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Effects of ageing on carbonyl stress and antioxidant defense in RBCs of obese Type 2 diabetic patients

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Abstract

In this study we investigated the effects of ageing on the carbonyl stress (protein carbonyls and 4-hydroxy-2-nonenal groups) and glutathione antioxidant defense in red blood cells (RBCs) of obese Type 2 diabetic patients with/without hypertensive complications. To this purpose the following methods were used: spectrophotometry (protein carbonyls, glutathione and glutathione peroxidase assays), immunofluorescence (4-hydroxy-2-nonenal localization), western blotting (immunodetection of carbonylated proteins). The results showed that compared to RBCs of healthy subjects, in obese Type 2 diabetics, ageing is associated with: (i) an increase in the concentration and expression of carbonylated proteins, a marker of oxidative stress; (ii) a decrease of both non-enzymatic and enzymatic endogenous glutathione defenses; (iii) a severely disturbed oxidant/antioxidant balance when obesity was associated with hypertension. The simultaneous insults of high blood pressure, obesity, and diabetes conducted to the highest carbonyl stress, exposure of 4-hydroxy-2-nonenal Michel adducts at the outer leaflet of RBCs plasmalemma, and the lowest glutathione antioxidant potential, particularly in elderly patients. These results can explain the gradual age-dependent diminishment of the detoxification potential of RBCs that at the old age can not overcome the deleterious effects of the high systemic oxidative stress.

Keywords: Type 2 diabetes • obesity • hypertension • ageing • carbonyl stress • 4-hydroxy-2-nonenal • glutathione • glutathione peroxidase

Introduction

While the systemic oxidative status was identified as an important contributor to ageing-related processes [1, 2], the modification of RBCs antioxidant defense during ageing is still controversial. Thus, RBCs catalase and glutathione peroxidase (GPx) activities were reported to be either positively correlated with ageing [3] or to have no significant modifications [4].

In Type 2 diabetes (compared to physiological conditions) RBCs were reported to have reduced glutathione (GSH) concentration [5], diminished values of reduced to oxidized glutathione ratio (GSH/GSSG) [6], and decreased GPx activity [7, 8]. However, no studies correlated the glutathione antioxidant protective effects with the ageing-related processes.

In obesity it was reported that RBCs have low activities of Cu, Zn - superoxide dismutase and GPx even in the absence of diabetes [9]. Interestingly, RBCs of obese persons expose phosphatidyl serine residues at the outer leaflet of their membrane, a feature contributing to the phagocytosis of senescent RBCs [10].

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Other modifications of RBCs properties occurr in hypertension. The osmotic fragility of the RBCs membrane was higher in hypertensive *vs.* normotensive subjects [11] and their deformability was reduced, causing impaired oxygen delivery within microcirculation [12]. In essential hypertension the protective antioxidant capacity of RBCs was diminished, as indicated by the significant depletion of endogenous GSH and the increased levels of GSSG [13, 14].

In this study we investigated the effects of normal ageing on red blood cells (RBCs) oxidant/antioxidant balance in Type 2 diabetes associated with obesity and hypertension.

Materials and methods

Patients

A number of 72 patients hospitalized at the Institute of Diabetes, Nutrition and Metabolic Diseases "N. Paulescu" and the Universitary Hospital (Bucharest, Romania), and 39 control volunteers participated in this study. Informed consent was obtained from each study subject. The patients were divided into three equivalent groups: (i) obese Type 2 diabetics (OD), (ii) obese Type 2 diabetics with arterial hypertension (ODH), and (iii) healthy controls (C). The patients included in groups OD and ODH were obese persons and those from group C were at normal weight. As a function of biological age, patients were divided into three groups: young (20–30 y; 25.54 ± 0.88 y) (n=21), mature (30–60 y; 40.22 ± 2.39 y) (n=27), and elderly (60–85 y; 72.11 ± 2.25 y) (n=24). Since Type 2 diabetes is usually a pathology of mature and old age, there were no young subjects in the OD and ODH groups. The anthropometric and blood pressure level measurements and routine biochemical assays were performed by the hospital staff, conducting to the inclusion of subjects in the OD, ODH and C groups.

Blood samples

After fast, blood was sampled on EDTA-coated tubes, spun down at 1,550 g for 10 min, plasma and leukocyte layers removed, and the packed RBCs layer used for the subsequent assays.

The quantitative assay of protein carbonyls in RBCs ghosts

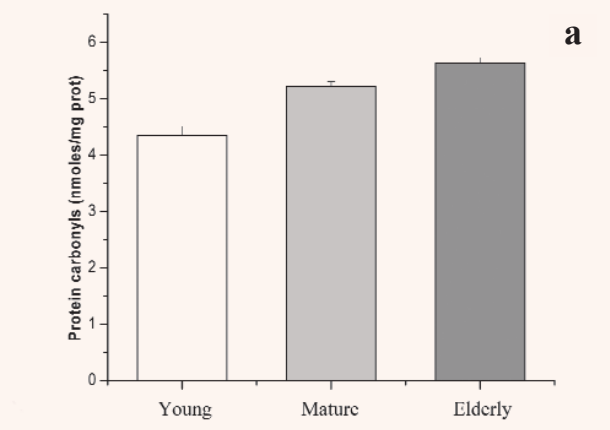
RBCs layer was lysed in hypotonic 5 mM phosphate buffer pH 8, containing 1 mM EDTA and 0.03 mM PMSF (phenylmethylsulphonyl fluoride), followed by 20 min centrifugation at 16,000 g, and washing of the sediment consisting of RBCs ghosts, till free of hemoglobin. In the latter, the protein carbonyls were assayed by the method reported by Levine *et al.* [15].

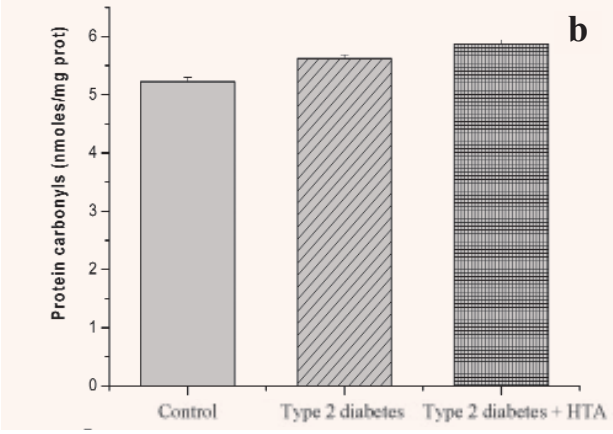
Immunoblotting detection of carbonylated proteins in RBCs ghosts

Ghosts solubilized in lysis buffer (10 mM Tris buffer pH 7.4 containing 5 mM EDTA, 25 μM PMSF, 1 μM benzamidine and 1% Triton X-100) were subjected to 10% SDS-PAGE, and electroblotted onto PVDF (polyvinylidene fluoride) membranes using a semi-dry procedure [16]. Immunodetection of protein carbonyl groups was performed as described in [17]. Carbonylated proteins were visualized using the enhanced chemiluminescence reagent kit (Pierce Inc., Rockford, IL, USA). In parallel, proteins of RBCs ghosts were separated by SDS-PAGE (as above) and stained with Coomassie brilliant blue.

Immunofluorescence localization of membrane 4-HNE Michael adducts

To find out the localization of 4-HNE (4-hydroxy-2-nonenal) - groups in RBCs plasmalemma, both ghosts and inside-out vesicles (IOVs) were used. IOVs were obtained from spectrin-depleted ghosts as described in [18]. Both preparations were incubated in 10 mM PBS (phosphate buffered saline) supplemented with 0.1 % BSA (bovine serum albumin), pH 7.4 and then exposed to a polyclonal rabbit 4-HNE-antiserum (Calbiochem, La Jolla, CA, USA), for 1h at 37°C. After washing, samples were reacted with goat anti-rabbit IgG labeled by TRITC (tetramethylrhodamine isothiocyanate) (Sigma, St. Louis, MO, USA), for 1h at 37°C. As controls, preparations reacted with the secondary antibody only, were used. The samples were examined for the presence of 4-HNE at λ_{exc}: 544 nm and λ_{em}: 572 nm with a NIKON fluorescence microscope.





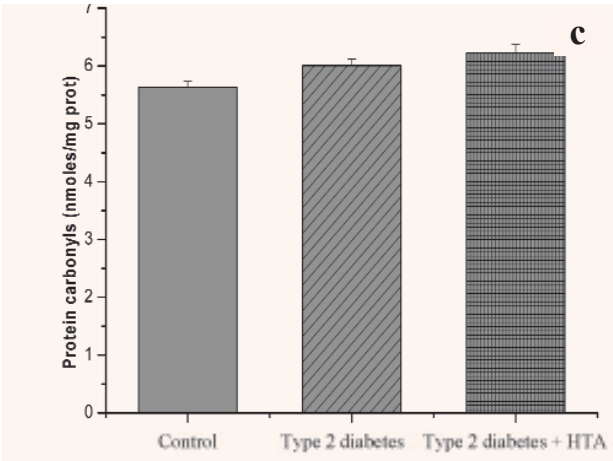


Fig. 1 The effect of biological ageing on the concentration of protein carbonyls in the RBCs membrane. a. the effect of ageing in physiological conditions (group C); b. protein carbonyls in RBCs of mature persons (~ 40 years old) in the groups C, OD (Type 2 diabetes) and ODH (Type 2 diabetes + HTA); c protein carbonyls in RBCs of elderly persons (~ 72 years old) in the same groups as at b.

Assay of RBCs glutathione

To measure the concentrations of reduced glutathione (GSH), total glutathione (tGSH) and oxidized glutathione (GSSG) the method reported by Anderson [19] was used. The antioxidant status of RBCs was evaluated by the GSH/GSSG ratio.

Assay of the activity of RBCs glutathione peroxidase

The GPx activity was assayed in RBCs hemolysates using the method described by Paglia *et al.* [20], and modified by Paget *et al.* [21].

Statistical analysis

For statistical analysis one way Anova was used. Results are given as mean ± standard error of the mean.

Results

Concentration of protein carbonyls in the RBCs membrane

In group C concentration of RBCs plasmalemma carbonyls was 4.355±0.154 nmoles/mg protein in young age, 5.226±0.075 nmoles/mg protein in the mature, and 5.627±0.103 nmoles/mg protein in the old-age (Fig. 1a). Thus, normal ageing is associated with an increase in RBCs membrane protein carbonyls of ~ 20% at the mature age ($p=0.001$) and ~ 29% at the old age ($p<0.001$). In OD group the concentrations of protein carbonyls in the RBCs membrane were 5.623±0.064 and 6.002±0.114 nmoles/mg protein in the mature and elderly patients, respectively (Fig. 1b, c). Thus, in OD group ageing was associated with higher levels of RBCs membrane carbonyls than those of age-matched controls. In the ODH group the concentrations of protein carbonyls in the RBCs membrane

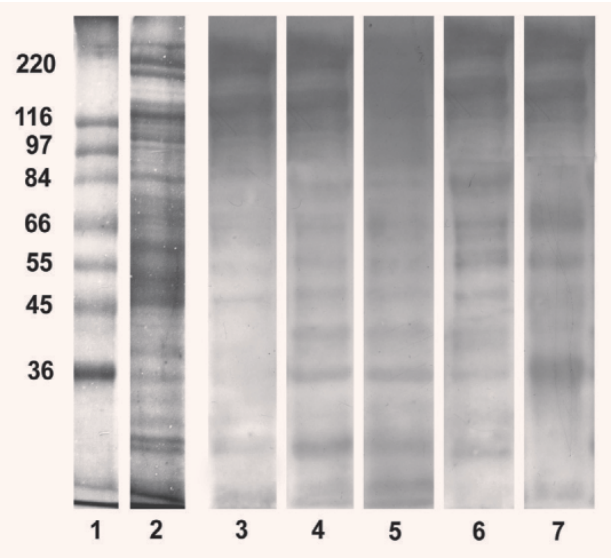


Fig. 2 SDS-PAGE (lane 2) and immunoblotting evaluation of carbonylated proteins in RBCs membrane: group C: young (lane 3), matures (lane 4) and elderly (lane 5); group OD: elderly (lane 6); group ODH: elderly (lane 7); lane 1 (molecular weight markers) and lane 2 are typical results of electrophoretic gels stained by Coomassie-brilliant blue of RBC proteins.

were 5.829±0.071 and 6.225±0.14 nmoles/mg protein in the mature and elderly patients, respectively (Fig. 1b, c). In comparison with age-matched controls, the ODH group contained the highest levels of RBCs membrane carbonyls, that were ~ 11% higher in the matures ($p<0.001$) and ~ 10% enhanced at the old aged ($p=0.007$). Taken together, the above results indicate that in the elderly persons the RBCs membrane proteins are the most damaged by carbonylation, and this occurs in the range ODH group > OD group > C group.

Immunodetection of carbonylated proteins in the RBCs membrane

The results of immunoblotting experiments showed that carbonyl groups were present in RBCs membrane at the high molecular weight bands in all samples from groups C, OD and ODH (Fig. 2). However, differences in carbonylation extent occured, and were influenced by both ageing or pathological condition. Thus, in group C the pattern of RBCs carbonylated proteins showed a faint reaction for the subjects at young age (Fig. 2, lane 3), and a stronger reaction at mature age associated with an increase in the number of carbonylated proteins (Fig. 2, lane 4), a pattern intensified by advanced ageing (Fig. 2, lane 5). In the group OD the largest number of carbonylated proteins were present in the RBCs of the elderly patients (Fig. 2, lane 6), while in the group ODH a change in the electrophoretic pattern of carbonylated proteins was found at the elderly patients, and this was associated with an increased bands intensity (indicative for higher oxidative insult) (Fig. 2, lane 7). These results show that ageing induced not only an increase in the number of RBCs carbonylated proteins but also an alteration of their expression.

Localization of 4-HNE Michael adducts in the RBCs plasmalemma

RBCs ghosts of young subjects in group C did not show any reaction for 4-HNE Michael adducts (Fig. 3a), while ghosts of both mature and elderly patients showed a very faint fluorescence reaction indicative for the occurrence of minute amounts of 4-HNE adducts (data not shown). In the OD group, RBCs ghosts of both mature and elderly patients expressed a very strong reaction for 4-HNE (Fig. 3c). A reaction of apparently similar in intensity was present in RBCs ghosts from mature and elderly patients in the ODH group (Fig. 3e). When the inside-out vesicles obtained from RBCs ghosts in groups C, OD and ODH were examined for the presence of 4-HNE, the reaction was absent in all the preparations (Fig. 3g). Therefore, one can safely conclude that 4-HNE adducts observed in groups OD and ODH (Fig. 3c, e) were situated at the outer leaflet of RBCs plasmalemma. Control preparations reacted with the secondary antibody only did not show any fluorescence for 4-HNE Michael adducts (not shown).

Concentration of RBCs glutathione, a non-enzymatic antioxidant defense

The concentration of GSH, GSSG and the values for GSH/GSSG ratio in the RBCs of groups C, OD, and ODH are shown in Table I. Compared to the levels measured in RBCs of young subjects in group C, the RBCs of healthy matures contained

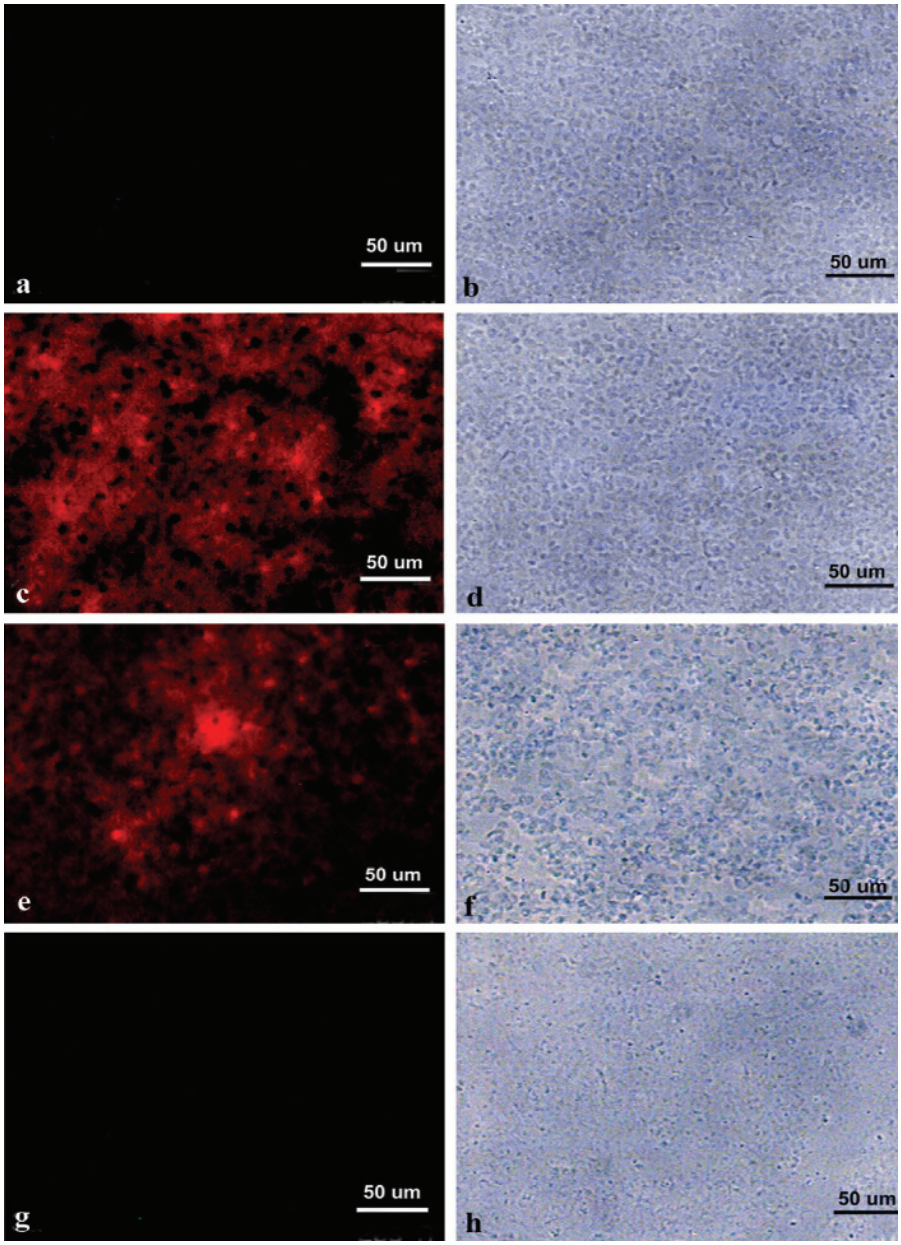


Fig. 3 Immunofluorescence localization of 4-HNE Michael adducts in the RBCs membrane (left pannels) and the same fields revealed by phase contrast microscopy (right pannels). a,b RBCs ghosts of a 27 years old subject in group C; c, d ghosts from a 42 y. obese Type 2 diabetic patient in group OD; e,f ghosts from a 50 y. hypertensive obese Type 2 diabetic patient in group ODH; g, h inside-out vesicles (IOV) prepared from the RBCs ghosts of an obese Type 2 diabetic patient. Magnification: x 20

~10% less GSH (not significant - n.s.) and showed a reduction of ~16 % for GSH/GSSG ratio (p=0.006) while the old persons exhibited ~13% decrease in GSH concentration (n.s.) and ~24 % reduced GSH/GSSG ratio (p=0.003). Thus, in healthy controls ageing diminished the non-enzymatic antioxidant defense exerted by RBCs glutathione. In the OD group (*vs.* age-matched controls) the matures had ~10.5% less GSH (n.s.) and ~ 20% reduced GSH/GSSG ratio (p=0.002), while RBCs of the elderly patients showed ~14% diminished GSH (n.s.) and ~29% reduced GSH/GSSG ratio (p<0.001). Thus, obesity and diabetes additionally lowered the ageing-associated decrease of endogenous RBCs glutathione. In ODH group, RBCs of matures contained ~26% less GSH (p=0.035) and ~45% reduced GSH/GSSG ratio (p<0.05) (*vs.* age-matched controls) while RBCs from the elderly patients showed ~33% less GSH (p=0.045) and ~47.5% reduced GSH/GSSG ratio (p<0.05) (*vs.* age-matched controls). These results indicate that hypertension associated with obesity in diabetic patients further diminished the ageing-associated decrease of antioxidant defense in RBCs. Thus, in comparison with the OD group, in ODH group ~18% decrease in GSH concentration

Table 1 Concentration of glutathione in the study groups

Concentration	C group			OD group		ODH group	
	Young	Mature	Elderly	Mature	Elderly	Mature	Elderly
GSH (nmol/ mg of Hb)	3.69 ± 0.08	3.33 ± 0.383	3.23 ± 0.55	2.98 ± 0.32	2.78 ± 0.48	2.45 ± 0.55	2.16 ± 0.38
GSSG (nmol/ mg of Hb)	0.28 ± 0.11	0.31 ± 0.04	0.325 ± 0.07	0.34 ± 0.02	0.40 ± 0.03	0.40 ± 0.03	0.41 ± 0.05
GSH/GSSG	13.16 ± 0.44	11.06 ± 0.63	10.05 ± 0.76	8.85 ± 0.70	7.12 ± 0.61	6.11 ± 1.58	5.27 ± 0.91

and ~31% ($p<0.05$) reduced GSH/GSSG ratio were recorded in the mature patients while the elderly group showed ~22% decreased GSH, and ~26% reduced GSH/GSSG ratio ($p<0.05$).

Activity of the RBCs glutahione peroxidase, an enzymatic antioxidant defense

In group C, the activity of RBCs GPx was 20.607±0.866 mU/mg Hb in the young, 15.862±0.533 mU/mg Hb in the matures, and 12.892±0.522 mU/mg Hb in the elderly persons (Fig. 4a). Thus, normal ageing led to a ~23 % decrease in GPx activity in RBCs of mature persons ($p=0.001$) and to a ~ 37% decrease in the elderly ($p<0.001$). In the OD group, the activity of GPx was 14.233±0.784 and 9.557±0.462 mU/mg Hb in RBCs of mature and elderly patients, respectively (Fig. 4b, c). In this group GPx activity was decreased (*vs.* age-matched controls) by ~10.3% in matures (n.s.) and by ~26 % in the RBCs of old-aged patients ($p=0.005$). In the ODH group, the activity of GPx was 11.479±0.388 and 9.121±0.322 mU/mg Hb in the RBCs of mature and elderly patients, respectively (Fig. 4b, c). Advancing in biological age in ODH group led to the lowest levels of GPx activity, that was lower with ~28 % in matures ($p<0.001$) and with ~29 % in the elderly group ($p=0.001$) in comparison with age-matched controls (Fig. 4b, c). Compared to the OD group, the GPx activity of RBCs in the ODH group was reduced by ~19.35 % and ~4.57 % for the matures and elderly, respectively ($p<0.05$). These results showed that hypertension further accentuates the obesity associated decline in GPx activity of RBCs in diabetic patients.

Discussion

Although there is a general interest in understanding the mechanisms of oxidant/antioxidant imbalances in cardiovascular pathology, little attention was paid to the ageing-induced perturbations of this balance in the RBCs of obese Type 2 diabetics and of hypertensive-obese diabetics. In this study we provide evidence that at the old age the antioxidant potential of RBCs can not overcome the deleterious effects of the high systemic oxidative stress, especially when hypertension complicates obesity in Type 2 diabetes.

A new finding of this study is that there is an age-dependent increase in the carbonylation of RBCs proteins; the latter may further initiate the formation of advanced glycated endproducts (AGEs). In diabetes, AGE-proteins of the RBCs membrane interact with specific receptors exposed by vascular endothelium [22] contributing to the augmented adhesion of RBCs [23].

Since RBCs contain more than 95% of the blood glutathione, it is obvious that the lower levels found in the RBCs of diabetic obese and of diabetic obese hypertensive patients contribute to the decline of the systemic antioxidant defense. In line with this conclusion is the low plasma GSH/GSSG ratio reported in hypertensive sub-

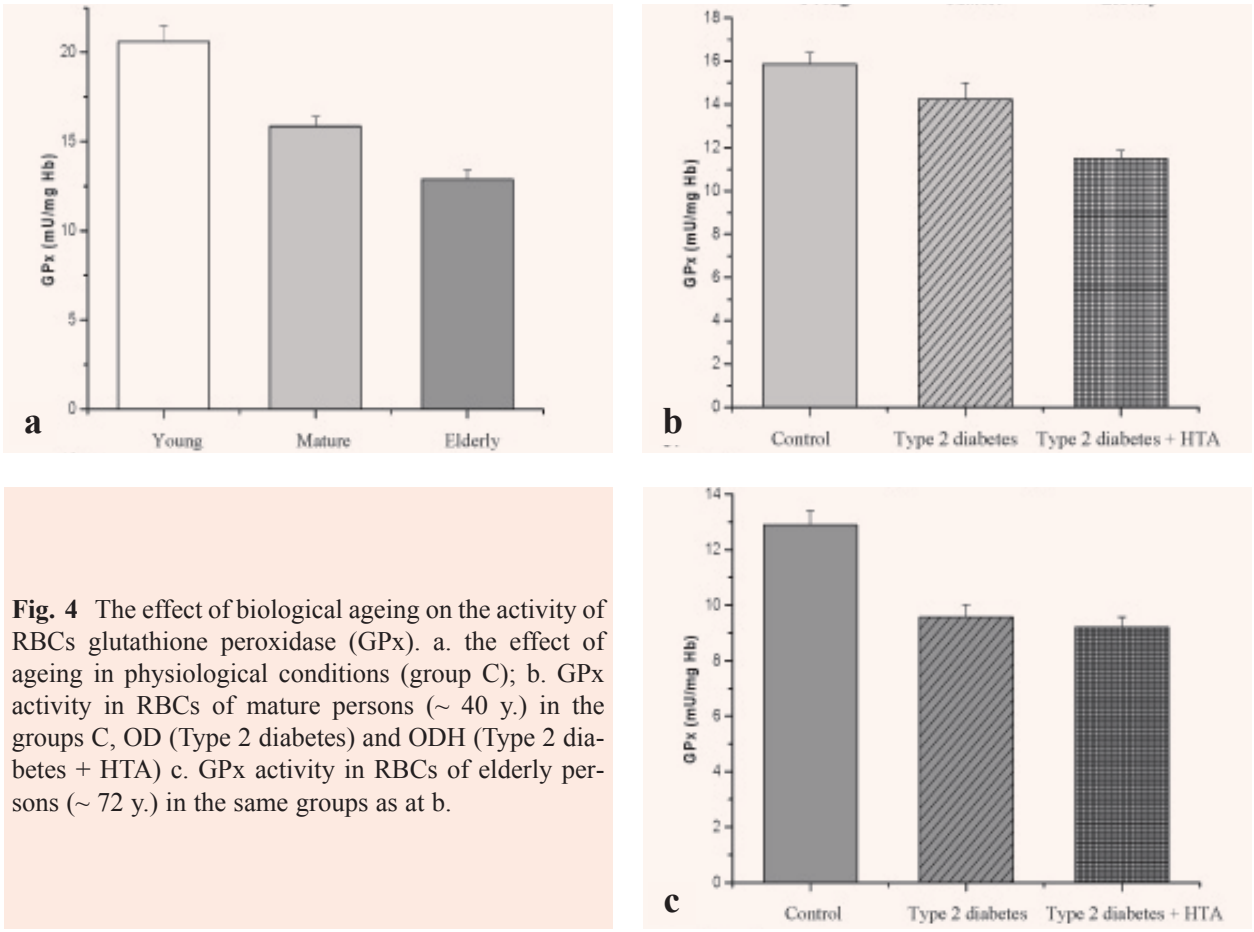


Fig. 4 The effect of biological ageing on the activity of RBCs glutathione peroxidase (GPx). a. the effect of ageing in physiological conditions (group C); b. GPx activity in RBCs of mature persons (~ 40 y.) in the groups C, OD (Type 2 diabetes) and ODH (Type 2 diabetes + HTA) c. GPx activity in RBCs of elderly persons (~ 72 y.) in the same groups as at b.

jects compared to the normotensive controls [24]. *In vitro*, rat pericytes exposed to diabetic conditions, such as high glucose, AGE-lysine, and angiotensin II showed a decrease in both GPx activity and GSH levels [25].

We demonstrate here for the first time that RBCs of obese Type 2 diabetics and of hypertensive-obese Type 2 diabetics contain 4-HNE Michael adducts at the outer leaflet of membrane; 4-HNE is a toxic and very reactive aldehyde obtained from lipid peroxidation [26], that can be detoxified by conjugation with GSH [27]. Since in diabetes the concentration of GSH is diminished [5], the detoxification of 4-HNE at the surface of RBCs may be impeded. In addition, accumulation of 4-HNE at the RBCs surface is evidence for an overload of circulating reactive oxygen species that oxidatively modify the RBCs membrane lipids.

The most impaired oxidant/antioxidant balance occurred in the RBCs of elderly hypertensive-obese diabetic patients. Thus, both ageing and high blood pressure accentuate the compromised functions of diabetic RBCs, such as a diminished life span [28], modified filtration characteristics (due to a higher rigidity of the membrane) [29], reduced deformability [30], and increased adherence to the endothelial layer [23], facilitating thrombotic events.

Taken together, the results of this study pointed out that at the old age RBCs membrane proteins were most affected by oxidative processes (*e.g.* protein carbonylation and formation of 4-HNE Michael adducts of lipids) while the endogenous glutathione defense (non-enzymatic and enzymatic) was severely compromised. These changes occurred in the range: ODH group > OD group > C group. Recently, in a double transgenic mice with Type 1 diabetes mellitus we reported that RBCs displayed an augmented sensitivity to the osmotic shock (compared to non-diabetic controls) assessing systemic oxidative stress [31]. The latter can be counterbalanced by the powerful antioxidant poten-

tial of RBCs that act as mobile detoxifying units [32]. Novel strategies to prevent and treat disorders associated with the oxidative stress such as diabetes, obesity, and hypertension are specifically targeted to diminish the deleterious effects of ROS [33, 34].

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