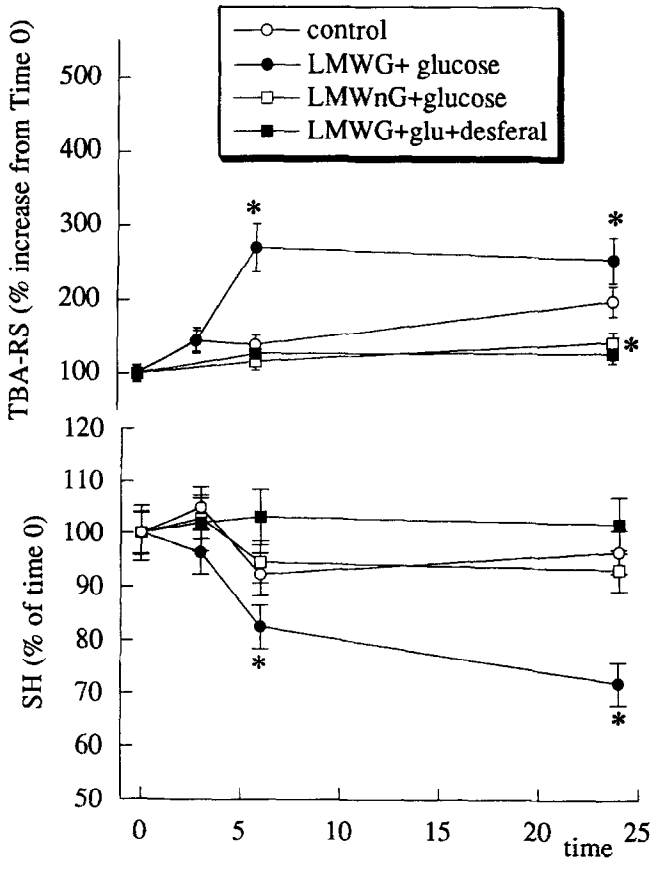

Figure 4 TBA-RS and -SH in ghosts incubated in PBS with the low molecular weight fraction of the lysate of RBC incubated 24 hours either with glucose (LMWG) or without glucose (LMWnG). The asterisk (*) indicates a significant difference (<0.05 by ANOVA) from the control at the same incubation time.

stress resulting in an increase of TBA-RS and free thiols depletion from ghosts that were cancelled by the addition of desferal ion the medium (see *Figure 4*). Lipid peroxidation at 24 hr incubation with desferal was significantly lower than that observed when ghosts were incubated with both LMWG and the control.

Conversely, the addition of LMWnG to glucose, did not induce any detectable oxidation of free sulphydril groups.

Discussion

Several mechanisms have been proposed for the toxicity of glucose. Glucose has been shown to react with free amino groups of proteins to form reversible Schiff bases and more stable Amadori products, as well as irreversible advanced glycosylation end products (AGE products).[18] Early glycosylation products are formed in proportion to blood glucose levels and return to normal levels in controlled diabetes. In contrast, the level of AGE products do not revert to normal when hyperglycaemia is corrected, and accumulate over the lifetime of the vessel-wall proteins.[19] Both early and end stage glycation products have several possible detrimental consequences in vivo by changing protein isoelectric points, protein conformation, or via formation of protein-protein or protein-lipid cross links. AGE products have been implicated in increased vascular permeability, vessel wall thickening, and decreased elasticity.[20–22] These are thought to result from permanent structural alteration in long-lived extracellular components, reduction in proteoglycan recognition sites and induction of various cytokines such as tumour necrosis factor, interleukin 1a via a macrophage AGE receptor site.[21]

It has been recently suggested that glucose autoxidation may also play an important role in diabetic complications. Simple monosaccharides have been shown to autoxidise under physiological conditions forming reactive dicarbonyl compounds, free radical intermediates, and hydrogen peroxide.[23] This reaction is thought to be transition metal-dependent[23] and is dependent on the rate of enediol formation from the monosaccharide substrate.[24]

Many studies have reported a free radical implication in diabetes. Wolff et al. have demonstrated that the aldehydes derived from glucose autoxidation can attach to the lysine groups of proteins and in addition, participate in protein-oxidative damage.[1] This was proposed to result from the production of hydroxyl radicals (or other radicals of similar reactivity) during glucose autoxidation.[1,23] It was also shown that glycated proteins are more susceptible to oxidation. There have also been several reports describing increases in lipid hydroperoxides and lipid decomposition products in the presence of glucose.[4,22] Diabetic individuals have been also reported to have increased levels of free

peroxides,[25,26] and decreased levels of many antioxidants such as ascorbic acid,[27] vitamin E,[6] as well as decreased activity of superoxide dismutase.[28]

The mechanisms by which monosaccharide autoxidation might play a role in lipid peroxidation have not been well characterised. It has been proposed that hyperglycaemia may augment lipid peroxidation by either generation of radical species from glucose autoxidation or glycated protein.[29]

Adventitious metal has been shown to form loose complexes with carbohydrates and glycated polylysine.[30] These complexes may also participate in lipid peroxidation. Hicks et al.[29] observed an increase in lipid peroxidation of fatty acid vesicles incubated with increasing concentrations of either glucose or glycosylated collagen. Lipid hydroperoxides have been proposed to react directly with the open chain configuration of glucose producing a glucose enediol radical and a lipid alkoxyl radical.[29] Glucose radical species have been suggested to participate in peroxidative chain reactions.[29]

Consistent with these hypotheses, is the central role of phospholipid-hydroperoxides. Though the presence of glucose does not appear to affect either the rate or extent of lipid peroxidation, its autoxidation and/or products cause a concomitant increase in lipid peroxidation as well as protein oxidation.[19] Lipid hydroperoxides may, in turn, augment the process of glucose autoxidation by increasing the availability of oxidized transition metal.

In our study, we had first a confirmation of previous studies addressing the pro-oxidant ability of high concentrations of glucose in RBC.[4,5] The presence of high glucose, at concentration up to those typical of non-controlled diabetes (35 mM), induced significantly higher lipid peroxidation than either no glucose or low glucose (5 mM), as indicated by the increase of TBA-RS. RBC lysis was also significantly affected by glucose concentration. These results confirmed those obtained by other authors[4,5] and also confirmed that high glucose is associated with lipid peroxidation on intact RBC. On the other hand, low glucose concentration (5 mM) seems to protect against lysis, with respect to the incubation with no glucose, probably providing an adequate substrate for GSH regeneration during incubation through the formation of NADPH.[4]

Membrane tocopherol decrease was significantly associated by glucose concentrations in the incubation medium, suggesting that a mild oxidative stress occurred, challenging RBC membrane.

A low level of both lipid peroxidation and lysis are detectable in absence of glucose, indicating that the incubation itself induces stress on isolated RBC, which is however significantly affected by high concentration of glucose.

We also explored the possibility that the increase in lipid peroxidation observed in intact RBC could be due to a rapid inactivation of antioxidant enzymatic defenses by glycation, by measuring the activity of two main antioxidant enzymes (SOD and GSHPx) at the beginning and at the end of the incubation time.

Both SOD and GSHPx activity remained stable after 24 hr of incubation either with high or low glucose concentrations.

To better understand the mechanism of glucose induced lipid peroxidation, we have repeated and expanded the experimental model by using ghosts instead of RBC, thus excluding any contribution of metabolic or enzymatic mechanism into the observed phenomena.

No significant increase of both lipid peroxidation and membrane thiols oxidation was observed in isolated ghosts incubated with a glucose concentration equal to that previously utilized in intact RBC. This suggests that not glucose itself, but metabolic byproducts generated during incubation of intact RBC with high glucose, might be possibly responsible for the observed lipid peroxidation.

In short, we assume that the lacking pro-oxidative agent may be some non-enzymatic byproduct of glucose (methylglyoxal), or trace level of transition metal (iron) or a combination of these two factors. Our experiments indicate that the addition of Fe-ADP complex both to glucose and methyl-glyoxal greatly potentiate the Fe-induced lipid peroxidation and free thiols loss in ghosts.

These experiments let the question open of the possible origin of "free catalytic iron" in intact erythrocytes. Previous studies have shown that oxidative stress induced by phenyl hydrazine in erythrocytes may results in the release of iron in diffusible and redox-active form.[31] On this basis, we have tried to assess if during high glucose incubation a similar release could occur. In this case, glucose induced lipid peroxidation observed in intact erythrocytes may be due to iron-containing molecules possibly released in the course of high glucose incubation.

The LMW filtrate, obtained from the incubation of intact RBC with hyperglycaemia-like concentration of glucose (LMWG), induced significant lipid peroxidation and protein oxidation in ghosts which was eliminated by desferal, indicating that ferric ions should be involved (see *Figure 4*). On the contrary no protein oxidation and lipid peroxidation were detectable in ghosts incubated with the LMW filtrate obtained from intact RBC incubated without added glucose (LMWnG) (see *Figure 4*).

These experiments suggest that hyperglycaemia induces a mild oxidative stress on RBC and may lead to the release of catalytic transition metals that, in turn, exacerbates oxidative stress. As well, dicarbonyl molecules, non-enzymatic byproducts of glucose metabolism (e.g., methyl-glyoxal), which have been shown to be increased in diabetes,[32] may have a cooperative effect with transition metals in the induction of the observed lipid peroxidation and thiol damage.

These results support the view of a free radical-mediated origin of many diabetic complications. The increase of transition metals availability and dicarbonyl molecules during hyperglycaemia appears to be a possible agents for the oxidative stress in diabetes.

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