Original Article

Influence of Platelet γ-Glutamyltransferase on Oxidative Stress and Apoptosis in the Presence of Holo-Transferrin

(platelet / γ-glutamyltransferase / transferrin / apoptosis / oxidative stress)

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Abstract. Several studies have documented that formation of oxidant mediators may induce apoptosis in nucleated and anucleated cells by modulating intracellular signalling pathways. Reactive oxygen species (ROS) play a very important role in the platelet function. γ-Glutamyltransferase (GGT), a novel source of cellular production of oxidants in the presence of iron and reduced glutathione (GSH), is also found on platelets. The role of platelet-bound GGT in platelet apoptosis and oxidative stress is unknown. The aim of our study was to determine the effects of platelet GGT activity on oxidative stress and apoptotic events *in vitro* via determination of lipid peroxidation (LPO), protein oxidation, GSH, catalase, caspase-3 activation and phosphatidylserine (PS) exposure in the presence of holo-transferrin (Tf). Stimulation of platelet GGT activity with GSH and glycylglycine (GlyGly) increased caspase-3 activation and PS exposure. A significant increase in lipid and protein oxidation and decrease in GSH and catalase levels was also observed in platelets with stimulation of GGT activity in the presence of Tf. Inhibition of GGT activity effectively reduced all the markers. These results suggest that generation of ROS by the GGT/GSH/Tf system can modify the platelets' redox environment and induce apoptosis in *in vitro* conditions.

Introduction

Platelets are among the most significant targets for oxidative stress (Stocker and Keaney, 2004). Platelets also have the ability to produce reactive oxygen species (ROS) from various intracellular enzymatic or non-enzymatic sources (Krötz et al., 2004). γ-Glutamyl transferase/transpeptidase (GGT, EC 2.3.2.2), a novel source of cellular production of oxidants, is a membrane-bound ectoenzyme in the cells. It has also been shown by enzymatic assays that human platelets express GGT (Sener and Yardimci, 2000, 2005a). The primary role of GGT is to metabolize extracellular reduced glutathione (GSH) allowing precursor amino acids to be assimilated and reutilized for intracellular GSH synthesis (Lee et al., 2004).

GSH cannot be taken up by most cells. First, it must be hydrolysed into its constituent amino acids; then these can be taken up by the amino acid transport proteins in the cell membrane. GGT is the only enzyme that can cleave intact glutathione. The enzyme initiates the breakdown of glutathione by cleaving the γ-glutamyl group, thus releasing cysteinylglycine. This dipeptide can be hydrolysed into cysteine and glycine by dipeptidases on the cell surface (McIntyre and Curthoys, 1982). Thus, ectoplasmic GGT favours the cellular supply of GSH, the most important non-protein antioxidant of the cell. It plays an important role in the maintenance of the intracellular redox state. However, recent studies have indicated a novel effect for GGT, suggesting that the extracellular cleavage of GSH by GGT, in the presence of transition metals, leads to ROS production. The pro-oxidant effects of GGT were shown to be mediated by reactive thiol dipeptide cysteinylglycine, a primary me-

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Abbreviations: ADP – adenosine diphosphate, CAT – catalase, DNPH – dinitrophenyl hydrazine, DTNB – 5-5-dithiobis-2-nitrobenzoic acid, DTT – dithiothreitol, GGT – γ-glutamyltransferase, GlyGly – glycylglycine, GSH – glutathione, LPO – lipid peroxidation, PBS – phosphate-buffered saline, PI – propidium iodide, PRP – platelet-rich plasma, PS – phosphatidylserine, RLU – relative light units, ROS – reactive oxygen species, SBC – serine/boric acid complex, SBD-F – 7-fluorobenzo-2-oxa-1,3-diazole 4-sulphonic acid ammonium, TBA – thiobarbituric acid, TBS – Tris-buffered saline, TCA – trichloroacetic acid, Tf – holo-transferrin, TIBC – total iron binding capacity.

tabolite of GSH catabolism. Cysteinylglycine (pKa = 6.4) can dissociate to thiolate anion more easily than GSH (pKa = 8.5) at physiological pH (Stark et al., 1989, 1993). Thiolate anion has a strong reducing capacity. Autoxidation of thiols leads to redox reactions, resulting in formation of the superoxide anion, hydrogen peroxide and other radicals in the presence of transition metals such as Cu^{+2} and Fe^{+3} (Tien et al., 1982; Paolicchi et al., 1997). Thus, GGT-mediated oxidative stress can induce oxidation of lipids (Stark at al., 1993; Paolicchi et al., 1997) and protein thiols (Dominici et al., 1999), and produce biological effects such as activation of transcription factors, proliferation and apoptosis in the cell (del Bello et al., 1999; Maellaro et al., 2000; Paolicchi et al., 2002). A great number of physiological functions are controlled by redox-responsive signalling pathways (Paolicchi et al., 2002). However, redox reactions can also be hazardous to cells. Pathological effects of altered redox regulation are implicated in various pathological conditions such as cardiovascular disease, cancer and diabetes (Valko et al., 2007).

Iron is an essential element necessary for life. It is an important component of many proteins and enzymes, including those participating in the generation of high energy metabolites (Brewer, 2010). On the other hand, it can also be toxic due to its ability to catalyse formation of free radicals, which can damage membranes and other essential biological substances. To avoid the hazardous effects of transition metals, extracellular metal cations are bound by proteins such as transferrin, ferritin and ceruloplasmin (Sener at al., 2009; Brewer, 2010). Transferrin, a main protein for iron transport, exists in the blood in either the ferric iron-bound state (holo-transferrin, Tf) or non-ferric iron-bound state (apo-transferrin). These forms do not usually cause lipid peroxidation (Prakash, 2007). In favour of this, apo-transferrin captures free iron released from the cells, thus showing anti-oxidant effects (Halliwell and Gutteridge, 1990; Prakash, 2007). However, various factors (ascorbate, thiols, reduced flavins, $O2^{\bullet-}$, mild acidic pH as seen in inflammation, influence of the vicinity of activated phagocytic cells, and glycosylation) may also cause reduction of the transferrin-bound ferric iron, thus releasing ferrous iron, which can catalyse Fenton-type generation of oxygen-derived free radicals (Drozdz et al., 1998; Prakash, 2007). ROS can play a modulatory role on the transduction of proliferative or apoptotic signals (Krötz, 2004).

Apoptosis, or programmed cell death, is the physiological mechanism that serves for controlled deletion of unwanted cells. Although platelets are anuclear cells, they show morphological marks of apoptosis, such as depolarization of the mitochondrial membrane potential ($\Delta\Psi m$), microparticle formation, activation of caspases, and exposure of phosphatidylserine (PS) on the platelet surface. Normally, PS is located in the inner phospholipid layer of the platelet membrane. It moves to the outer surface of the cell membrane following platelet activation, and thus serves as an anchor upon which the prothrombinase complex assembles (Leytin et al., 2009).

The aim of our study was to determine the effects of platelet GGT activity on oxidative stress and apoptotic events *in vitro* via determination of lipid peroxidation (LPO), protein oxidation, GSH, catalase (CAT), caspase-3 activation, and PS exposure in the presence of Tf.

Material and Methods

Chemicals

Annexin V-FITC, propidium iodide (PI) and the binding buffer were obtained from BD Biosciences (San Diego, CA). The Caspase-3 kit was obtained from Chemicon (Temecula, CA). Holo-transferrin, phosphate-buffered saline (PBS), reduced glutathione (GSH), Triton X-100, 5-5-dithiobis-2-nitrobenzoic acid (DTNB), adenosine diphosphate (ADP), 7-fluorobenzo-2-oxa-1,3-diazole 4-sulfonic acid ammonium (SBD-F), cysteinylglycine, cysteine, glycylglycine, luminol and apyrase were purchased from Sigma Chemical Co. (St. Louis, MO). Guanidine hydrochloride, thiobarbituric acid (TBA), dinitrophenyl hydrazine (DNPH), dithiothreitol (DTT) and other chemicals were obtained from Merck (Darmstadt, Germany).

Subjects

Platelet concentrations were obtained from the whole blood of medication-free healthy volunteers (for measurements of thiols and ROS production, N = 6 and female/male ratio = 3/3; for measurements of oxidative stress and apoptosis, N = 12 and female/male ratio = 6/6). All subjects gave written informed consent to participate in the study. This study was performed in accordance with the Declaration of Helsinki. None of the subjects had been receiving medications such as acetylsalicylic acid or other platelet-active drugs for at least two weeks prior to sampling. Table 1 shows plasma GSH and serum iron characteristics of the participants. The serum GSH levels were determined using the HPLC method as mentioned below. The levels of serum iron, total iron binding capacity (TIBC) and ferritin were determined using test kits with a Hitachi 917 analyzer (Roche Diagnostic, Holliston, MA). Transferrin saturation (%) was calculated by the formula (transferrin saturation = 100 × serum iron (μg/dl)/TIBC (μg/dl)). Plasma GSH levels and iron parameters of all the participants were within the normal ranges.

Table 1. Plasma GGT, GSH levels and serum iron parameters of participants

N: 12	
Plasma GGT (U/l)	29.0 ± 5.6
Plasma GSH (μM)	8.7 ± 1.3
Serum Iron (μg/dl)	80.3 ± 25.7
Serum Ferritin (μg/dl)	50.7 ± 22.4
TIBC (μg/dl)	30.5 ± 31.2
Transferrin Saturation (%)	26.0 ± 6.4

Platelet isolation

Venipunctures were performed without stasis, using a 21-gauge butterfly needle. Blood samples were collected into 3.8% trisodium citrate and 1 U/ml apyrase-containing tubes. Samples were centrifuged at 150 × *g* for 10 min to separate platelet-rich plasma (PRP). PRP was transferred to another tube. More than 99 % of the cells in PRP, measured by an automated cell counter Beckman Coulter LH750 (Beckman Coulter, Fullerton, CA), consisted of platelets. PRP was centrifuged at 2,000 × *g* for 10 min at room temperature. The pellet was washed twice in Tris-buffered saline (TBS) containing EDTA (0.03 M Tris; 0.12 M NaCI, 5 mM EDTA, pH 7.4) with 1 U/ml apyrase. After centrifugation, platelet pellets were suspended with TBS containing glucose (0.03 M Tris, 0.12 M NaCI, 5 mM glucose, pH 7.4) for incubations.

Inhibition of GGT activity

To inhibit GGT activity we used the specific reversible competitive inhibitor L-serine/boric acid complex (SBC). Platelets were incubated in two different concentrations of SBC (2.5 mM L-serine/5mM boric acid, 5mM L-serine/10 mM boric acid) in TBS at 37 °C for 60 min. After treatment, the platelet pellet was suspended in distilled water. Platelet suspensions were sonicated. After centrifugation, platelet-GGT activity was determined.

Measurement of GGT activity

Platelet-GGT activity was assayed according to Szasz's method (Szasz, 1969), using γ-glutamyl p-nitroanilide as donor substrate and glycylglycine (GlyGly) as glutamate acceptor for the transpeptidation reaction. Standard assay included final reagent concentrations of 4 mM γ-glutamyl p-nitroanilide, 40 mM glycylglycine in 185 mM Tris-HCl buffer, pH 8.2. The results were calculated using a molar extinction coefficient of p-nitroaniline at 405 nm of 9,900 M^{-1} cm^{-1} and expressed as mU/mg protein.

Measurement of extracellular thiols

Platelet GGT-mediated GSH metabolism in the incubation medium was monitored by HPLC measurement of thiols. Platelets were incubated with 2.5 mM GSH and 25 mM GlyGly with or without 5/10 mM SBC for 1 h at 37 °C in TBS. Thiol levels were detected by the method of Pfeiffer et al. (1999) with minor modifications. For assay of thiols in the extracellular medium, a mixture of 50 µl of the sample and 25 µl of PBS, pH 7.4, was incubated with 50 µl of 1 mM DTT for 30 min at room temperature. Trichloroacetic acid (TCA, 5 g/dl) was added for deproteinization. After the sample had been centrifuged for 10 min at 10,000 × *g*, 50 µl of the supernatant was added to the tube containing 10 µl of 1.55 mol/l NaOH; 125 µl of 0.125 mol/l borate buffer, pH 9.5; and 50 µl of 1 g/l SBD-F in the borate buffer (0.125 mol/l pH 9.5). The mixture was then incubated for 60 min at 60 °C. A 10 µl sample was later injected into an Agilent 1100 series HPLC system (Agilent, Mississauga, Canada) with a fluorescence detector (385 nm excitation, 515 nm emission) and 5 µm Kromasil C18 column (15 cm × 4.6 mm, Hi Chrom, Berkshire, UK). Acetic acid-acetate buffer (0.1 mol/l, pH 5.5, containing 30 ml/l methanol) was used as the mobile phase. The flow rate and column temperature were 0.7 ml/min and 29 °C, respectively. The concentrations of thiols were calculated using standard solutions of GSH, cysteinylglycine and cysteine and were expressed as µM.

Chemiluminescence measurements

For the generation of reactive oxygen species related to platelet GGT and thiols, luminol chemiluminescence was used as the indicator of radical formation. Luminol detects a group of reactive species including $OH^{\bullet}$, H_2O_2, HOCl. The levels of ROS in washed platelets in the presence of 2.5 mM GSH + 25 mM GlyGly or 2.5 mM cysteinylglycine or 2.5 mM cysteine and 80 µM Tf were recorded by the method of Kröl et al. (1990). The chemiluminescence signals were evaluated by a luminometer (Glomax, Promega, Sunyvale, CA) after addition of 10 µl of 2 mM luminol solution in buffered saline. Results were expressed as relative light units (RLU).

Platelet incubations for oxidative stress and apoptosis

To determine oxidative events, platelets were suspended in plastic tubes by 1 ml TBS, each containing 500,000 platelets per mm^3. To stimulate the GGT activity, platelets were incubated with the substrate, 2.5 mM GSH and co-substrate, 25 mM GlyGly in the presence or absence of Tf for 1 h at 37 °C. To inhibit GGT activity, platelets were pre-treated with 5/10 mM SBC for 15 min. Platelet suspensions (1 ml) were incubated at final concentrations as follows:

1. Platelets+buffer (Control)
2. Platelets+GSH-GlyGly (2.5 mM-25 mM)
3. Platelets+Tf (80 µM)+GSH-GlyGly
4. Platelets+Tf+SBC (5/10 mM)+GSH-GlyGly

At the end of incubation, platelets were centrifuged in order to remove the incubation medium and washed once in ice-cold buffer. Platelets were resuspended in distilled water. Then, platelet suspensions were sonicated. After centrifugation for 10 min at 2,500 × *g*, the supernatant was used to determine the GSH levels, GGT and CAT activities. The precipitate was used to determine LPO and protein oxidation. The same incubations were also prepared as mentioned above for caspase-3 and flow cytometric analyses.

Determination of platelet GSH levels

GSH determinations were performed using the method described by Mergel and Anderman (1979) with colorimetric assay using DTNB. This method is based on protein precipitation by metaphosphoric acid (15%) and spectrophotometric assay of the yellow derivative at

412 nm. GSH concentration was calculated using a standard solution of GSH. The results were expressed as μmol GSH/10^9 platelets.

Determination of LPO and protein carbonyl (PCO) contents

For measurements of membrane lipid peroxidation and protein oxidation, the precipitate was solubilized with 100 mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100 and then centrifuged for 10 min at 10,000 × *g*.

LPO levels were measured by monitoring thiobarbituric acid-reactive substance formation as described previously (Buege and Aust, 1978). Platelet samples were treated with 1 ml TBA reagent (containing 0.37% TBA, 0.25 N HCl, 15% TCA) and incubated for 15 min in a boiling water bath and then cooled. The absorbance of the clear supernatant was measured against the reference blank at 535 nm using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$. The results were expressed as nmol LPO/mg protein.

The contents of PCO for protein oxidation were assayed according to the method of Levine et al. (1990) with minor modifications. Supernatants were incubated with 10 mM DNPH in 2 N HCl for 1 h at room temperature. Protein hydrazone derivatives were precipitated with 20% TCA and the precipitates were then washed three times with ethanol : ethyl-acetate (1 : 1). The final pellet was resuspended in 6 M guanidine hydrochloride and incubated for 15 min at 37 °C. The absorbance was measured at 360 nm, using a molar extinction coefficient of 2.2×10^4 $M^{-1}cm^{-1}$. The results were expressed as nmol/mg protein.

Measurement of CAT activity

The method for the measurement of CAT levels, which is an important enzymatic mechanism against oxidative stress, is based on the catalytic activity of the enzyme which catalyses the decomposition reaction of H_2O_2 to give H_2O and O_2 (Aebi, 1984). The reaction mixture (1.5 ml) contained 1 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml of the sample and 0.4 ml of 2 M H_2O_2. The reaction was terminated by addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid mixed in 1 : 3 ratio). Then the absorbance was read at 620 nm. Lyophilized bovine liver CAT (Sigma C1345) was used as positive control. One unit of CAT is defined as the amount needed to decompose 1 nmol H_2O_2 per minute and the specific activity is expressed as U/mg protein.

Determination of caspase-3 activity

Caspase activity was measured using a caspase-3 colorimetric assay kit (Chemicon, Cat No: APT165) following the manufacturer's protocol. This method uses a colorimetric assay to monitor cleavage of an acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) substrate, which resembles the caspase-3 cleavage site. First, platelets were lysed in the lysis buffer at room temperature for 15 min. The platelet lysates were then centrifuged at 2,000 × *g* for 10 min at 4 °C to precipitate cellular debris. Cell lysates were incubated with substrate solution Ac-DEVD-pNA for 1–2 h at 37 °C. In the experiments, N-Ac-Asp-Glu-Val-Asp-CHO (Ac--DEVD-CHO) was used as the caspase-3 inhibitor and shown to completely block the release of p-nitroaniline (pNA). Lyophilized caspase-3 (Sigma C5974) was used as positive control. The absorbance was read at 405 nm using a microplate reader. The caspase-3 activity was calculated from the cleavage of the specific colorimetric substrate (Ac-DEVD-pNA). The results were expressed as nmol pNA/mg protein.

Analysis of apoptosis by flow cytometry

Apoptosis was measured by annexin-V kit (BD Pharmingen, San Diego, CA, Cat. No: 556547) using annexin-V-labelled FITC, which has a high affinity for binding to PS. Annexin-V-FITC (25 μg/ml) and PI (50 μg/ml) were added to the samples which were incubated with GSH, GlyGly, SBC and Tf as described above (under the heading of "platelet incubations"). The sample tubes were kept on ice until analysis for 10 min in the dark. After addition of an equal volume of cold binding buffer (0.1 M HEPES/NaOH buffer, pH 7.4, containing 1.4 M NaCl, 25 mM $CaCl_2$, diluted 1 : 9 with distilled water), flow cytometric analysis was made in the FACS Calibur flow cytometry system (Beckman Coulter) (Overbeeke et al., 1998).

To estimate non-specific and background fluorescence, inactivated samples were stained with an isotype FITC--conjugated immunoglobulin G (IgG) control. The analysis of all the samples was carried out in the flow cytometer. The system was equipped with 488 nm argon ion laser. CaliBrite beads (BD Biosciences, San Jose, CA) were used for daily quality control. In each tube, 50,000 cells were counted and the results represented the mean value of the duplicate samples. Platelets were identified by staining the platelets with FITC-conjugated CD41a, which was gated from logarithmic scaled forward scatter/side scatter scattergram. The results were expressed as the percentage of positive cells. The negative control cursor was set to 2 % of cells on histograms.

Protein determination

Protein content was analysed according to the Bradford's method (1976) using bovine serum albumin as the standard and expressed as mg protein per ml platelet suspension.

Statistical analysis

All data represented mean values with standard deviation (SD) obtained from at least 2–3 independent experiments. Statistical analysis was carried out using the Instat statistical package (GraphPad Software, San Diego, CA). For statistical analyses, first the degree of normality was investigated. Friedman test was used for the nonparametric data and repeated-measures analysis

of ANOVA was used for the parametric data. A P value < 0.05 was considered statistically significant.

Results

Determination of inhibitor concentrations

First, we analysed the platelet GGT activity after incubation of platelets for 15 min with two different concentrations of the inhibitor. As shown in Table 2, SBC inhibited 93 % of platelet GGT activity at 5 mM L-serine and 10 mM boric acid concentrations. This concentration of the inhibitor was used for the analysis.

Extracellular GSH and cysteinylglycine levels

The GGT activity-dependent production of cysteinylglycine was monitored by HPLC in the extracellular medium with or without the presence of GGT inhibitor. After stimulation of GGT activity with 2.5 mM GSH and 25 mM GlyGly, cysteinylglycine and cysteine levels were detected at maximum levels when compared to the control group (498 ± 61 μM, 140 ± 20 μM, respectively, P < 0.001, Fig. 1). The extracellular GSH level decreased from 2.5 to approximately 2 mM. The inhibition of platelet GGT activity with SBC significantly decreased production of cysteinylglycine as compared to the GSH-GlyGly group (P < 0.001).

Table 2. Inhibition of GGT activity. Platelets were incubated with SBC for 15 min and GGT activity was measured.

	GGT activity (mU/mg protein)	Inhibition (%)
Control	19.0 ± 3.5	0
SBC (2.5/10 mM)	11.6 ± 2.0	39
SBC (5/10 mM)	1.34 ± 0.60	93

Values are expressed as the means ± SD; N = 12 in each group

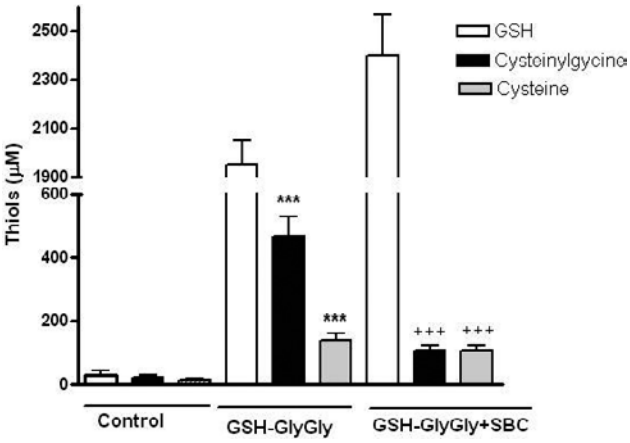

Fig. 1. Levels of thiols in extracellular medium. Cells were suspended in 1 ml of solution containing 2.5 mM GSH, 25 mM GlyGly for 1 h at 37 °C in TBS. Experiments were run with or without SBC. Values are expressed as the mean ± SD; N = 6 in each group. (***P < 0.001 vs. control, $^{+++}$P < 0.001 vs. GSH-GlyGly)

ROS generation

In our experimental conditions testing whether the thiols possess similar oxidant properties, platelets were incubated with GSH-GlyGly in the presence of Tf; and ROS generation was monitored by chemiluminescence of luminol for 5 min (without an enhancer, i.e., H_2O_2, HOCl or platelet agonists) (Fig. 2A). Addition of GSH and GlyGly in platelet suspensions enhanced the intensity of chemiluminescence as compared to the control group. Fig. 2B also shows ROS generation by platelets which were incubated with GSH, cysteinylglycine or cysteine for 3 min. Cysteinylglycine markedly increased the intensity of chemiluminescence as compared to GSH and cysteine (P < 0.001). GSH also increased the

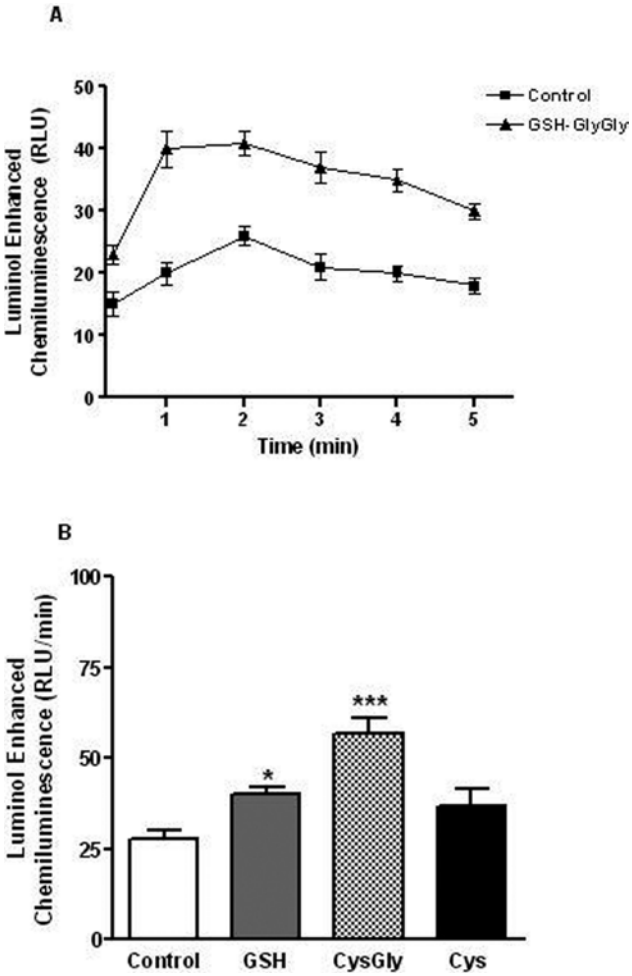

Fig. 2. (**A**) Effects of GGT activity on ROS generation. Washed human platelets were stimulated for 5 min with 2.5 mM GSH and 25 mM GlyGly in the presence of 80 μM holo-transferrin and 2 mM luminol. (**B**) Platelet-GGT and thiol-induced ROS generation from platelets in the presence of Tf. The reaction mixture contained 2.5 mM GSH + 25 mM GlyGly or 2.5 mM cysteinylglycine (CysGly) or 2.5 mM cysteine (Cys). Experiments were run with 2 mM luminol and 80 μM Tf. Results are expressed as mean ± SD; N = 6. (*P < 0.05 vs. control, ***P < 0.001 vs. control, GSH and cysteine)

intensity of chemiluminescence as compared to the control group ($P < 0.05$).

LPO levels

In our study, we investigated whether the lipid peroxidation process stimulated by platelet-bound GGT might be propagated to platelet lipids in the presence of Tf. High concentrations of GSH and GlyGly were used in order to provide the Vmax of GGT activity.

Fig. 3A shows LPO levels mediated by platelet GGT. When washed platelets were incubated with GSH, GlyGly and/or Tf, LPO levels of the Tf+GSH-GlyGly group increased significantly as compared to the control and GSH-GlyGly groups in the platelet crude membranes ($P < 0.001$). After inhibition of GGT activity by SBC, the LPO levels decreased significantly as compared to the Tf+GSH-GlyGly group ($P < 0.05$).

PCO contents

As shown in Fig. 3B, the platelet PCO amounts increased significantly with GGT activity stimulation in the presence of Tf ($P < 0.01$). On the other hand, following incubation of GGT substrates and Tf with platelets that had been pre-incubated with GGT inhibitor, the PCO amounts decreased significantly ($P < 0.001$).

GSH levels

The platelet GSH contents increased significantly after stimulation of GGT in the absence of Tf as compared to the control group ($P < 0.001$, Fig. 4A). However, after addition of Tf, GSH levels decreased as compared to the GSH-GlyGly group ($P < 0.001$). When GSH, GlyGly and Tf were added to platelets that had been pre-incubated with SBC, GSH contents increased significantly ($P < 0.001$).

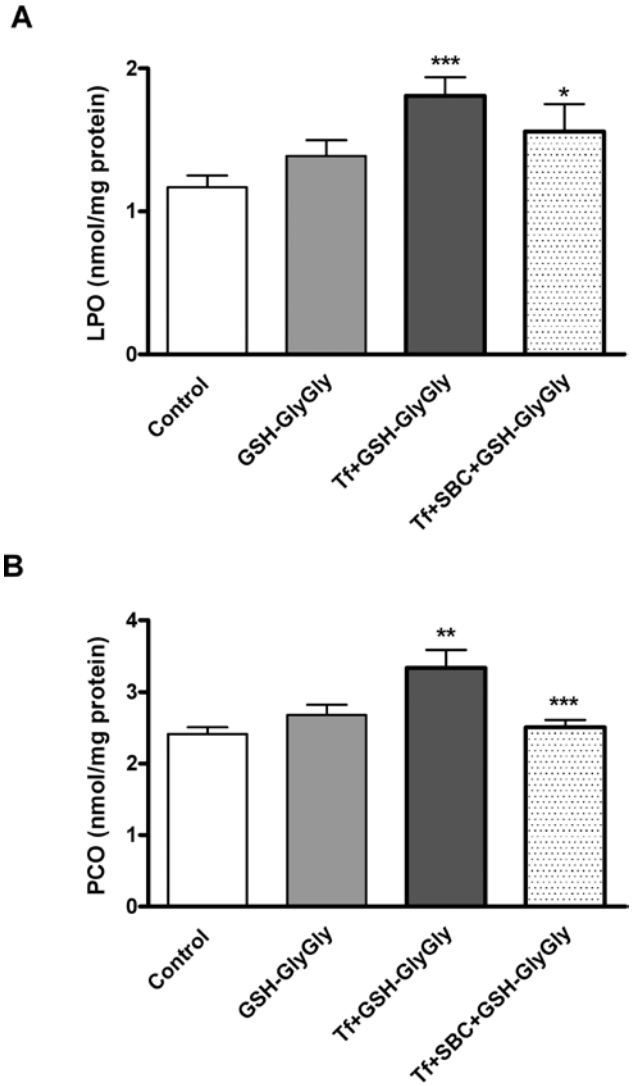

Fig. 3. Oxidative stress damage after incubation with agents for 1 h. Values are expressed as the mean ± SD; N = 12 in each group. (**A**) Platelet LPO levels (***$P < 0.001$ vs. GSH-GlyGly and control, *$P < 0.05$ vs. Tf+GSH-GlyGly). (**B**) Platelet PCO levels (**$P < 0.01$ vs. GSH-GlyGly and control, ***$P < 0.001$ vs. Tf+GSH-GlyGly)

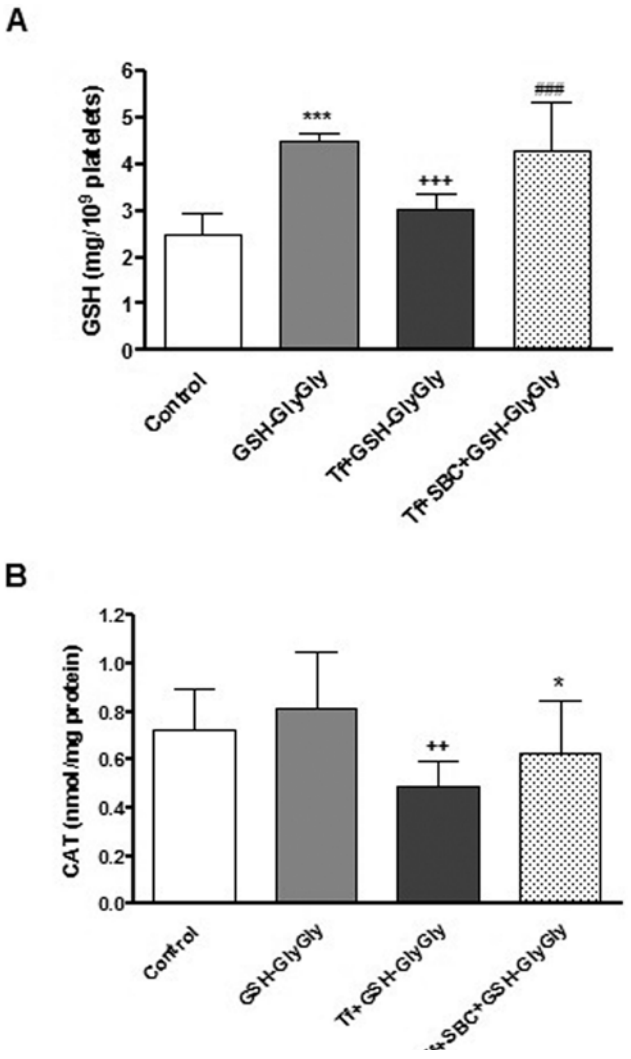

Fig. 4. Bar graphs showing GSH and CAT levels after incubation with agents for 1 h. Values are expressed as the mean ± SD; N = 12 in each group. (**A**) Platelet GSH levels (***$P < 0.001$ vs. control, $^{+++}P < 0.001$ vs. GSH-GlyGly, $^{\#\#\#}P < 0.001$ vs. Tf+GSH-GlyGly) (**B**) Platelet CAT activities ($^{++}P < 0.01$ vs. control and GSH-GlyGly, *$P < 0.05$ vs. Tf+GSH-GlyGly)

CAT activity

The platelet CAT activity decreased significantly after stimulation of GGT in the presence of Tf ($P < 0.01$, Fig. 4B). After addition of inhibitors, the CAT activity increased significantly compared to the group treated with Tf+GSH-GlyGly ($P < 0.05$).

PS exposure

Platelet samples stained with annexin V-FITC are represented by histograms in Fig. 5A. The percentage of PS exposure in the Tf+GSH-GlyGly group was significantly different from that of the GSH-GlyGly group ($P < 0.001$). The inhibition of GGT activity with SBC prevented Tf+GSH-GlyGly-mediated increases in PS ($P < 0.001$, Fig. 5B).

Caspase-3 activity

After incubation with GSH, GlyGly and Tf, platelet caspase-3 levels increased significantly as compared to the control group and GSH-GlyGly group ($P < 0.01$, Fig. 6). On the other hand, following incubation of GSH, GlyGly and Tf with platelets that had been treated with inhibitors, the caspase-3 levels decreased significantly with SBC as compared to the group treated with Tf+GSH-GlyGly ($P < 0.01$).

Discussion

Pro-oxidant products which are generated by redox reactions during GGT-mediated GSH catabolism were first shown by Stark et al. (1993). Additionally, it has been shown that cysteinylglycine liberated during the destruction of GSH by GGT in the presence of an iron source is also primarily responsible for the pro-oxidant effect (Drozdz et al., 1998). The first step in our study was to investigate platelet GGT-mediated metabolism of GSH. GSH is present intracellularly in mM concentrations and extracellularly in μM concentrations. A high concentration of GSH (more than 2.5 mM Km and over the serum concentration level) was used to provide a maximal GGT activity. Thus, we obtained maximal cysteinylglycine and cysteine formation and also quantifiable oxidative modifications. These conditions were optimal for the *in vitro* study of the pro-oxidant properties of GGT (Drozdz et al., 1998). Our findings demonstrated that 1 h incubation of platelet GGT with 2.5 mM GSH resulted in significant production of extracellular cysteinylglycine and cysteine. Inhibition of the platelet GGT activity blocked consumption of extracellular GSH and reduced production of cysteinylglycine (approximately 79 %). In our previous study, we observed that transition metals must be present for GGT-associated redox reactions (Sener et al., 2005b). In the present study, we used Tf as a physiological source of iron. We

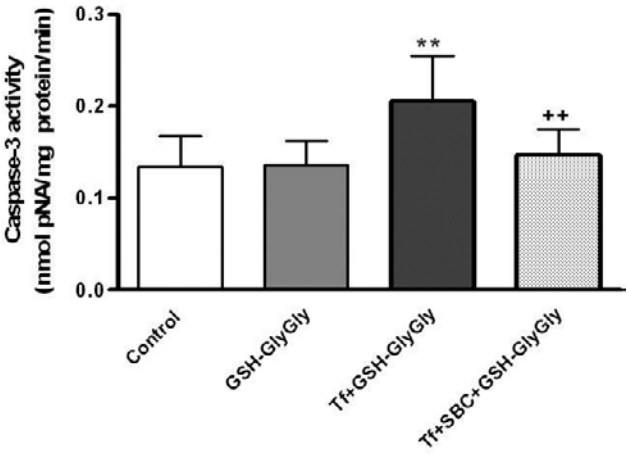

Fig. 6. Platelet caspase-3 levels. Values are expressed as the mean ± SD; N = 12 in each group. (**$P < 0.01$ vs. control, and GSH-GlyGly, $^{++}P < 0.01$ vs. Tf+GSH-GlyGly)

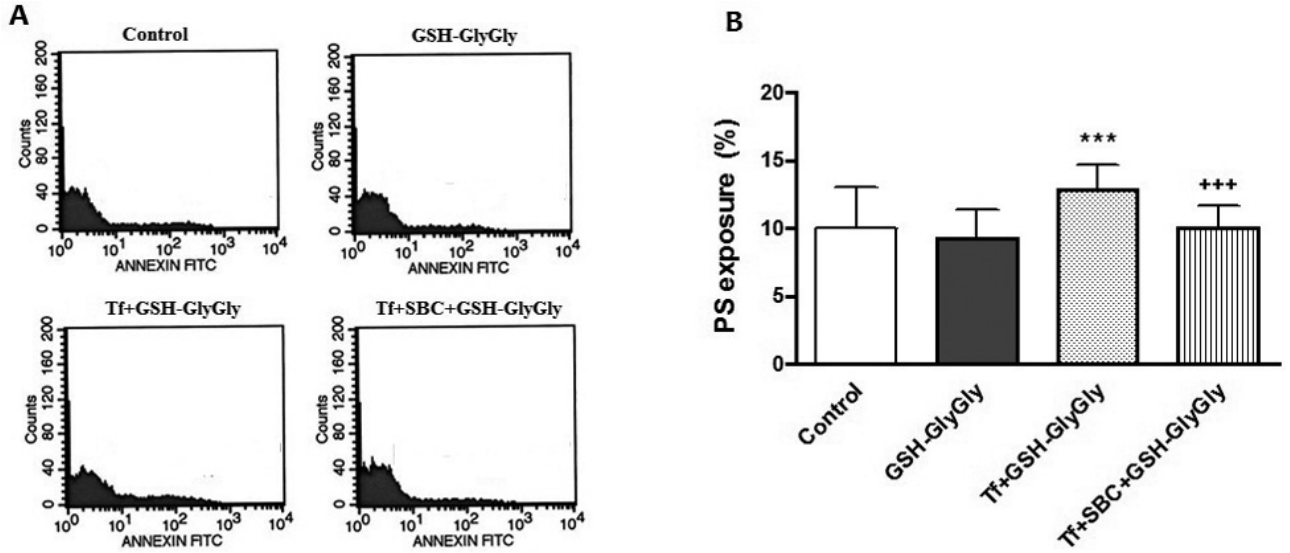

Fig. 5. Platelet PS levels. (**A**) Histogram of annexin-V-FITC-labelled platelet samples. Annexin-V FITC-positive platelets are in the area marked as M1. (**B**) Bar graphs showing PS exposure after incubation with agents for 1 hour. Values are expressed as the mean ± SD; N = 12 in each group. (***$P < 0.001$ vs. GSH-GlyGly, $^{+++}P < 0.001$ vs. Tf+GSH-GlyGly)

observed that at *in vitro* conditions and in the presence of Tf, cysteinylglycine is markedly more effective than both GSH and cysteine on platelet ROS generation. This finding may result from the fact that the iron-reducing effect of cysteinylglycine is stronger when compared to GSH and cysteine (Drozdz et al., 1998). GSH also increased ROS production to some extent. This increase may be due to cysteinylglycine released as a result of the platelet GGT activity.

In the second part of our study, we investigated the relationship between platelet GGT-mediated cysteinylglycine formation and platelet oxidative stress. Platelet GGT-mediated GSH catabolism caused increases in the oxidative process (lipid peroxidation, protein oxidation) and reductions in the intracellular GSH levels and CAT activity. The stimulation of GGT activity without holotransferrin did not cause any increase in oxidative events. Our previous findings demonstrated that platelet GGT/GSH/Fe^{+3} systems can also induce lipid peroxidation, protein oxidation and consumption of intracellular antioxidant capacity in platelets (Sener et al., 2011). Inhibition of the GGT activity also blocked the decrease in intracellular GSH and CAT levels in this study. These events may contribute to the atherosclerotic process by changing membrane specifications and increasing platelet thrombotic response. A study conducted using erythrocytes showed that the GGT/GSH/iron system can oxidize isolated erythrocyte membranes and cause dysfunctions. Erythrocytes are thought to be important targets of pro-oxidant molecules produced by the serum GGT activity (Aberkane et al., 2002). It is considered that serum GGT activity is largely derived from the liver (Dufour et al., 2000). However, it was observed that platelets can contribute to GGT activity and the oxidative modifications in the plasma (Bolodeoku et al., 1997; Sener and Cevik 2009). In circulation, human platelets produce and release ROS, such as hydrogen peroxide (H_2O_2), under physiological stimulation. At the same time, they are also significant targets for ROS. High concentrations of ROS may cause platelet activation, loss of function in platelets and shorten their life span (Krötz et al., 2004). H_2O_2 may induce platelet activation and platelet aggregation by causing tyrosine phosphorylation in the intracellular site of platelet membrane glycoprotein Gp IIb/IIIa, which has a significant role in platelet activation (Law et al., 1996). Free thiol groups in Gp IIb/IIIa are also controlled by the ratio of extracellular GSH/GSSG (Essex et al., 2004). Thus, besides the intracellular and extracellular ROS concentrations, the GSH/GSSG ratio is also an important regulator of platelet functions. Therefore, addition of exogenous GSH is expected to have an anti-platelet effect. However, it has been reported that exogenous GSH addition to platelets may affect platelet aggregation in a different way depending on the concentration. It has been indicated that GSH addition has an anti-oxidant effect in millimolar concentrations but an anti-aggregant effect at micromolar concentrations (Essex and Li, 2003; Essex, 2009). Although the concentration that we used in this study was at the millimolar level, contrary to expectations, it caused an increase in oxidative reactions in the presence of Tf and iron (Sener et al., 2011). Prevention of the increase in oxidative reactions by addition of the GGT inhibitor denotes that GGT has a role in redox reactions associated with GSH/Tf or iron (Sener et al., 2011). Moreover, the fact that cysteinylglycine levels in the inhibited samples were very low shows that cysteinylglycine is more effective than GSH in redox reactions.

In the third step, we analysed the effects of platelet GGT activity on platelet apoptosis by determination of cytosolic caspase-3 levels and externalization of PS. In our study, stimulation of GGT activity increased the caspase-3 levels in the presence of holo-transferrin as compared to the control. The inhibition of GGT activity partially blocked this increase. When we analysed PS translocation as another apoptosis marker, we observed that while PS in platelets increased as a result of GGT stimulation, they decreased following GGT inhibition. In several studies, it has been shown that the GGT activity stimulation is effective on apoptosis and cell proliferation in certain types of cells. It has been shown that the additional exogenous GSH in GGT-transfected Ramos cells (without inclusion of transition metals) increases cell viability compared to cells not transfected with GGT to provide the cysteine (Karp et al., 2001). The stimulation of GGT activity with 200 μM GSH in melanoma cells is observed to be approximately 15 nmol $H_2O_2/10^6$ cells/h (Maellaro et al., 2000). H_2O_2 originating as a by-product during GGT activity is capable of acting as a signal molecule. It has been shown that blocking low level H_2O_2 generation in U937 monoblastoid cells using GGT inhibitors increases cell death and DNA fragmentation (del Bello et al., 1999). In spite of these studies, Lopez et al. (2007) have shown that platelet exposure to exogenous H_2O_2 in a dose-dependent manner results in activation of caspase-3 and PS exposure. The ROS that increased during the GSH metabolism may be responsible for the increased apoptosis levels that we observed in our study. Excess ROS may trigger apoptosis and activation through several signal pathways in the platelets (Krötz et al., 2004). In our previous study, we showed that the increase of platelet oxidative modifications and apoptosis occurs simultaneously in individuals with hypercholesterolaemia (Sener et al., 2005c). We obtained similar findings in this study as well.

Conclusions

Here, in *in vitro* conditions, we report that the stimulation of membrane bound-GGT activity in washed platelets triggers oxidative modifications and induces platelet apoptosis in the presence of Tf. Our observations may be helpful in suggesting possible mechanisms for the regulation of platelet activation and life span and in improving the antithrombotic therapy in several clinical conditions such as iron overload after blood transfusion, inflammation and cardiovascular diseases.

Study limitations

In our study, we used washed platelets. Thus, this study provided unaltered information about the relation between platelet GGT activity and oxidative stress that was not affected by plasma antioxidant capacities. The used GSH concentration exceeds the plasma concentration. Consequently, these phenomena do not represent physiological conditions but may constitute a significant basis for our and other ongoing studies on platelet GGT in physiological conditions.

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References

Aberkane, H., Stoltz, J. F., Galteau, M. M., Wellman, M. (2002) Erythrocytes as targets for γ-glutamyltranspeptidase initiated pro-oxidant reaction. *Eur. J. Haematol.* **68**, 262-271.

Aebi, H. (1984) Catalase *in vitro*. *Methods Enzymol.* **105**, 121-126.

Bolodeoku, J. A., Ganotakis, E. S., Mikhailidis, D. P., Winder A. F. (1997) Correlation between serum γ-glutamyl transferase activity and the platelet count. *Platelets* **8**, 333-335.

Bradford, M. M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254.

Brewer, G. J. (2010) Risks of copper and iron toxicity during aging in humans. *Chem. Res. Toxicol.* **23**, 319-326.

Buege, J. A., Aust, S. D. (1978) Microsomal lipid peroxidation. *Methods Enzymol.* **52**, 302-310.

del Bello, B., Paolicchi, A., Comporti, M., Pompella, A., Maellaro, E. (1999) Hydrogen peroxide produced during γ-glutamyl transpeptidase activity is involved in prevention of apoptosis and maintenance of proliferation in U937 cells. *FASEB J.* **13**, 69-79.

Dominici, S., Valentini, M., Maellaro, E., del Bello, B., Paolicchi, A., Lorenzini, E., Tongiani, R., Comporti, M., Pompella, A. (1999) Redox modulation of cell surface protein thiols in U937 lymphoma cells: the role of γ-glutamyl transpeptidase-dependent H_2O_2 production and S-thiolation. *Free Radic. Biol. Med*. **27**, 623-635.

Drozdz, R., Parmentier, C., Hachad, H., Leroy. P., Siest, G., Wellman, M. (1998) γ-Glutamyltransferase dependent generation of reactive oxygen species from a glutathione/transferrin system. *Free Radic. Biol. Med.* **25**, 786-792.

Dufour, D. R., Lott, J. A., Nolte, F. S., Gretch, D. R., Koff, R. S., Seeff, L. B. (2000) Diagnosis and monitoring of hepatic injury. I. Performance characteristics of laboratory tests. *Clin. Chem.* **46**, 2027-2049.

Essex, D. W, Li, M. (2003) Redox control of platelet aggregation. *Biochemistry* **42**, 129-136.

Essex, D. W., Li, M., Feinman, R. D., Miller, A. (2004) Platelet surface glutathione reductase-like activity. *Blood* **104**, 1383-1385.

Essex, D. W. (2009) Redox control of platelet function. *Antioxid. Redox Signal.* **11**, 1191-1225.

Halliwell, B., Gutteridge, J. M. C. (1990) The antioxidants of human extracellular fluids. *Arch. Biochem. Biophys.* **280**, 1-8.

Karp, D. R., Shimooku, K., Lipsky. P. E. (2001) Expression of γ-glutamyl transpeptidase protects Ramos B cells from oxidation-induced cell death. *J. Biol. Chem*. **276**, 3798-3804.

Kröl, W., Czuba, Z., Scheller, S., Gabrys, J., Grabiec, S., Shani. J. (1990) Antioxidant property of ethanolic extract of propolis (EEP) as evaluated by inhibiting the chemiluminescence oxidation of luminol. *Biochem. Int.* **21**, 593-597.

Krötz, F., Sohn, H. Y., Pohl, U. (2004) Reactive oxygen species players in the platelet game. *Arterioscler. Thromb. Vasc. Biol*. 24, 1988-1996.

Law, D. A., Nannizzi-Alaimo, L., Phillips, D. R. (1996) Outside-in integrin signal transduction. α IIb β 3-(GP IIb IIIa) tyrosine phosphorylation induced by platelet aggregation. *J. Biol. Chem.* **271**, 10811-10815.

Lee, D., Blomhoff, R., Jacobs, D. R. (2004) Is serum γ-glutamyltransferase a marker of oxidative stress? *Free Radic. Res.* **38**, 535-539.

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S., Stadtman, E. R. (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol*. **186**, 464-478.

Leytin, V., Allen, D. J., Mutlu, A., Gyulkhandanyan, A. V., Mykhaylov, S., Freedman, J. (2009) Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. *Lab. Invest.* **89**, 374-384.

Lopez, J. J., Salido, G. M., Gomez-Arteta, E., Rosado, J. A., Pariente, J. A. (2007) Thrombin induces apoptotic events through the generation of reactive oxygen species in human platelets. *J. Thromb. Haemost*. **5**, 1283-1291.

Maellaro, E., Dominici, S., del Bello, B., Valentini, M. A., Pieri, L., Perego, P., Supino, R., Zunino, F., Lorenzini, E., Paolicchi, A., Comporti, M., Pompella, A. (2000) Membrane γ-glutamyl transpeptidase activity of melanoma cells: effects on cellular H_2O_2 production, cell surface protein thiol oxidation and NF-κB activation status. *J. Cell Sci.* **113**, 2671-2678.

Mclntyre, T., Curthoys, N. P. (1982) Renal catabolism of glutathione: Characterization of a particulate rat renal dipeptidase that catalyzes the hydrolysis of cysteinylglycine. *J. Biol. Chem*. **257**, 11915-11921.

Mergel, D., Andermann, G., Andermann, C. (1979) Simultaneous determination of oxidized and reduced glutathione in human rabbit red cells. *Methods Find. Exp. Clin. Pharmacol*. **1**, 277-283.

Overbeeke, R., Steffens-Nakken, H., Vermes, I., Reutelingsperger, C., Haanen, C. (1998) Early features of apoptosis detected by four different flow cytometry assays. *Apoptosis* **3**, 115-121.

Paolicchi, A., Tongiani, R., Tonarelli, P., Comporti, M., Pompella, A. (1997) γ-Glutamyl transpeptidase-dependent lipid peroxidation in isolated hepatocytes and HepG2 hepatoma cells. *Free Radic. Biol. Med*. **22**, 853-860.

Paolicchi, A., Dominici, S., Pieri, L., Maellaro, E., Pompella, A. (2002) Glutathione catabolism as a signaling mechanism. *Biochem. Pharmacol*. **64**, 1027-1035.

Pfeiffer, C. M., Huff, D. L., Gunter, E. W. (1999) Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. *Clin. Chem*. **45**, 290-292.

Prakash, M. (2007) Role of non-transferrin-bound iron in chronic renal failure and other disease conditions. *Indian J. Nephrol.* **17**,188-193.

Sener, A., Yardimci, T. (2000) Lectin affinity chromatography and electrophoretic properties of human platelet γ-glutamyl transferase. *Platelets* **11**, 325-330.

Sener, A., Yardimci, T. (2005a) Activity determination, kinetic analyses and isoenzymes identification of γ-glutamyltransferase in human neutrophiles. *J. Biochem. Mol. Biol.* **38**, 343-349.

Sener, A., Ozsavci, D., Yanikkaya-Demirel, G., Aksoy, H., Oba, R., Uras, F., Yardimci K. T. (2005b) The role of γ-glutamyltransferase (GGT) activity on early platelet apoptotic process. *J. Thromb. Haemost.* **3 (Suppl. 1)**, 899-899.

Sener, A., Ozsavci, D., Oba, R., Yanikkaya-Demirel, G., Uras, F., Yardimci, K. T. (2005c) Do platelet apoptosis, activation, aggregation, lipid peroxidation and platelet-leukocyte aggregate formation occur simultaneously in hyperlipidemia? *Clin. Biochem.* **38**, 1081-1087.

Sener, A., Cevik, O. (2009) Can platelet activation influence plasma γ-glutamyltransferase activity? *Turk. J. Biochem.* **34 (Suppl. 1)**, 79-79.

Sener. A., Ozsavci, D., Bingol-Ozakpinar, O., Cevik, O., Yanikkaya-Demirel, G., Yardimci, T. (2009) Oxidized-LDL and Fe^{3+}/ascorbic acid-induced oxidative modifications and phosphatidylserine exposure in human platelets are reduced by melatonin. *Folia Biol. (Praha)* **55**, 45-52.

Sener, A., Cevik, O., Ozsavci, D., Yanikkaya-Demirel, G. (2011) The pro-oxidant effect of platelet γ-glutamyltransferase in the presence of iron(III). *Marmara Pharm. J.* **15**, 30-37. (in Turkish)

Stark, A. A., Arad, A., Siskindovich, S., Pagano, D. A., Zeiger, E. (1989) Effect of pH on mutagenesis by thiols in Salmonella typhimurium TA102. *Mutat. Res.* **224**, 89-94.

Stark, A. A., Zeiger, E., Pagano, D. A. (1993) Glutathione metabolism by γ-glutamyl transpeptidase leads to lipid peroxidation: characterization of the system and relevance to hepatocarcinogenesis. *Carcinogenesis* **14**, 183-189.

Stocker, R., Keaney, J. F. (2004) Role of oxidative modifications in atherosclerosis. *Physiol. Rev.* **84**, 1381-1478.

Szasz, G. (1969) Kinetic photometric method for serum γ-glutamyl transpeptidase. *Clin. Chem.* **15**, 124-136.

Tien, B. M., Bucher, J. R., Aust, S. D. (1982) Thiol dependent lipid peroxidation. *Biochem. Biophys. Res. Commun.* **107**, 279-285.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **39**, 44-84.