IN VITRO EFFECT OF REDUCED GLUTATHIONE ON PLATELET FUNCTION

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ABSTRACT

Background. Generation of reactive oxygen species has been suggested to represent an important regulatory mechanism of platelet reactivity in both physiological and pathological conditions, and free-radical scavengers may inhibit platelet activation. The purpose of the present study was to investigate the effect of reduced glutathione (GSH) on different platelet functions stimulated by ADP, collagen or PAF.

Methods. Platelet aggregation was investigated by Born's method. TxB_2 and PDGF levels were measured by radioimmunoassay.

Results. GSH at the lowest dose (1 mM) did not significantly modify aggregation, TxB_2 production or PDGF release induced by ADP or PAF, while at higher concentrations (3 mM or 10 mM) it significantly inhibited all parameters. Collagen-induced platelet activation was remarkably less sensitive to GSH, since aggregation was not significantly reduced, while TxB_2 production was reduced by GSH when employed at concentrations of 3 mM or 10 mM, and PDGF release was inhibited only by the highest dose (10 mM). IC_{50} for inhibition of platelet aggregation, TxB_2 production and PDGF release were between 1.43 and 2.36 mM when platelets were stimulated with ADP, between 2.23 and 8.90 mM when PAF was used and between 8.00 and 16.30 mM when collagen was the agonist.

Conclusions. Our data suggest that GSH may act as a physiological inhibitor of platelet activation and may therefore contribute to the regulation of platelet reactivity.

Key words: glutathione, platelets, aggregation, thromboxane B_2, PDGF

Reactive oxygen species (ROS), such as H_2O_2 and the free radicals O_2- and OH°, have potentially deleterious effects on biological systems and may be involved in various pathological entities, including atherosclerosis and its complications.[1-3] An important mechanism through which ROS may play a role in vascular and thrombotic diseases could be related to their well-documented ability to activate platelet function.[4-8] Conversely, platelets stimulated with different agonists are themselves a source of hydrogen peroxide[9] as well as of superoxide anions[10] and hydroxyl radicals,[11] and therefore ROS generation has been suggested to represent an important regulatory mechanism of platelet reactivity in both physiological and pathological conditions.[5,7] Consistent with this hypothesis is the observation that free-radical scavengers may inhibit platelet activation.[7,12,13]

Reduced glutathione (GSH), a naturally occurring tripeptide, plays a key role as a defense mechanism against excessive ROS accumulation because it acts as a substrate for the enzyme glutathione peroxidase and also possesses direct anti-oxidant activity.[1,7] The effect of GSH on platelet function has not yet been investigated in detail. Available evidence suggests that GSH inhibits platelet aggregation induced by peroxynitrite, an oxidant agent formed from the reaction of nitric oxide and superoxide;[14] furthermore, GSH was been shown to enhance the antiplatelet activity of S-nitroso-proteins, probably through the formation of S-nitroso-glutathione, which acts as a donor of the antiplatelet agent nitric oxide.[15] It is also known that a GSH derivative, (1,2-dicarboxyethyl) glu-

		GSH				
	Control	*1 mM*	*3 mM*	*10 mM*	*chi-square*	*p*
Aggregation (%)	33.50	15	10.5*	0*	17	< 0.01
TxB$_2$ (ng/mL)	26.15	22	10.2*	0*	12.05	<0.01
PDGF (ng/mL)	30.05	17.55	9.5*	0*	17	<0.01

Table 1 Effect of GSH on platelet aggregation, TxB$_2$ production and PDGF release induced by ADP (1 µM) (median values of 6 independent experiments).

Significant differences versus control by multiple comparisons.

		GSH				
	Control	*1 mM*	*3 mM*	*10 mM*	*chi-square*	*p*
Aggregation (%)	62	47	49	50	1.98	n.s
TxB$_2$ (ng/mL)	119.6	109.5	77.1*	53.3*	14.8	<0.01
PDGF (ng/mL)	109.5	103	94	66.5*	13.85	<0.01

Table 2. Effect of GSH on platelet aggregation, TxB$_2$ production and PDGF release induced by collagen (5 µg/mL) (median values of 6 independent experiments).

Significant differences versus control by multiple comparisons.

		GSH				
	Control	*1 mM*	*3 mM*	*10 mM*	*chi-square*	*p*
Aggregation (%)	68	57	55*	27*	11.16	0.01
TxB$_2$ (ng/mL)	62.2	50	39*	0*	14.46	<0.01
PDGF (ng/mL)	58	52	37*	13*	15	<0.01

Table 3. Effect of GSH on platelet aggregation, TxB$_2$ production and PDGF release induced by PAF (1.8 x 10^{-7}M) (median values of 6 independent experiments).

Significant differences versus control by multiple comparisons.

tathione, inhibits collagen-induced platelet aggregation through an increase in platelet cAMP levels.[16]

The purpose of the present study was to investigate the effect of GSH on different platelet functions stimulated by the physiological agonists ADP, collagen and PAF. We provide evidence that GSH inhibits platelet aggregation, thromboxane B$_2$ production and PDGF release induced by these agents and that the extent of inhibition is greatest when platelets are stimulated with ADP, intermediate when PAF is employed and lowest if collagen is the inducer.

Materials and Methods

Platelet aggregation studies

Blood was obtained from apparently healthy subjects aged 20-35 years by puncture of the antecubital vein and gently mixed with trisodium citrate (9 volumes of blood and 1 volume of 3.8% trisodium citrate) in plastic tubes. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained as previously described.[17] When necessary, PRP was diluted with PPP to give a final platelet concentration between 2.5 and 3.0×10^8/mL.

Platelet aggregation was investigated using a *Platelet Ionized Calcium Aggregometer* (Chrono-Log, Havertown, PA). PRP (0.5 mL) was pipetted in a siliconized glass cuvette and placed in the appropriate compartment of the aggregometer at 37°C. After 3 min the agonist was added and aggregation was recorded. Platelet aggregation inducers were used at the following final concentrations: ADP (Sigma, St. Louis, MO) 1 µM and 10 µM, collagen (Mascia Brunelli, Milan, Italy) 5 µg/mL and PAF (Sigma) 1.8×10^{-7} M. In other experiments platelets were preincubated for 3 min in the aggregometer cuvette with GSH (Sigma) at final concentrations ranging from 10^{-3}M to 5×10^{-2} M before addition of the agonist. Platelet aggregation was expressed as the percentage change in light transmission.

Table 4. Concentrations of GSH able to produce 50% inhibition (IC$_{50}$) of the platelet activation induced by ADP, collagen or PAF.

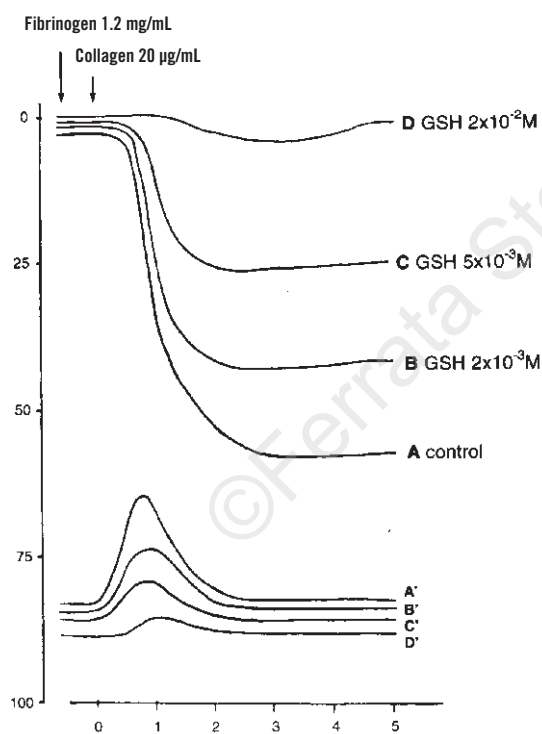| Agonist	Platelet function	IC$_{50}$ (mM)
ADP 1 μM	Aggregation	1.43
	TxB$_2$ production	2.35
	PDGF release	2.36
Collagen 5 μg/mL	Aggregation	16.30
	TxB$_2$ production	8.00
	PDGF release	11.20
PAF 1.8 x 10^{-7} M	Aggregation	8.90
	TxB$_2$ production	2.23
	PDGF release	3.10

Figure 1. Effect of GSH on platelet aggregation and Ca^{++} movement induced by collagen. Washed, aequorin-loaded platelets (1 mL) were placed in the cuvette of the aggregometer and activated with collagen in the presence of trace amounts of fibrinogen (arrows). A: light transmission and A': aequorin related luminescence (uncorrected instrument readings) after addition of collagen alone; B-D: light transmission and B'-D': aequorin related luminescence in the presence of GSH at increasing concentrations.

The effect of GSH on platelet aggregation and Ca^{++} movement was tested in washed, aequorin-loaded platelets. For this purpose platelets were washed and loaded with the photoprotein aequorin (Mascia Brunelli, Milan, Italy) according to a procedure already described in detail[17] and activated with collagen (20 μg/mL) in the presence of trace amounts of fibrinogen (1.2 mg/mL).

Thromboxane B$_2$ and PDGF assays

Thromboxane B$_2$ (TxB$_2$) and PDGF levels were determined by radioimmunoassay using commercially available reagents (Amersham Italia, Milan, Italy). Samples of PRP collected 6 min after addition of the agonists, when aggregation was complete, were centrifuged at 11,000 x g for 2 min in an Eppendorf centrifuge, and the levels of TxB$_2$ and PDGF were determined in the supernatants. From these values we subtracted the levels of TxB$_2$ or PDGF in the supernatants of PRP treated in exactly the same way but after addition of buffer alone without the agonists.

Statistical analysis

Statistical analysis of data was performed through non parametric analysis of variance of repeated measurements (Friedman test); multiple comparisons between control values and those observed with different concentrations of GSH were performed.

Concentrations of GSH able to produce half-maximum inhibition of platelet aggregation, TxB$_2$ production and PDGF release (IC$_{50}$) were calculated by log-probit analysis.

Results

The effect of GSH at different concentrations on platelet activation induced by 1 μM ADP is reported in Table 1. GSH at the lowest dose (1 mM) did not significantly modify aggregation, TxB$_2$ production or PDGF release, while at higher concentrations (3 mM or 10 mM) it significantly inhibited all parameters.

Collagen-induced platelet activation was remarkably less sensitive to GSH (Table 2). In fact, aggregation was not significantly reduced,

while TxB_2 production was reduced by GSH when employed at concentrations of 3 mM or 10 mM, and PDGF release was inhibited only by the highest dose (10 mM).

Table 3 reports the effect of GSH on PAF-induced platelet aggregation, TxB_2 production and PDGF release. All parameters were significantly reduced by GSH when added at concentrations of 3 mM or 10 mM.

Our data suggest that GSH added *in vitro* to platelets inhibits aggregation, arachidonic acid metabolism and release reaction from α-granules. Inhibition is more pronounced if platelets are stimulated with ADP or PAF, while it is less evident when collagen is the inducer.

Concentrations of GSH able to produce a 50% suppression of platelet activation (IC_{50}) are reported in Table 4. These values were between 1.43 and 2.36 mM when platelets were stimulated with ADP, between 2.23 and 8.90 mM when PAF was used and between 8.00 and 16.30 mM when collagen was the agonist. Among the platelet function tests investigated, the highest sensitivity to GSH inhibition was observed for aggregation induced by ADP, and the lowest for aggregation induced by collagen. Furthermore, when platelets were stimulated with collagen or PAF, inhibition by GSH of TxB_2 production was greater than inhibition of PDGF release or aggregation.

The stronger effect of GSH on ADP-induced platelet activation could be due to the low dose of the agonist employed (1 μM). Therefore ADP was used at a higher concentration (10 μM) in additional experiments, and the IC_{50} value for inhibition by GSH of aggregation was found to be 2.83 mM, confirming the higher sensitivity to GSH of platelet activation induced by ADP.

Furthermore, in order to verify whether the observed inhibition of platelet function by GSH was due to a direct antiplatelet effect or to indirect activity through glutathione peroxidase[18] or other mechanisms, experiments were repeated in washed, aequorin-loaded platelets. Results are reported in Figure 1. GSH inhibited collagen-induced platelet aggregation and Ca^{++} movement in a dose-dependent manner, indicating that it possesses an antiplatelet effect which is independent of plasma glutathione peroxidase activity.

Discussion

Our study has shown that the antioxidant agent GSH is an inhibitor of platelet activation induced by the physiological agonists ADP, collagen or PAF. GSH showed this effect when added *in vitro* to platelets at high concentrations, since the IC_{50} was in the millimolar range when platelets were stimulated with ADP and even higher when PAF and in particular collagen were the inducers. This is not surprising, since it is known that GSH levels in platelet cytosol are very high, ranging from 3 to 5 mM.[18] In other words, platelets physiologically contain large amounts of GSH and this agent has to be added at high concentrations to suppress platelet function effectively.

An important question arising at this point is whether externally added GSH may have penetrated inside the platelets. This is very unlikely since GSH is known to pass through cell membranes poorly;[7] furthermore, incubation time was very short (3 minutes) and extracellular concentrations were not much higher than estimated intracellular levels. Therefore the effect of GSH observed is likely to be related to its interference with cell-to-cell messengers or mediators, leading to an inhibition of platelet activation. It has recently been suggested that ROS and in particular hydroxyl radicals act as extracellular *second messengers* during the initial phase of the platelet activation process.[8] GSH, a free-radical scavenger, may have interrupted ROS-related amplification of platelet activation, leading to decreased aggregation, TxB_2 production and PDGF release.

The extent of inhibition of platelet activation by GSH depends on the agonist employed; it is maximal if platelets are stimulated with ADP, intermediate when PAF is used and much lower if collagen is the inducer. PAF and especially collagen are known to be stronger agonists than ADP; accordingly, in the present investigation we found that ADP essentially stimulated aggregation, with a slight effect on TxB_2 production and PDGF release (Table 1), while PAF and collagen were much stronger inducers (Tables 2 and 3). The different extent of the inhibiting effect of GSH therefore seems to be related to the strength of the agonists.

It is interesting that when the strong inducers collagen or PAF were used inhibition of TxB_2 production was greater than inhibition of aggregation or PDGF release. This suggests that a decrease in the levels of free radicals generated during platelet activation affects mainly arachidonic acid metabolism, and to a lesser extent aggregation and release reaction from α-granules. With a different approach our data confirm the results of previous investigations, which showed that TxB_2 generation plays an essential role in platelet activation induced by hydroxyl radicals,[5,8] and that generation of oxidized glutathione during platelet activation correlates with TxB_2 production rather than with aggregation.[19]

Although the observed inhibition of platelet activation is most probably related, as discussed above, to an extracellular effect of added GSH, the GSH already physiologically present in platelet cytosol may well exert similar effects and may therefore represent a mechanism of control of arachidonic acid metabolism and platelet activation. Evidence of the involvement of GSH and its metabolism in platelet function is already available. It is known that GSH through the formation of the intermediate compound S-nitroso-glutathione may favor the release and the availability of nitric oxide, a powerful antiplatelet agent.[20,21] Furthermore, in human platelets the selenoenzyme glutathione peroxidase, which converts GSH to the oxidized form GSSG, acts as a powerful scavenger of the peroxides generated during the burst of arachidonic acid metabolism,[22] and the intraplatelet levels of this enzyme were found to be decreased in elderly subjects[23] and in patients with coronary heart disease, and increased platelet aggregability.[24] Our data demonstrate that, apart from these indirect effects on platelet function, GSH may directly inhibit platelet activation.

It is always difficult to extrapolate from results obtained *in vitro* to the *in vivo* situation, but it is possible to speculate that increased platelet function during oxidative stress could be related, at least in part, to GSH depletion and that recostitution of high intracellular GSH levels through GSH administration could be beneficial not only in restoring the imbalance between ROS and anti-oxidant defenses but also in reducing increased platelet reactivity. Further studies specifically addressing these points are obviously needed to verify this hypothesis, which may have important clinical implications.[25,26]

In conclusion, GSH added *in vitro* to platelets at concentrations of the same order of magnitude as those physiologically present in platelet cytosol inhibits platelet acivation induced by ADP, and to a lesser extent platelet activation induced by PAF or collagen; when these last two agonists are employed, inhibition of TxB_2 production is greater than inhibition of aggregation or PDGF release. Therefore GSH may act as a physiological inhibitor of platelet activation and may contribute to the regulation of platelet reactivity.

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