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Erythrocytes as targets for gamma-glutamyltranspeptidase initiated pro-oxidant reaction

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Abstract: Gamma-glutamyltranspeptidase (GGT) is a well known cell plasma membrane and serum circulating enzyme. In clinical chemistry, GGT is used as a marker of alcohol consumption and drug uptake. Serum GGT activity varies in hepatobiliary diseases and cancer. This enzyme is involved in glutathione (GSH) metabolism, which is generally associated with antioxidant properties. However, in recent years, findings from our group and from others showed that GGT-catalysed extracellular metabolism of GSH leads, in the presence of iron, to the generation of reactive oxygen species (ROS). It was demonstrated that those highly reactive species oxidise lipids, cell surface protein thiols or activate transcriptional factors such as Nuclear Factor κB (NFκB). The objective of the present work is to determine whether the red blood cells are targets for plasma GGT-initiated pro-oxidant reaction. The results obtained demonstrate that the GGT/GSH/iron system oxidises isolated erythrocyte membranes. A significant release of haemoglobin and a decrease of erythrocyte deformability are also observed. In addition, *in vivo* studies showed a relationship between plasma GGT activity and erythrocyte deformability in 20 studied subjects. In conclusion, GGT-mediated ROS production is able to oxidise erythrocytes and thus disturbs their functions.

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Key words: gamma-glutamyltranspeptidase; red blood cells; lipid peroxidation; haemolysis; erythrocyte deformability

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Increasing evidence suggests that oxidative damage to cell components may have an important physiopathological role in several human diseases (1, 2). In most of these diseases, an enhancement of the oxidative stress is supposed to result from an increase of the free radical load and/or a decrease in the efficiency of the antioxidant systems. Red blood cells (RBCs) are particularly sensitive to oxidative damage as a result of the high poly-unsaturated fatty acid content of their membranes and the high cellular concentrations in oxygen and in haemoglobin, a potentially powerful promoter of oxidative processes (3, 4). In addition, it has been suggested that iron is released from iron stores under conditions in which oxidative stress is involved both *in vitro* (5, 6) and in isolated perfused organs (7).

It has been shown that the susceptibility to lipid peroxidation is increased in erythrocyte membranes of patients in the case of diabetes mellitus (8, 9), haemodialysis (10), chronic alcoholism (11) or during cardiovascular diseases (12).

Except in RBCs, which do not express it, gamma-glutamyltranspeptidase (GGT) is an ubiquitous cell membrane and plasma circulating enzyme which is generally considered as a useful laboratory marker for chronic alcohol consumption or drug induction (13). Therefore, this enzyme has been regarded as a marker for the diagnosis of hepato-biliary diseases or hepatic cancer (14, 15). A high GGT activity is also associated with obesity and elevated blood pressure (16).

GGT is a key enzyme in glutathione (GSH) metabolism. It initiates the breakdown of extra-cellular GSH by removing the γ-glutamyl moiety, therefore providing amino-acid precursors and especially cysteine for the intracellular *de novo* synthesis of GSH. As GSH is commonly known as

the major antioxidant in the organism, GGT was long considered as a protective enzyme contributing to the maintenance of cellular redox status via GSH synthesis (17, 18). However, recent studies have shown that in the presence of chelated transition metals (iron, copper or transferrin), pro-oxidant species can originate during the GGT-mediated metabolism of GSH (19, 20). This process was explained by the autoxidation of the GGT-generated first metabolite of GSH, cysteinylglycine (CysGly), which is able to form thiyl and oxygen radicals by reacting with Fe^{3+} and O_2. The major reactive oxygen species (ROS) produced are hydrogen peroxide, superoxide anion and hydroxyl radical. As has been demonstrated in recent years, GGT-dependent ROS production induces lipid peroxidation (LPO) of several substrates, e.g. polyunsaturated fatty acids (21), human low-density lipoproteins (LDL) (22), liver microsomes (23) or HepG2 cells (23). The involvement of GGT in oxidative mutagenesis (24) as well as in the redox modulation of cell surface protein thiols (25) was also documented. Indeed, recent studies in our laboratory show the role of GSH metabolism of GGT in the activation of the transcriptional factor NFκB (26).

Against this background, we hypothesised that the ability of GGT to produce ROS via GSH metabolism might also promote erythrocyte oxidation and thus contribute to alter its physiological functions.

Material and methods

Chemicals

All reagents were purchased from Sigma (St Louis, MO, USA). Ultra-pure quality water at 18 MΩ cm resistivity was used throughout.

Bovine lactoferrin as apoprotein was kindly provided by Morinaga Milk Industry Co. Ltd. (Kanagawa, Japan).

Samples

Human EDTA blood was withdrawn by venipuncture from unrelated supposedly healthy people coming to the Centre for Preventive Medicine in Nancy for a periodic health examination. These people belonged to the Stanislas cohort described by Siest *et al.* (27). All subjects gave written free and informed consent. The protocol was approved by the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lorraine.

For the study of the GGT/TBARS relationship the population consisted of 134 subjects (69 men aged 45.8 ± 2.19 yr and 65 women aged 39.4 ± 2.19 yr), with body mass index 25.42 ± 0.416 kg m^{-2}. Subjects were selected as a function of their GGT activity, which ranged from 6.3 to 442 U L^{-1}.

For GGT-elongation index study the population consisted of 10 subjects aged 43 ± 2.6 yr with plasma GGT activity higher than 50 U L^{-1} (136 ± 45.4 U L^{-1}) and 10 paired control subjects aged 45 ± 2.3 yr with a normal plasma GGT activity level (26.3 ± 8.7 U L^{-1}).

For *in vitro* studies (haemolysis and erythrocyte deformability) a single sample with a normal GGT level was tested three times.

Subjects with high fasting glucose ($\geqslant 10$ mmol L^{-1}), high total cholesterol ($\geqslant 6$ mmol L^{-1}), high triglycerides ($\geqslant 4$ mmol L^{-1}) or treated with drugs known to induce GGT activity were excluded for these different studies.

ROS production

Quantification of reactive oxygen species (ROS) was performed using the dihydrorhodamine 123 (DHR-123) probe, which is oxidised by various ROS into the fluorescent rhodamine 123 (Rh-123) (28). Fluorescence was measured using a microplate fluorimeter (Cytofluor 2350, Millipore, Bedford, MA, USA) at excitation and emission wavelengths of 485 and 530 nm, respectively.

Preparation of red blood cells and erythrocyte membrane

Fresh human red blood cells were isolated by centrifugation at 1000 *g* for 10 min at 4°C and washed three times with NaCl 9 g L^{-1}. Erythrocytes were then resuspended in 152 mM NaCl, 10 mM sodium phosphate pH 7.4.

Haemoglobin-free ghosts were prepared according to the method of Doge *et al.* with some modifications of Chan *et al.* (29). Washed red cells were haemolysed in 40 volumes of 5 mM sodium phosphate buffer (pH 8.0) and centrifuged at 25 000 *g* for 20 min at 4°C. The pellet was washed repeatedly until the ghosts no longer contained haemoglobin.

GGT-dependent lipid peroxidation

Erythrocyte membranes (0.5 mg mL^{-1}) were incubated in 50 mM phosphate buffer pH 7.4 at 37°C in the presence of purified GGT (Sigma) (0–200 U L^{-1}), 2 mM GSH, 20 mM GlyGly, and either 1.5–0.15 mM ADP-Fe^{3+} or 80 μM Fe^{3+}-saturated lactoferrin as source of iron. The reaction was started by adding GSH. Lipid peroxidation was monitored for 90 min by thiobarbituric acid-reactive substance (TBARS) assay

according to the method of Stocks and Dormandy (30). 1,1′,3,3′-Tetraethoxypropane (TEP) was used as a standard to construct a calibration curve of equivalent malondialdehyde (MDA).

In some of the experiments, the antioxidant Trolox C was added at a concentration of 2 mM in DMSO (0.1% final concentration).

Haemolysis

A 10% suspension of RBCs in phosphate buffer was incubated at 37°C with the following oxidant system: 200 U L^{-1} GGT, 2 mM GSH, 20 mM GlyGly, 1.5–0.15 mM ADP-Fe^{3+}. At specific intervals, the degree of haemolysis (%) was determined from the absorbance of the supernatant at 540 nm due to the release of haemoglobin. The value of 100% haemolysis was determined from the supernatant of 1 vol of erythrocyte cells with 9 vol of ice-cold water.

Deformability measurements

Erythrocyte deformability was measured with a laser scattering method (31) using LORCA (Laser-Assisted Optical Rotational Cell Analyser). Treated erythrocytes in 5.5% polyvinylpyrrolidone (PVP) in PBS were submitted to several shear stresses: 0.3, 0.53, 0.95, 1.69, 3, 5.33, 9.45, 16.85, 29.92 and 53.65 Pa. In this study we considered the elongation index (EI) at 30 Pa and at 37°C.

Other determinations

Plasma GGT activity measurement was performed on an Olympus AU 640 apparatus according to the method of Szasz (32), using γ-glutamyl-3-carboxy-4-nitroanilide as donor substrate.

The protein content was determined by the method of Lowry *et al.* (33).

Statistical methods

For the *in vivo* studies of the relation between plasma GGT and TBARS and between GGT and erythrocyte deformability, ANOVA and the unpaired Mann–Whitney test were used, respectively.

For the *in vitro* studies the paired Student's *t*-test was employed.

Results

ROS generation by GGT/GSH/iron

The GGT-dependent breakdown of GSH into CysGly (first product) and Cys has been previously demonstrated in our laboratory by HPLC measurements of the resulting thiols (20).

As enzymatic activity can be affected by metal ions, we further tested the influence of iron on GGT activity. The concentration of iron (0.15 mM) used in the present study does not affect GGT activity (data not shown).

GGT-dependent ROS production was assayed using a membrane-permeable leucodye DHR-123, which is unspecifically oxidised by various ROS into the fluorescent Rh-123 (28). As shown in Fig. 1, after 60 min incubation the mixture containing 200 U L^{-1} GGT, 0.1–2 mM GSH and 0.15 mM ADP-chelated iron exhibits a significant increase in the amount of probe oxidation (11.2-fold vs. control for 2 mM GSH). In the absence of GGT, a very low level of Rh-123 was measured (data not shown). This indicates that the GGT metabolites of GSH, but not GSH itself, are responsible for ROS production, thereby confirming previous results obtained in our laboratory (20).

The amount of ROS produced is dependent on GSH concentration. Even at the lowest level (0.1 mM), a significant oxidation of DHR-123 already occurs (53.4% of relative increase). It becomes more important for higher GSH concentrations (up to 91% of relative increase).

GGT-dependent lipid peroxidation of ghosts

In order to test whether GGT-dependent ROS production is able to induce lipid peroxidation in erythrocyte membranes, ghosts were exposed to 2 mM GSH in a mixture containing different levels of GGT activity and a constant amount of chelated iron (0.15 mM). The kinetics of LPO as a function of GGT activity was monitored for 90 min using the

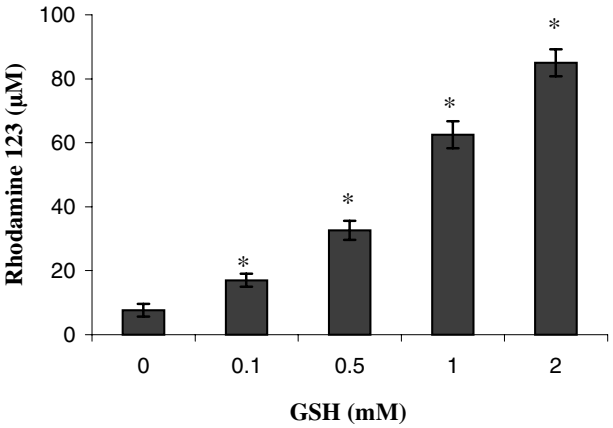

Fig. 1. ROS production by the GGT/GSH/iron system measured as DHR-123 oxidation. The reaction mixture contains: 200 U L^{-1} GGT, 0, 0.1, 0.5 and 2 mM GSH, 20 mM glycylglycine (GlyGly), 1.5–0.15 mM ADP-Fe^{3+} and 250 μM DHR-123 in 50 mM phosphate buffer pH 7.4 incubated for 60 min at 37°C. The data are means ± SD of three independent experiments. * Statistically different from the control, $P < 0.05$.

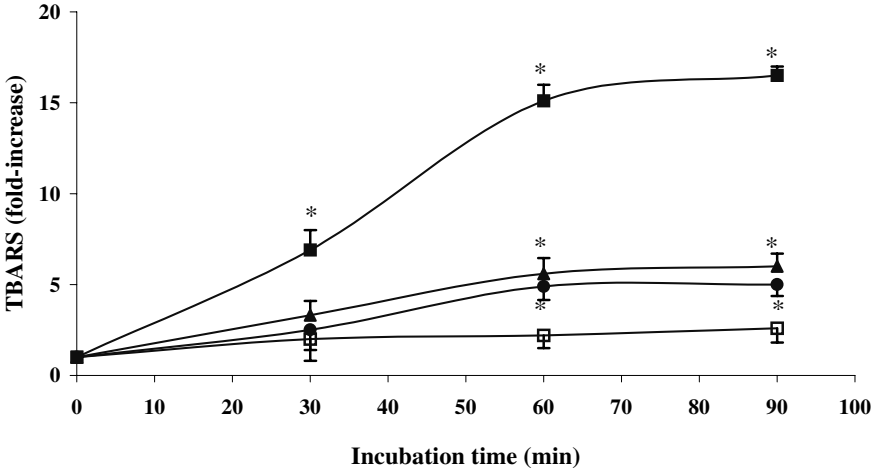

Fig. 2. Kinetics of GGT-dependent lipid peroxidation of erythrocyte membranes. The reaction mixture contains: ghosts (0.5 mg protein), 2 mM GSH, 20 mM glycylglycine (GlyGly), 1.5–0.15 mM ADP-Fe^{3+} and various GGT activities (■): 200 U/L (▲): 100 U/L (●): 50 U/L and (□): 0 U/L in 50 mM phosphate buffer pH 7.4 incubated for 30, 60 and 90 min at 37°C. LPO was monitored using TBARS assay as described in Material and Methods. The results are expressed as percentage of control (non-oxidised ghosts).The data are means ± SD of three independent experiments. * Statistically different from the control, $P < 0.05$.

TBARS assay. As shown in Fig. 2, ghosts incubated only with GSH and ADP-chelated ferric iron exhibit a very slight increase of TBARS level. In contrast, the addition of GGT to the incubation mixture greatly stimulates the process. The extent of oxidation is indeed dependent on GGT activity. After 60 min incubation, lipid peroxidation of the erythrocyte membrane in the presence of 200 U L^{-1} GGT is seven-fold higher as compared to that observed in the absence of GGT.

We further measured the GGT-dependent LPO as a function of GSH concentration. The intensity of LPO stimulation shows a dependency of dose on the GSH concentration. Even at the lowest studied GSH concentration (0.01 mM), TBARS level of GGT-oxidised ghosts is higher than the control (Table 1).

In the following experiments, we substituted ADP-Fe^{3+} with Fe^{3+}-saturated lactoferrin as a physiological source of iron. The results presented on Table 2 show that the GGT/GSH/lactoferrin mixture also oxidises erythrocyte membranes (*ca.* nine-fold compared to control). The presence of 2 mM Trolox C partially inhibits LPO caused by GGT/GSH/lactoferrin (or ADP-Fe^{3+}). The inhibition ratio is 47% or 67% when either lactoferrin or ADP-Fe^{3+} is used as a source of iron, respectively.

The dependency of GGT-induced LPO of erythrocyte membrane on iron concentration was also tested. The results obtained demonstrate that 0.015 mM Fe^{3+} is sufficient to enhance the GGT/GSH-dependent ghost oxidation as measured by TBARS (eight-fold vs. control). Increasing iron concentration in the mixture (up to 0.15 mM) leads to an enhanced LPO (13-fold vs. control).

Table 1. GSH-dependence of the GGT-mediated lipid peroxidation of erythrocyte membranes

GSH (mM)	TBARS (nmol mg^{-1} protein)	*n*-Fold increase
0	1.5 ± 0.90	1.0
0.01	2.3 ± 0.85	1.5
0.05	3.2 ± 0.90	2.1
0.1	4.0 ± 0.60	2.7
0.5	8.5 ± 0.80	5.7
1	13.0 ± 0.19	8.7
2	23.0 ± 0.64	15.3

The reaction mixture contains: ghosts (0.5 mg protein), 200 U L^{-1} GGT, 0, 0.01, 0.05, 0.5, 1 and 2 mM GSH, 20 mM glycylglycine (GlyGly),1.5–0.15 mM ADP-Fe^{3+} in 50 mM phosphate buffer pH 7.4 incubating for 60 min at 37°C. LPO was then measured using TBARS assay as described in Material and Methods. The data are means ± SEM of three independent experiments.

Table 2. Influence of iron source and of Trolox C on GGT-mediated lipid peroxidation of erythrocyte membranes

	TBARS nmol mg^{-1} protein
Control	1.5 ± 0.50
GGT + GSH + GlyGly + Lactoferrin	13.7 ± 0.70*
GGT + GSH + GlyGly + Lactoferrin + Trolox C	7.3 ± 0.83†
GGT + GSH + GlyGly + ADP-Fe^{+3}	21.6 ± 0.65*
GGT + GSH + GlyGly + ADP-Fe^{+3} + Trolox C	6.3 ± 0.54†

Incubations were carried out for 60 min at 37°C in 50 mM phosphate buffer pH 7.4 containing: ghosts (0.5 mg protein), 200 U L^{-1} GGT, 2 mM GSH, 20 mM glycylglycine (GlyGly),1.5–0.15 mM ADP-Fe^{3+} or 80 μM lactoferrin in the presence or the absence of 2 mM Trolox C. LPO was then measured using TBARS assay as described in Material and Methods. The data are means ± SD of three independent experiments. * Statistically different from the control; † statistically different from the oxidized sample, $P < 0.05$.

GGT/TBARS correlation

The population studied was divided into four groups as a function of their GGT activity. Group 1 : 0–10 U L^{-1}, group 2 : 11–50 U L^{-1}, group 3 : 51–100 U L^{-1} and group 4: > 100 U L^{-1} (Fig. 3). Lipid peroxidation as expressed by TBARS did not differ significantly in groups 1, 2 and 3. However, the TBARS level is significantly higher ($P < 0.001$) in group 4, corresponding to the highest GGT activity level. A strongly significant positive correlation between GGT activity and TBARS was obtained in plasma ($r = 0.7$).

GGT-dependent haemolysis

Incubation of human erythrocytes in the presence of oxidative agents such as the radical initiator AAPH [2,2′-azo-bis(2-amidinopropane) dihydrochloride] or H_2O_2 is known to initiate haemolysis. Therefore we tested the haemolysis effect of ROS produced by the GGT-dependent system.

When human RBCs were incubated in air at 37°C as a 10% suspension in buffered saline solution, they were stable and exhibited only a low level of haemolysis after 4 h incubation (5 ± 1.5% haemolysis, Fig. 4). A high concentration of H_2O_2 (10 mM) caused a rapid and strong increase of haemolysis (data not shown). When the GGT-generated ROS system was added to the RBC suspension, it induced haemolysis in a time-dependent manner. However, the kinetics of haemolysis is much slower in the case of the GGT-dependent system than after addition of H_2O_2. Under these experimental conditions, the

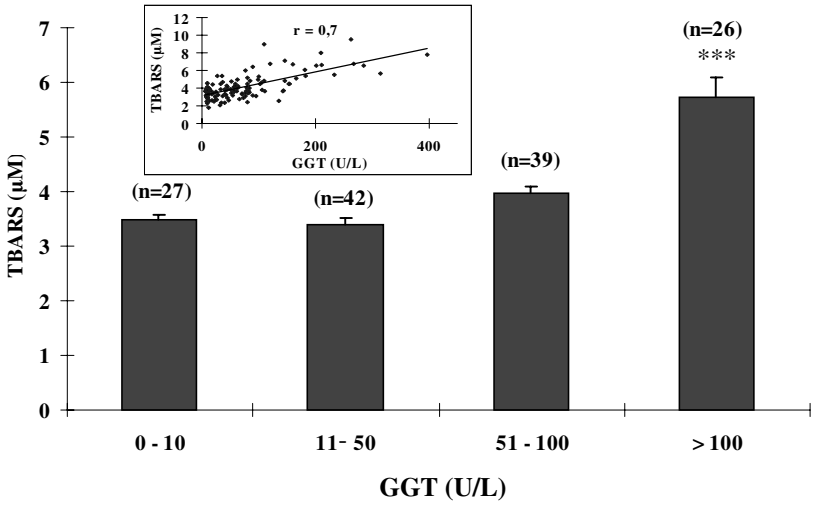

Fig. 3. Relation between GGT activity and lipid peroxidation in plasma. Values indicate mean of GGT activity in each group. *** Statistically different from groups 1, 2 and 3, $P < 0.001$, $n = 134$ subjects.

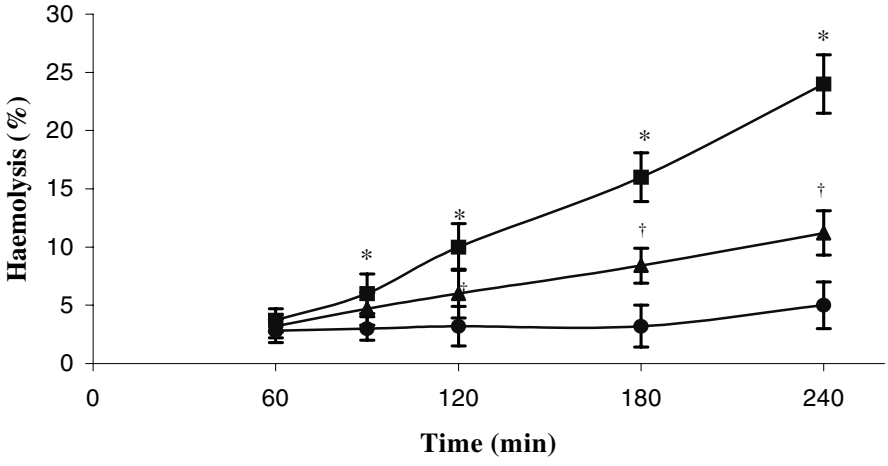

Fig. 4. GGT/GSH/Fe^{+3} system induces haemolysis of human RBCs. A 10% suspension of RBCs was incubated with 200 U L^{-1} GGT, 2 mM GSH, 20 mM GlyGly and 1.5–0.15 mM ADP-Fe^{3+} in the absence (■) or in the presence (▲) of 2 mM Trolox C at 37°C for 60, 120, 180 and 240 min The control sample contains only RBCs in saline buffer (●). The value of 100% haemolysis was determined from the supernatant of 1 vol of erythrocyte cells with 9 vol of ice-cold water. The data are means ± SEM of three independent experiments. * Statistically different from the control; † statistically different from the oxidized sample, $P < 0.05$.

onset of oxidative haemolysis corresponds to 120 min incubation. For the same incubation time, Trolox C partially inhibits the GGT-induced haemolysis (56% inhibition at 120 min).

Erythrocyte deformability

As a consequence of LPO, polymerisation of membrane components increases the membrane rigidity and is supposed to decrease the cell deformability (4, 34). In order to test this assessment, we measured the deformability of erythrocytes incubated with GGT/GSH/Fe^{3+}.

Erythrocyte deformability was assayed in control and oxidised RBCs (GGT/GSH/Fe^{3+} or H_2O_2) by laser diffractometry using LORCA. This is a new and sensitive instrument that is capable of detecting small changes in erythrocyte deformability (31).

Incubation of RBCs with a GGT oxidation mixture for 1 h at 37°C causes a significant decrease of the elongation index, reflecting a reduced erythrocyte deformability (Fig. 5). This effect is partially prevented by the addition of 2 mM Trolox C (50% inhibition).

As expected, 10 mM hydrogen peroxide causes a more severe decrease in the elongation index. Trolox C also protects RBCs against H_2O_2-induced loss of deformability.

We have further measured erythrocyte deformability of patients with variable plasma GGT activity. The results obtained (Fig. 6) indicate that in patients with high plasma GGT activity level (>50 U L^{-1}), erythrocyte deformability is significantly decreased compared to control patients ($GGT<50$ U L^{-1}). These data demonstrate an increase in membrane rigidity of erythrocytes in patients with high plasma GGT activity.

Discussion

GGT is a membrane-bound enzyme metabolising extracellular GSH. It is indeed the unique enzyme able to initiate the extracellular GSH breakdown by removing the γ-glutamyl moiety. A new pro-oxidant role for this enzyme has been described by Stark *et al.* (19). They showed that GGT can divert GSH metabolism into an oxidative pathway, generating reactive oxygen species. GGT-dependent oxidation has been demonstrated on several substrates. Previous studies (19) have shown that the pK_a of the thiol group is significantly lower in cysteinylglycine than in GSH (6.4 vs. 8.56, respectively). Therefore the ability of GGT to induce LPO in the presence of GSH might reflect the formation of cysteinylglycine, bearing a thiol moiety that dissociates more rapidly than GSH at near-neutral pH 7.4 and participates in the formation of oxygen radicals by reaction with transition metals (19, 20).

In this work, we tested if RBCs can be a target for this oxidation.

On the one hand, RBCs are endowed with efficient defence mechanisms such as GSH or α-tocopherol that are able to prevent oxidation of iron in the haem group and labile (sulfydryl) groups on the haemoglobin molecule (35). On the other hand, RBCs are vulnerable to oxidative stress by free radicals because they are loaded with iron and exposed to high oxygen pressure (36).

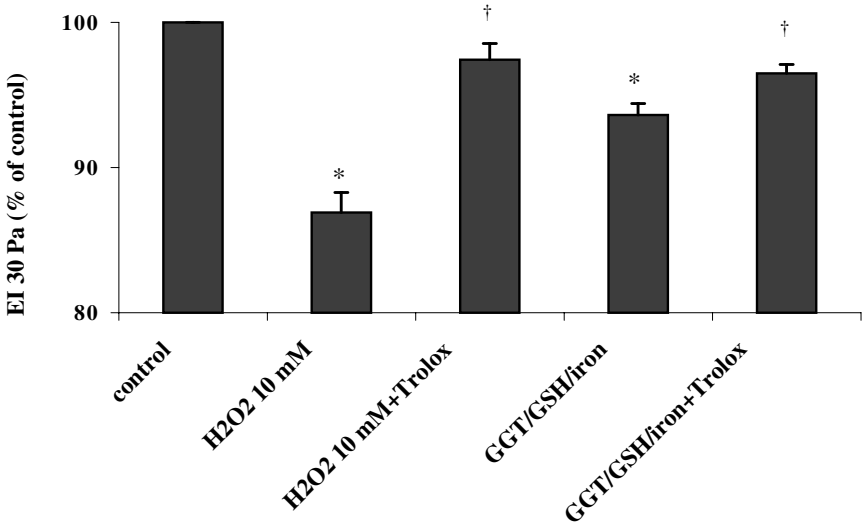

Fig. 5. Influence of oxidant system and Trolox C on erythrocyte deformability. The RBCs were incubated with 10 mM H_2O_2 or GGT/GSH/Fe^{3+} (200 U L^{-1}/2 mM/150 μM) at 37°C for 60 min. Erythrocyte oxidation was performed in the absence or in the presence of 2 mM Trolox C. A 10-μL aliquot of the reaction mixture was then used for LORCA measurements. The data are means ± SD of three independent experiments. * Statistically different from the control; † statistically different from the oxidized sample, $P<0.05$.

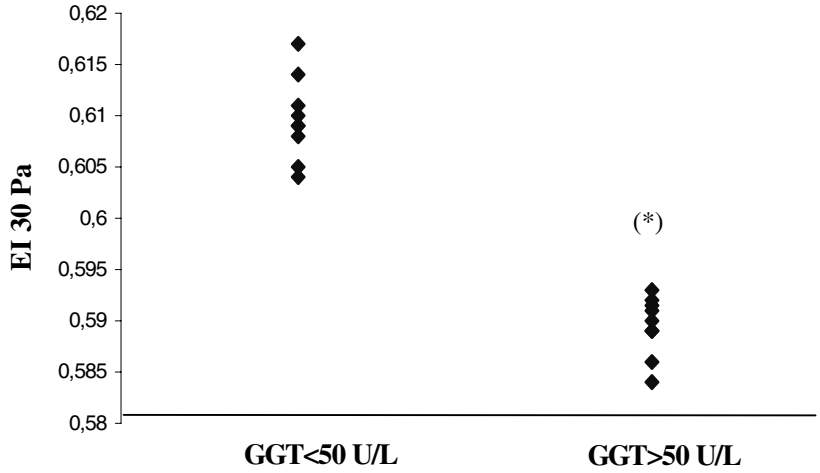

Fig. 6. Comparison of erythrocyte elongation index of patients with high or normal plasma GGT activity. Erythrocyte deformability was measured in the blood of 10 patients with plasma GGT activity higher than 50 U L^{-1} and 10 control patients with plasma GGT activity lower than 50 U L^{-1}. Means of EI values at a shear stress of 30 Pa are 0.589 ± 0.004 and 0.609 ± 0.004 for high GGT activity plasma and control, respectively. * Statistically different from the control.

There has been some controversy about the presence of GGT in human erythrocytes. However, the studies of Suzuki *et al.* (37) showed that the absence of GGT in RBCs and GGT activity measured in human erythrocytes by other authors was due to the existence of white blood cell contamination in red cell preparations.

Our results show that the incubation of erythrocyte membranes with a GGT/GSH/iron system leads to an increase in the TBARS level. GGT-induced LPO of ghosts depends on GGT activity level and on iron and GSH concentrations in the incubation medium.

Human RBCs do not express the membrane enzyme GGT (37); however, GGT activity can be provided by the blood (plasma, white cells) circulating enzyme. The reference value of GGT activity in plasma at 37°C ranges from 7 to 50 U L^{-1} for healthy adults (38). GGT activity can be greatly increased during several physiopathological situations, e.g. liver diseases, cancer, obesity, drug induction or alcohol consumption, and can reach values higher than 1500 U L^{-1} (15).

Iron plays a central role in generating harmful ROS through the Fenton reaction. In order to achieve this, it must be reduced to the redox-active form Fe^{2+}. The results of our work and of others (20, 23) show that the ability of GSH to reduce Fe^{3+} into Fe^{2+} is dependent on GGT-catalysed cysteinylglycine formation. In normal conditions, iron is transported and stored by specific proteins (ferritin, transferrin, lactoferrin and haem proteins), which prevent or minimise its reaction with reduced oxygen derivatives (39). However, release of iron from its protein storage can be facilitated in several conditions such as mildly acidic pH, exposure to reducing agents and binding to specific receptors (40, 41). Under conditions in which oxidative stress is involved, iron can also be released from its storage protein. Ferrali *et al.* (42) demonstrated that oxidising agents such as phenylhydrazine, divicine and isouramil induce iron release from haemoglobin in erythrocytes.

Previous studies in our laboratory (20) have shown that GGT stimulation of lipid peroxidation can be sustained by transferrin alone. In this work, we tested lactoferrin as the physiological source of iron during erythrocyte oxidation. Lactoferrin is an iron-binding glycoprotein of the transferrin family (43). It is involved in Fe^{+2} autoxidation and thereby in OH generation (44). Lactoferrin is present in most of the exocrine secretions, mainly in milk, but it is also a major component of the secondary granules of polymorphonuclear neutrophils (PMNs). During inflammation, lactoferrin is released by PMNs in the injured tissues, and its concentration in blood rises up to 20 mg L^{-1} (45). Like that obtained with transferrin, GGT-dependent lipid peroxidation can also be sustained by lactoferrin alone.

The GSH concentration used in this study is higher than that measured in human plasma. The GSH and GlyGly concentrations (2 and 20 mM, respectively) were chosen based on the described K_m values of GGT for its substrate, in order to optimise the conditions for GGT activity and thus facilitate the detection of its pro-oxidant effects (23).

However, GGT-dependent lipid oxidation of ghosts exhibits a linear dependence on GSH concentration (Table 1), down to GSH concentrations (50–10 μM) in the range of those detectable in rat or human plasma (46).

In humans, a significant amount of GSH is released into the blood from periportal hepatocytes. GSH can thus reach higher local concentrations. GGT, the unique enzyme able to cleave the γ-glutamyl moiety from GSH, produces CysGly, the active metabolite in GGT-dependent ROS production (20).

It was established that GSH can be released by erythrocytes in response to an oxidative stress, presumably to protect the essential thiol groups on the surface of membrane proteins (47). GSH is also released from human RBCs stored under standard blood banking conditions in a time-dependent manner (48). GSH can thus reach higher local concentrations available for GGT-mediated oxidation. In these cases, a GGT-dependent LPO can also contribute to and worsen oxidation in plasma.

High plasma GGT activity is known to be associated with obesity and elevated blood pressure. GGT activity also increases after myocardial infarction, and epidemiological studies have revealed a positive correlation between GGT activity level and overall mortality (49). The pro-oxidant role of GGT is suggested as a mechanism-based explanation of the observed relationship supporting the hypothesis that GGT contributes to vascular damage by oxidising LDL. Indeed, recent biochemical evidence shows that GGT activity directly participates (22) in the oxidative events related to the progression of atheromatous plaque (50, 51).

In addition a 6-yr follow-up of patients known to present angiographically documented coronary artery diseases demonstrated that the patients with plasma GGT activity >40 U L^{-1} were at higher risk of cardiac mortality (25.2%) than those with plasma GGT activity <40 U L^{-1} (13.9%) (52).

The significant positive correlation we obtained between GGT activity level and TBARS levels in the plasma of 134 healthy subjects follows the same logic.

Several authors proposed the addition of GSH or its amino acid precursors (*N*-acetyl-L-cysteine) to the conventional additive solution in order to protect red blood cells from oxidation during storage and thus improve the quality of post-transfusion. We recommend more attention to this approach because, paradoxically, GSH (like other antioxidant molecules) can lead to the unwanted generation of free radicals. As demonstrated in our studies, this phenomenon requires the enzymatic activity of GGT for its activation.

Lipid peroxidation of RBC is always associated with haemolysis. LPO of erythrocytes forms pores in cell membranes, which lead to haemoglobin release. We demonstrated that the GGT/GSH/Fe^{+3} system causes erythrocyte cytolysis. Other molecules than haemoglobin, such as GSH, can be released from erythrocytes and thus support the possibility of oxidation reaction by the GGT/GSH/Fe^{+3} system.

Erythrocytes deform in order to traverse narrow capillaries with diameters smaller than that of the red blood cells in order to supply tissues with oxygen and remove carbon dioxide. As a result of oxidation by GGT/GSH/Fe^{+3}, RBCs not only become permeable and leaky as we just stated, but also show an increase in their shear rigidity. Our results indeed show that exposing RBCs to GGT/GSH/Fe^{+3} reduces their deformability as measured by LORCA. This lack of deformability hampers the physiological function of erythrocytes. To investigate further the implication of GGT in this process, we measured the erythrocyte deformability in patients with variable GGT activity levels. The relationship between those two parameters indicates the possible role of GGT in the loss of RBC deformability *in vivo*. Patients with hypertension or diabetes mellitus exhibit a higher GGT activity than the healthy population. In these pathological populations, an increase in the susceptibility to oxidation of RBCs and a decrease of their deformability are also well documented (8, 9, 53). It is likely that GGT pro-oxidant reaction helps to increase the oxidative stress and to disturb the erythrocyte functions in the case of these diseases.

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