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Cell surface thiol isomerases may explain the platelet-selective action of S-nitrosoglutathione

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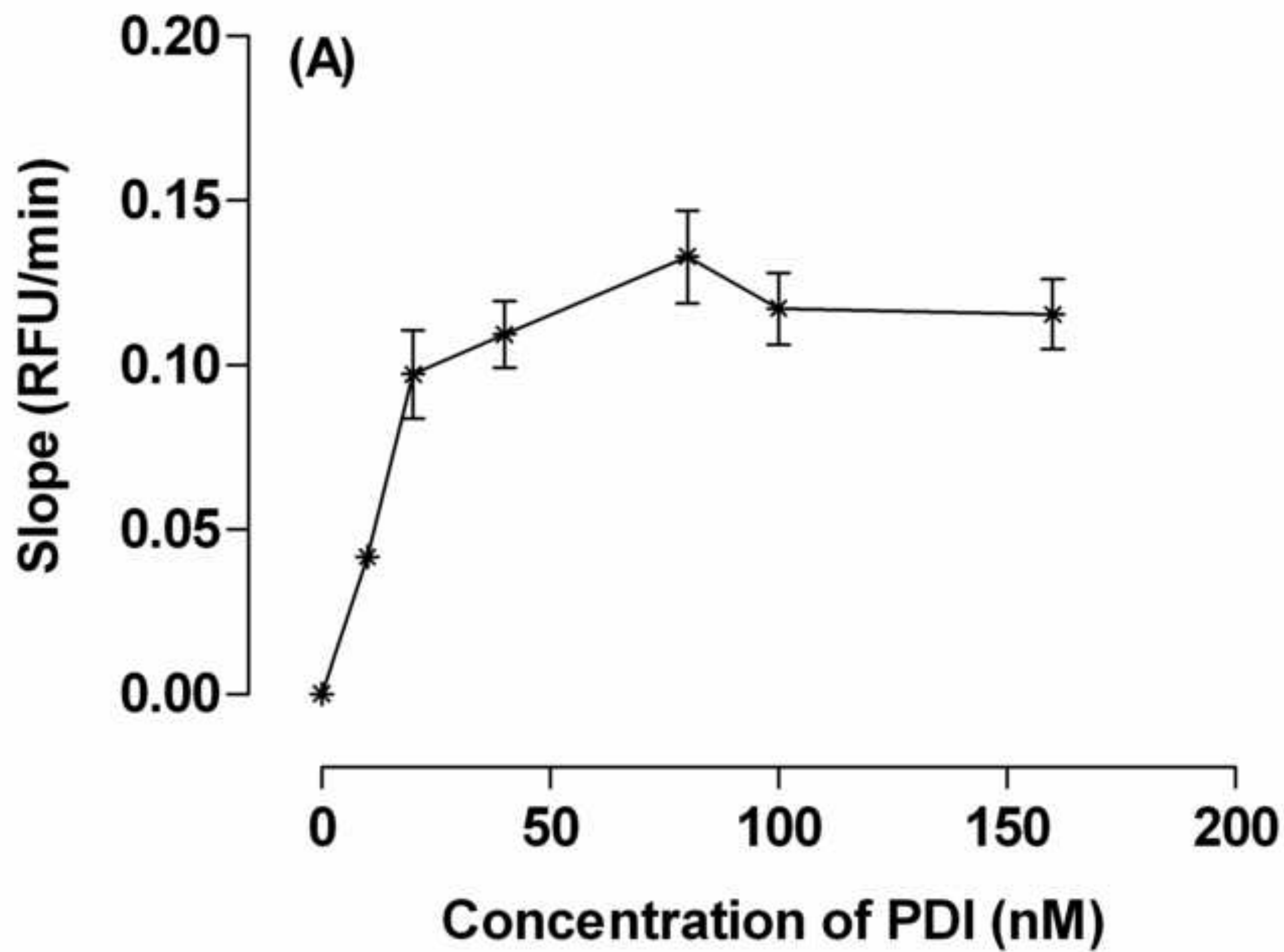

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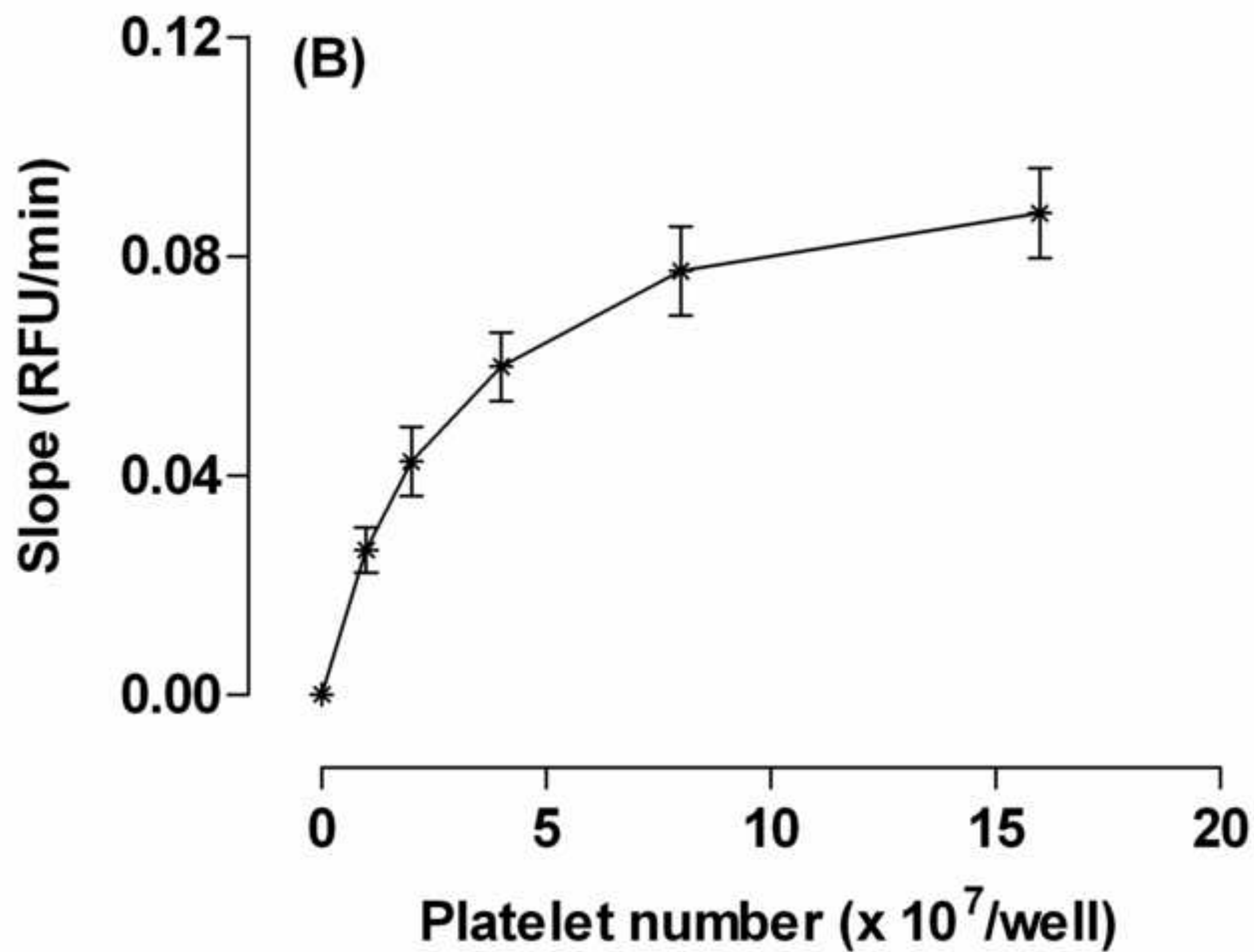

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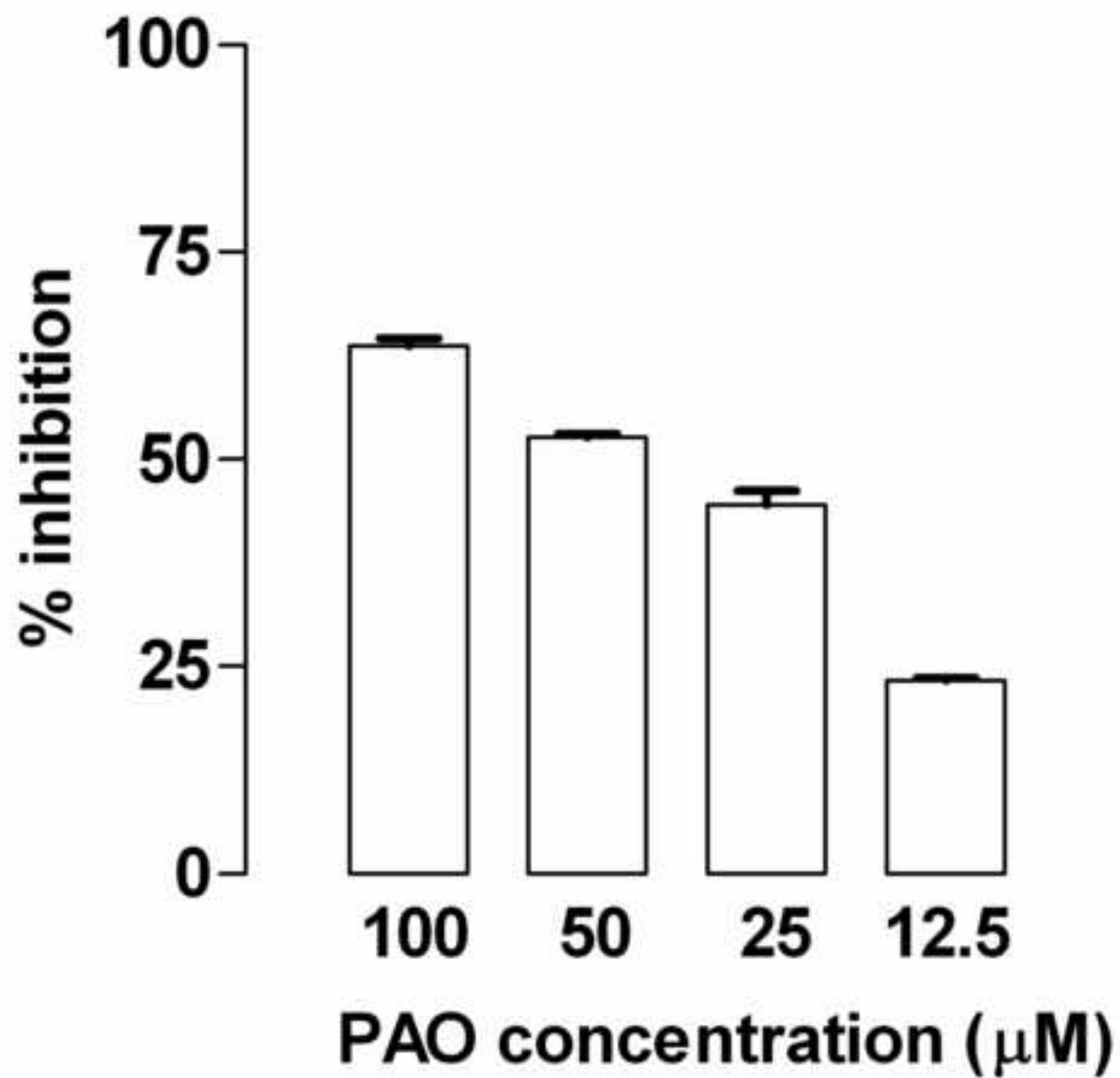

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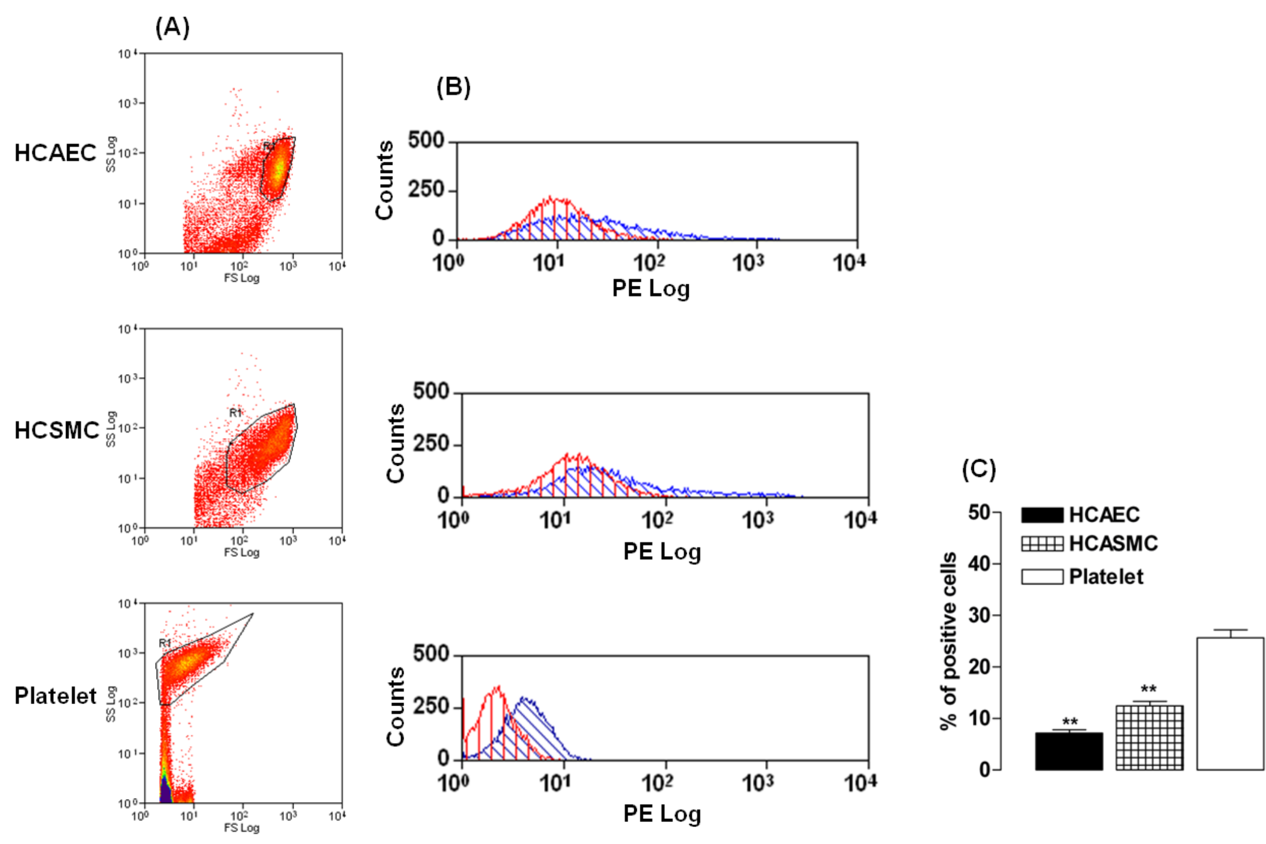

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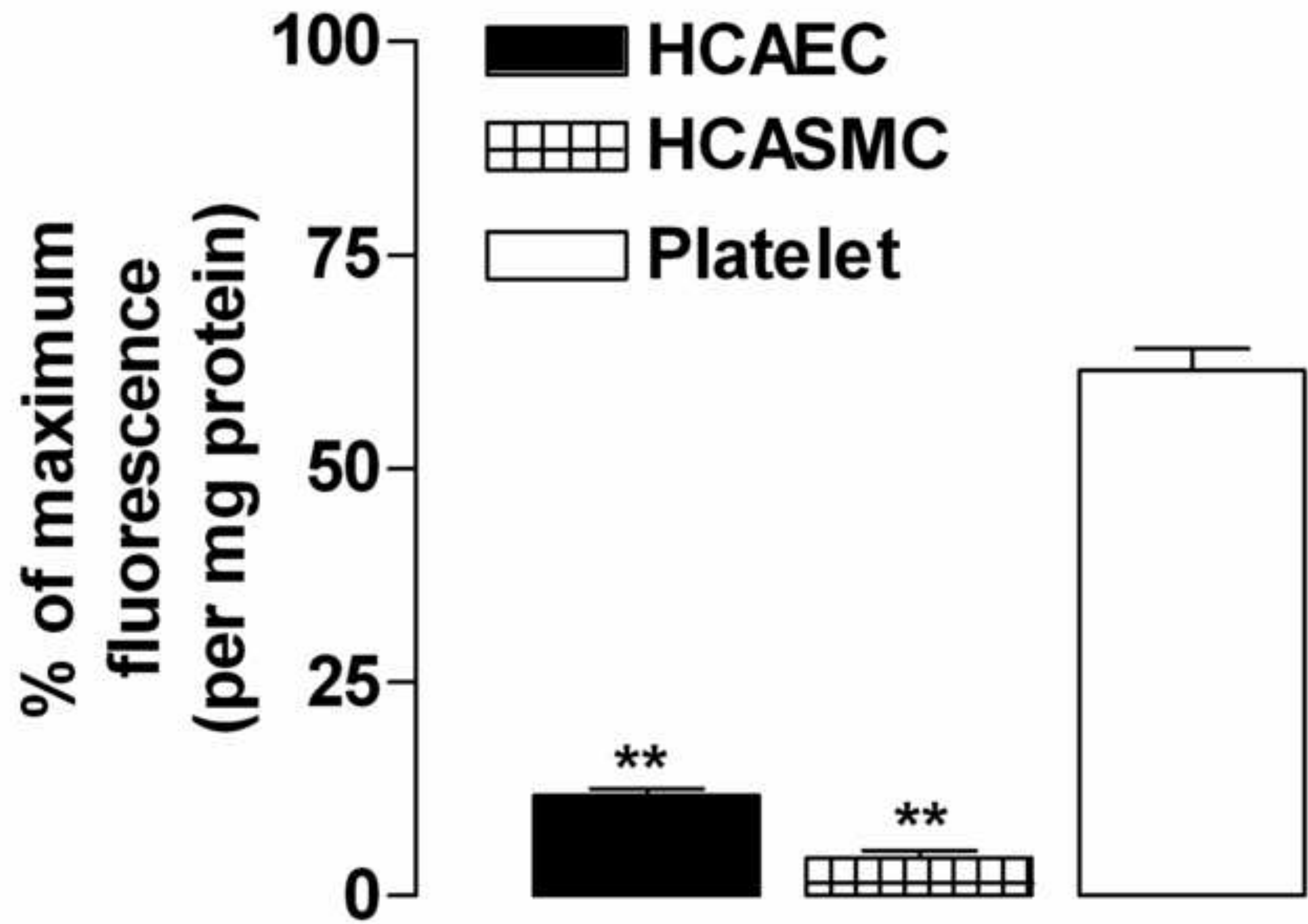

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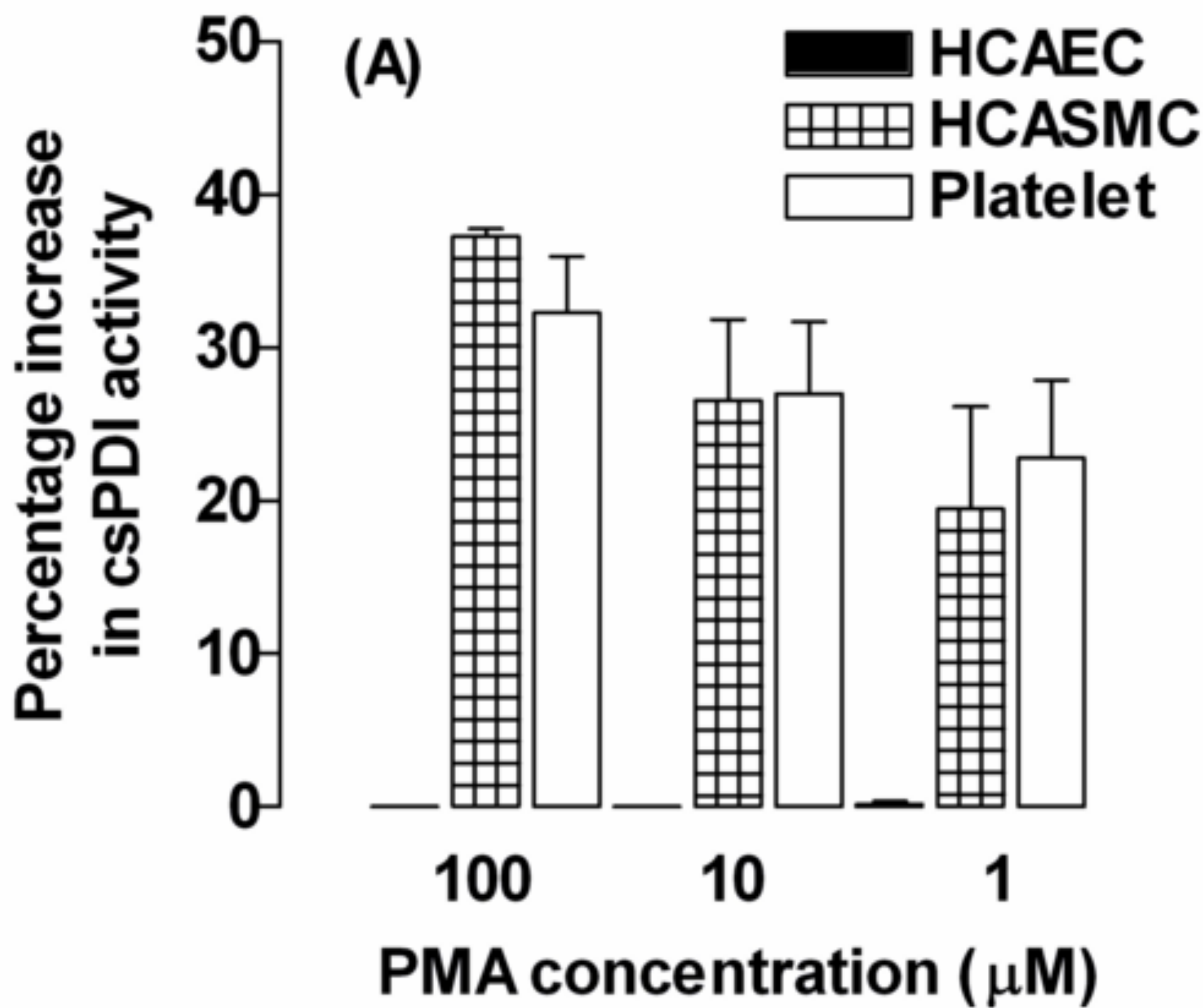

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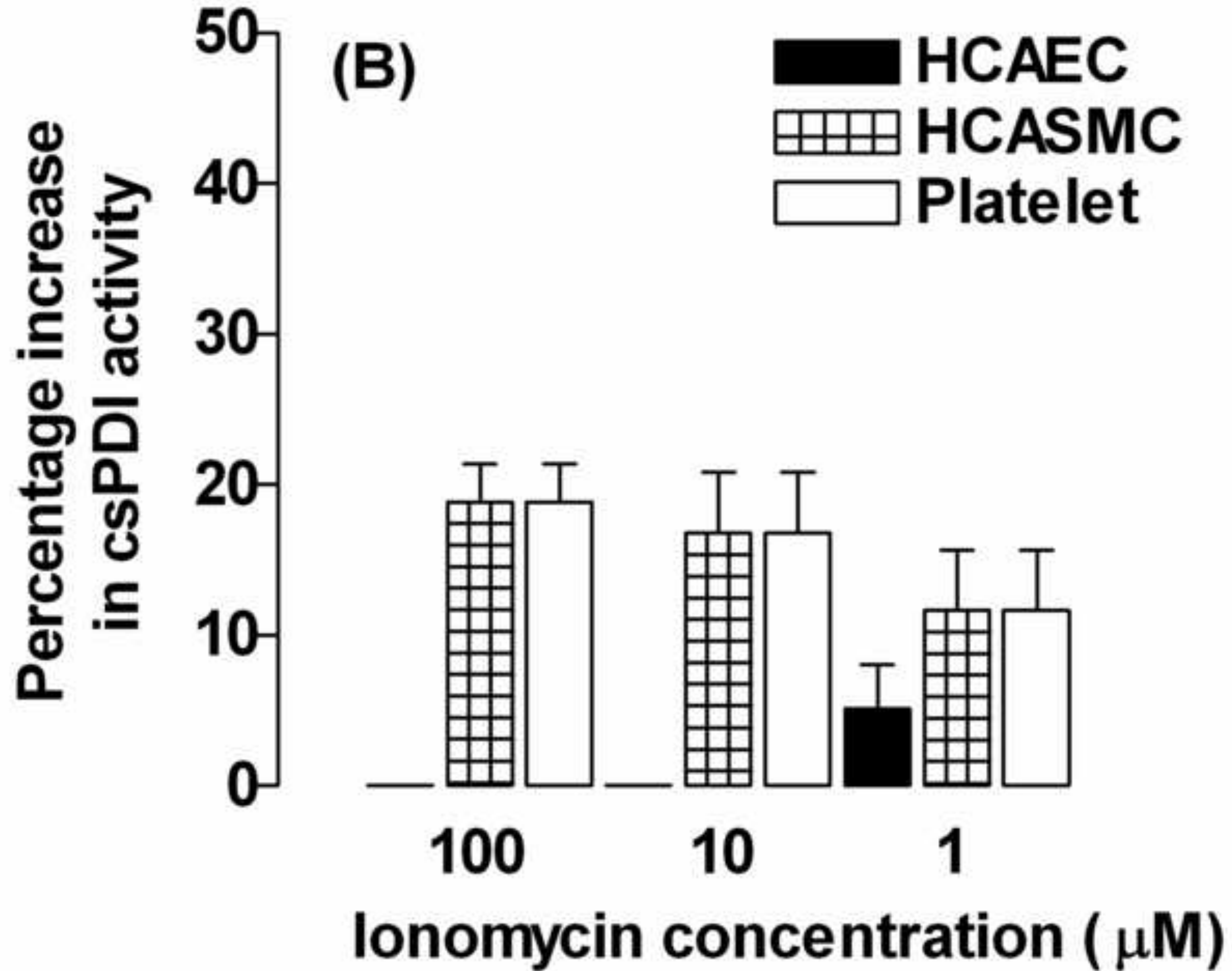

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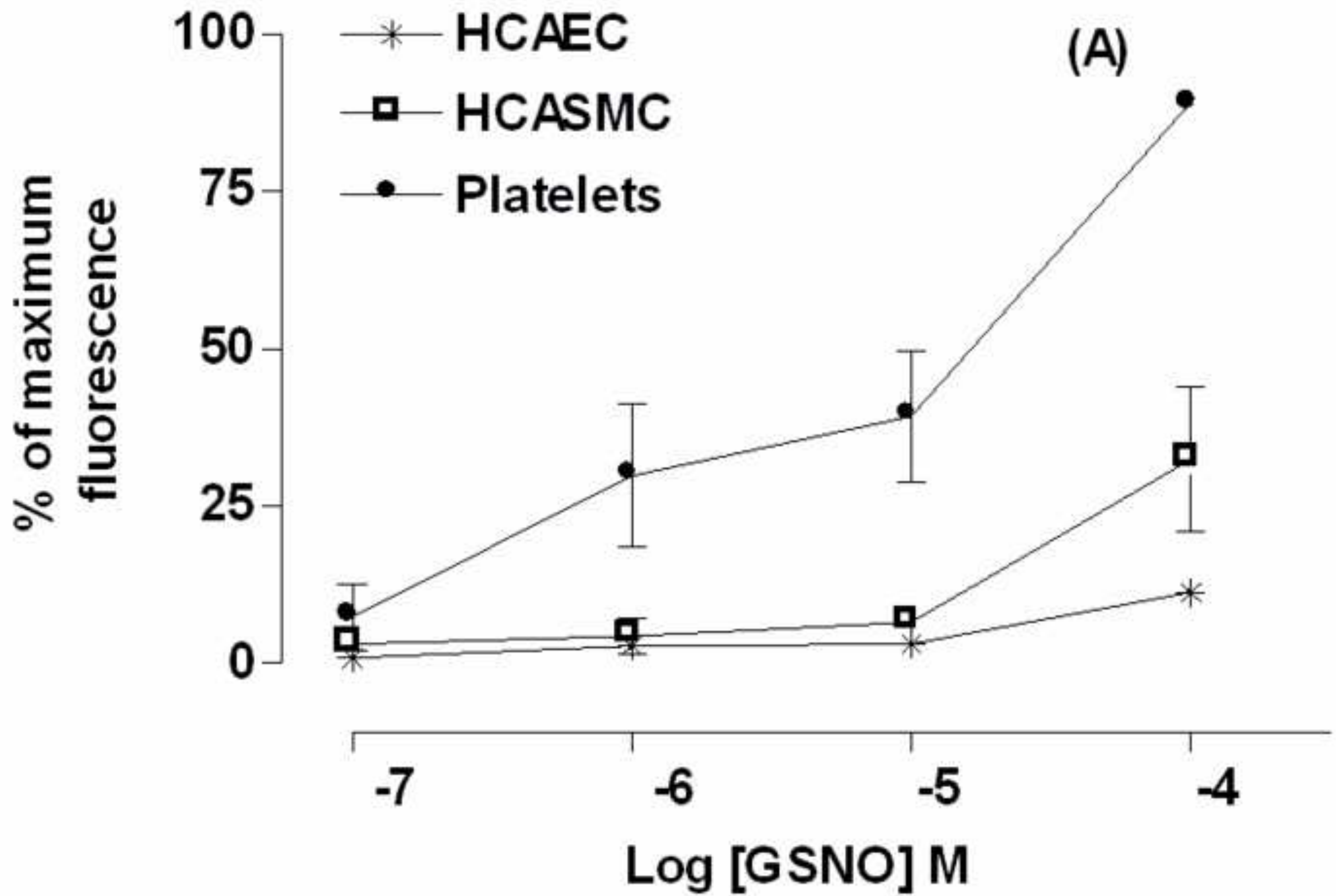

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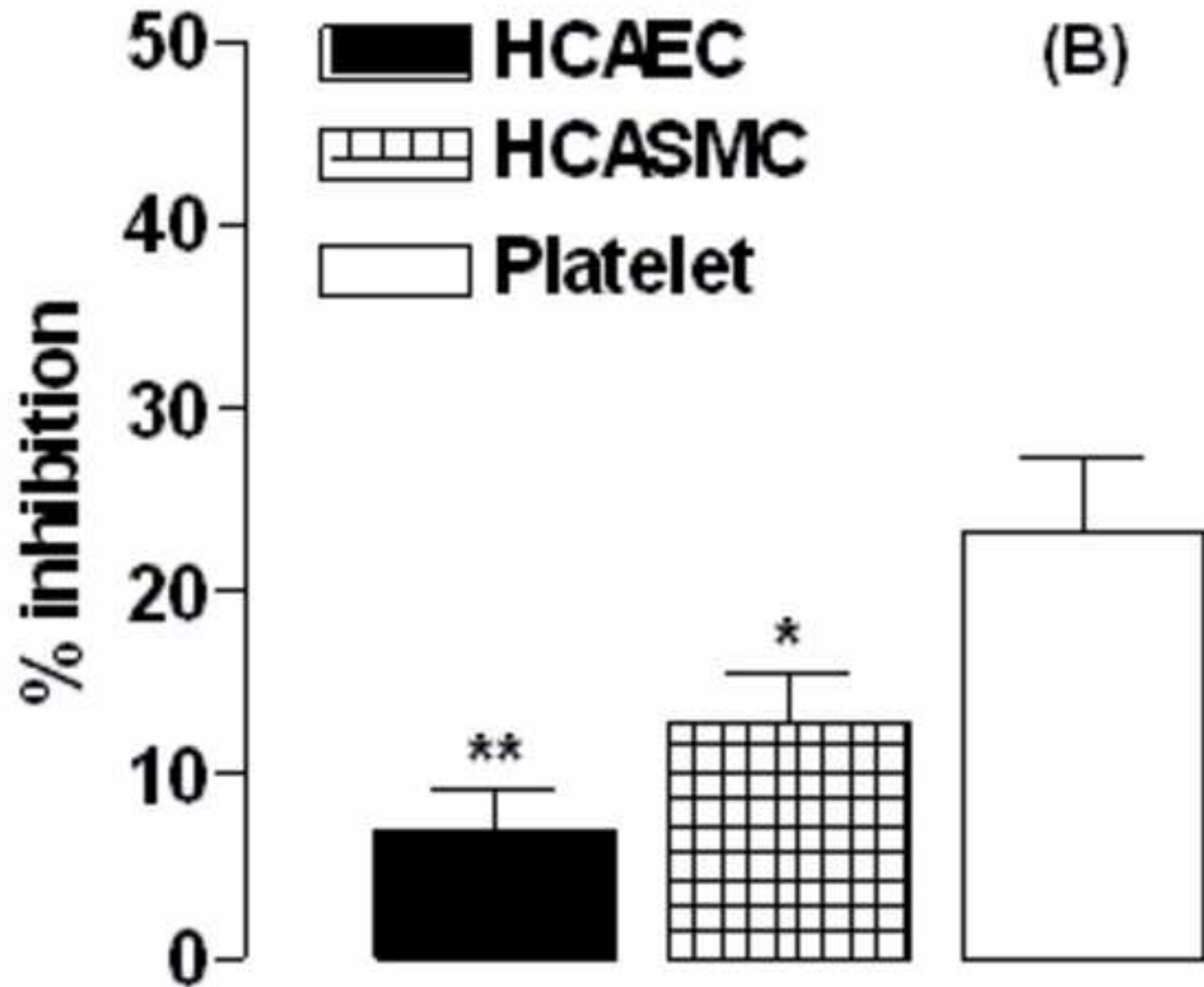

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CELL SURFACE THIOL ISOMERASES MAY EXPLAIN THE PLATELET-SELECTIVE ACTION OF S-NITROSOGLUTATHIONE

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Abstract

S-nitrosoglutathione (GSNO) at low concentration inhibits platelet aggregation without causing vasodilation, suggesting platelet-selective nitric oxide delivery. The mechanism of this selectivity is unknown, but may involve cell surface thiol isomerases, in particular protein disulphide isomerase (csPDI) (EC 5.3.4.1). We have now compared expression and activity of csPDI on platelets, endothelial cells and vascular smooth muscle cells, and the dependence on thiol isomerase activity of these cell types for NO uptake from GSNO.

csPDI expression was measured by flow cytometry and its reductase activity using the pseudosubstrate dieosin glutathione disulphide. This activity assay was adapted and validated for 96-well plate format. Flow cytometry revealed csPDI on all three cell types, but percentage positivity of expression was higher on platelets than on vascular cells. Consistent with this, thiol isomerase-related reductase activity was higher on platelets ($p < 0.01$), and cellular activation (with either phorbol myristate acetate or ionomycin) increased csPDI activity on both platelets and smooth muscle cells, but not on endothelium. Intracellular NO delivery from GSNO was greater in platelets than in vascular cells ($P < 0.002$), and was more sensitive to thiol isomerase inhibition using phenylarsine oxide ($P < 0.05$).

Increased surface thiol isomerase activity on platelets, compared with cells of the vascular wall, may explain the platelet-selective actions of GSNO and help define its antithrombotic potential.

Short Title: Thiol isomerase-mediated NO delivery to platelets and vascular cells

Keywords: Platelets; endothelial cells; smooth muscle cells; S-nitrosoglutathione (GSNO); thiol isomerases; protein disulphide isomerase (PDI)

1. Introduction

Thiol isomerases comprise the thioredoxin superfamily of oxidoreductases, characterised by the presence of one or more thioredoxin-like domains containing the CXXC motif [1].

Over the last decade thiol isomerases located at the cell surface, most prominently protein disulphide isomerase (EC 5.3.4.1), have emerged as regulators of platelet function [2-6], and of tissue factor activity [7-9]. Cell surface protein disulphide isomerase (csPDI) influences both fibrin generation and platelet thrombus formation in mouse models (8), confirming that csPDI is relevant to *in vivo* haemostatic regulation.

A further cellular action of csPDI relevant to haemostasis and vascular regulation is to facilitate delivery across the plasma membrane of nitric oxide (NO) signalling [10,11]. Our own previous studies have shown that csPDI is required for the delivery into platelets of a range of NO redox derivatives [12] and that during this process csPDI undergoes thiol modification [13]. NO supplementation by means of donor drugs is a potentially useful antithrombotic strategy in patients suffering from cardiovascular disease due to endothelial dysfunction [14]**,** and the anti-thrombotic action of S-nitrosothiols (RSNOs) has attracted particular interest because certain of these compounds show evidence of platelet-selective action [15]. For example, administration of low doses of S-nitrosoglutathione (GSNO) to human patients produced platelet inhibition without significant vasodilation [16]. This suggests preferential NO signalling into platelets, perhaps via differences in GSNO metabolism between platelets and cells of the blood vessel wall. Because of the role of csPDI in NO delivery, it is important to establish whether there are differences in the expression or activity of this enzyme between platelets and vascular cells.

PDI displays both oxidase and reductase, as well as thiol isomerase activity [1], however since the mechanism of its S-denitrosation of GSNO involves electron donation by the active site thiols [17], measurement of its reductase activity is most relevant in this context. A novel fluorescent assay sufficiently sensitive to measure csPDI reductase activity has recently been published [18] and we have therefore used this technique, adapted in 96 well plate format, to compare csPDI on platelets, endothelial cells and vascular smooth muscle cells. Our aim was to identify differences in csPDI expression between the cell types and to determine whether preferential NO delivery into platelets might be explained on this basis.

2. Materials and Methods

2.1 Chemicals

DAF-FM (4-amino-5-methylamino-2'7' difluorofluorescein) diacetate was obtained from Molecular Probes (Paisley, UK). GSNO (S-Nitroso-L-glutathione) was obtained from Alexis Biochemicals (Exeter, UK). Mouse anti-PDI antibody (1D3) conjugated R-Rhycoerythrin (PE) and PE-labeled isotype-matched control antibody (mouse IgG1) were obtained from Assay Designs (Exeter, UK). Live/Dead Fixable Dead Cell Stain Kits was obtained from Invitrogen (Paisley, UK). BCA protein assay kit was obtained from Thermo Scientific (Northumberland, UK). PD-10 Desalting columns were obtained from GE Healthcare (Buckinghamshire, UK). Dulbecco's Phosphate Buffered Saline (D-PBS and Fetal bovine serum (FBS) were obtained from Lonza Workingham Ltd (Slough, UK). All other chemicals were purchased from Sigma (Poole, UK).

2.2 Preparation of dieosin glutathione disulphide (di-E-GSSG)

The synthetic pseudosubstrate dieosin glutathione disulphide (Di-E-GSSG) was generated by incubation of GSSG (100 μM) with a 10-fold molar excess of eosin isothiocyanate (1 mM) in phosphate buffer (100 mM potassium phosphate and 2mM EDTA, pH 8.8) overnight at room temperature as previously described [18] with minor modification. One hundred microliter aliquots were taken and passed down a PD-10 desalting column (Sephadex G-25) using 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM EDTA, and one millilitre aliquots were collected. The eluted fractions were tested for fluorescence increase before and after the addition of DTT (10 mM) by monitoring at 545 nm with excitation at 525 nm. All fractions showing at least 10-fold increase in fluorescence were pooled and stored at -20°C.

2.3 Preparation of washed platelets

Washed platelets were prepared as previously described [12]. Briefly, whole blood (20 ml) was obtained with informed consent, according to the Declaration of Helsinki, from healthy volunteers and drawn into 25 ml container containing 3 ml of acid citrate dextrose. Platelet-rich plasma (PRP) was separated from other cellular components of blood by centrifugation twice at 170 g for 10 min, keeping the upper layer each time. PRP was acidified with 0.5 M citric acid to pH 6.2-6.5, and prostaglandin E_1 (1.5 μM) and apyrase (2 units/ml) were added to prevent aggregation during subsequent centrifugation at 1000 g for 12 min. The platelet pellet was then resuspended in 1 ml HEPES-buffered saline (HBS) containing 140 mM NaCl, 2.7 mM KCl, 5mM Glucose and 10 mM HEPES (pH 7.3) and loaded on to a Sepharose 2B column (1.45 x 5.0 cm). The platelet fraction was eluted with HBS and the platelet count was adjusted to 10^8/ml before use.

2.4 Cell culture

Human coronary artery endothelial cells (HCAEC), human coronary artery smooth muscle cells (HCASMC), and their culture mediums were purchased from PromoCell (Heidelberg, Germany), and cultured according to manufacturer's instructions. Cells were passaged using a solution of 0.125% trypsin and 0.02% EDTA in Dulbecco's PBS (D-PBS). Experiments were performed after 3-5 passages, and the cell count adjusted to 10^5 / ml before use.

2.5 Assay of PDI reductase activity

Reductase activity of cell surface thiol isomerases was monitored in a 96-well microplate fluorescence assay. Authentic PDI (10-100 nM) was incubated with Di-E-GSSG (150 nM) in HBS containing 5 μM DTT (PDI assay buffer). The increase in fluorescence was monitored at excitation and emission wavelengths (Ex 510nm / Em 550nm) which were optimised for the microplate reader (Gemini EM, Molecular Devices, Wokingham UK). The same protocol was used to monitor reductase activity of csPDI on variable numbers of platelets (1 – 16 x 10^7 per well). In further experiments, platelets (2 x 10^7/well) were incubated with the thiol isomerase inhibitor phenylarsine oxide (PAO) (12.5 - 100 μM) at 37^0C for 30 min before addition of Di-E-GSSG (150 nM). To compare the reductase activity of csPDI in the different cells, cellular total protein was measured in parallel using BCA kit according to the manufacturer's protocol, and results normalized to the amount of cell protein. To determine the effect of cell activation, the three cell types were incubated with phorbol myristate acetate or ionomycin at concentrations of 1-100 μM, and cell surface thiol isomerase activity was then monitored over 45 min, as described above.

2.6 Flow cytometry analysis

Cultured cells were collected by trypsinization and washed twice in cold D-PBS by centrifugation at 150 g for 6 min. The density of washed cells and isolated platelets was then adjusted to 2 x 10^7/ml with D-PBS. 100 µl of the adjusted cells were incubated with 1 µl of near-IR fluorescent reactive dye provided with Live/Dead Fixable Dead Cell Stain Kits for 30 min to distinguish the live and dead cells. The dye-loaded HCAEC and HCASMC were then washed twice with D-PBS by centrifugation at 150 g for 6 min and the dye-loaded platelets were finally washed in D-PBS containing 5 mM EDTA by centrifugation at 1000 g for 6 min. Cells were then resuspended in D-PBS containing 2% FBS and incubated with PE-PDI antibody (1:50 dilution) on ice for 30 min. PE-labeled isotype-matched control antibody (mouse IgG1) was used in parallel to set the fluorescence threshold for primary antibody. The expression of csPDI on the cells was analyzed in a CyAn ADP Analyzer (DakoCytomation, Denmark). Forward (FSC) and side scatter (SSC) were set at logarithmic gain and triggering was set at FSC. Live cells were gated and analyzed.

In separate experiments, platelets were subjected to the trypsinisation protocol described above for detachment of vascular cells, and the effect of this on csPDI expression was assessed to see whether expression might be affected by differences in cell preparation procedure.

2.7 Measurement of the entry of NO_x (reactive nitrogen species) into cells

DAF-FM diacetate, a membrane-permeant fluorescent probe, was used for detecting intracellular NO_x as previously described [12]. Cells were suspended in HBS and then

incubated with DAF-FM diacetate (5 μM) at 37^0C for 30 min. The DAF-FM-loaded platelets were then separated from free probe on a Sepharose 2B column. Excess DAF-FM was separated from HCAEC and HCASMC by centrifugation. S-Nitroso-L-glutathione (GSNO) at concentrations of 10^{-7} – 10^{-3} M was then added to the cells (10^5 /mL) in PDI assay buffer. Fluorescence intensity at 515 nm with excitation at 480 nm was measured over 30 min using the microplate reader. To take account of higher fluorescence in the larger cells, data for each cell type were expressed as a percentage of the maximum value, obtained using 1 mM GSNO. In experiments to determine the role of PDI in NO delivery, cells were pre-incubated with 100 μM phenylarsine oxide (PAO), a thiol isomerase inhibitor, at 37^0C for 30 min before addition of 1 mM GSNO.

2.8 Statistical analysis

All data are expressed as mean ± SEM. One-way ANOVA was used for difference between data groups and dose response curves were compared using two-way ANOVA followed with Bonferroni post-hoc correction for multiple comparisons. Differences were considered significant when $p < 0.05$.

3. Results

3.1 Reductase activity of authentic PDI and platelet cell surface thiol isomerases

The fluorescent PDI reductase assay employing Di-E-GSSG, first described by Raturi and Mutus [18] was successfully adapted for use in 96 well microplate format, as shown by reproducible and dose dependent activity of authentic PDI (Fig 1A) and of cell surface thiol

isomerase on platelets (Fig 1B), which was inhibited in a dose dependent manner by the thiol isomerase inhibitor PAO (Fig 2).

3.2 Comparison of csPDI expression and thiol isomerase activity on platelets, endothelial cells and vascular smooth muscle cells

csPDI was detected by flow cytometry on platelets, HCAEC and HCASMC (Fig 3A). Both endothelial and smooth muscle cells showed more variable expression than platelets (Fig 3B) and when compared in terms of percentage positive cells, the level of the expression on platelets was significantly higher than on HCAEC and HCASMC (Fig 3C, $P < 0.01$). Platelet csPDI expression was unchanged by exposure to the trypsin reagent used to detach vascular cells from culture flasks (data not shown), indicating that our results were not due to differences in cell preparation prior to flow cytometric analysis. Consistent with this, csPDI reductase activity was significantly greater on platelets than on HCAEC and HCASMC ($P < 0.01$), when results were normalized for cell protein, with platelets showing approximately 6-fold and 15- fold higher activity than HCAEC and HCASMC, respectively (Fig 4). To take into account the effect of differences in surface area between the cell types, we measured the number of cells required to provide an equal amount (1 μg) of protein as 68000, 380 and 540 for platelets, HCAEC and HCASMC, respectively. Estimates of platelet surface area range from 14 μm^2 [19] to 22 μm^2 [20]. The diameter of trypsinised HCAEC and HCASMC ranges from 10 – 25 μm (personal communication, Promocell, Heidelberg), so taking a mean value of 17.5 μm, and assuming a spherical shape, for these cells, their average surface area can be estimated as 962 μm^2. Taking this value, and a mean platelet value of 18 μm^2, it can be calculated that the excess of platelet surface area in our experiments was 3.3-fold compared

with HCAEC and 2.4-fold compared with HCASMC. Thus it appears that discrepancy in cell surface area does not fully account for the higher csPDI activity seen in platelets.

In experiments to determine the effect of cell activation on csPDI activity, stimulation with PMA and ionomycin significantly increased the reductase activity on platelets and HCASMC ($p < 0.01$), but no changes were detected on HCAEC (Fig 5A, 5B).

3.3 GSNO mediated NO_x entry into platelets, HCAEC and HCASMC

Compared with both HCAEC and HCASMC, intracellular NO_x delivery from GSNO was greater in platelets ($P < 0.001$) (Fig 6A). Because of differences in absolute DAF-FM fluorescence values between the different cell types, NO_x delivery was normalized to the percentage of maximal signal obtained following exposure to a GSNO concentration of 1mM. The results showed a greater proportional increase in platelets than in the other cell types, indicating that platelets are better able than HCAEC and HCASMC to receive NO_x at low concentrations of GSNO**.** When csPDI was blocked using PAO, the percentage inhibition of DAF-FM fluorescence following GSNO exposure was higher for platelets than for endothelial cells or smooth muscle cells ($P < 0.05$) (Fig 6B), indicating that platelets rely more heavily on csPDI for NO_x delivery.

4. Discussion

We have established techniques to measure the expression and reductase activity of surface thiol isomerases on different cell types. PDI is a prominent member of this surface population, however the Di-E GSSG assay and the PAO inhibitor used in our experiments are non-specific, so the functional activity detected may derive from other isomerases in addition

to PDI. Flow cytometric determination of csPDI showed the highest percentage positivity on platelets, whereas both vascular smooth muscle and endothelial cells displayed a wider spread of expression, with some cells showing high activity but the majority being negative. Functionally active thiol isomerase was higher on platelets than on either of the two vascular cell types, and this heightened activity on platelets was not fully accounted for by increased availability of cell surface area. In addition, platelets showed an increase in thiol isomerase-dependent NO delivery.

The presence of csPDI has been documented on a variety of cell types [21] however the processes governing localisation of PDI to the cell surface are not fully determined. In hepatocytes PDI secretion is mediated by microtubular activity and attachment to the plasma membrane appears to involve electrostatic interactions. Secreted PDI continues to display the KDEL endoplasmic reticulum (ER) retention signal and it is speculated that secretion may arise via leakage from a salvage pathway whose function is to recycle proteins to the ER [22].

Several groups have shown that PDI is present inside endothelial cells and that it is up-regulated under conditions of hypoxia [22-25], during which it becomes a target for S-nitrosylation [26]. It is not yet clear whether PDI becomes upregulated on the cell surface in hypoxia. Endothelial cells in culture also express PDI on their exofacial surface in a punctuate distribution, possibly reflecting a concentration at cellular projections [9,27]. This csPDI regulates the adhesive properties of both thrombospondin [27] and of its binding partner integrin $\alpha_v\beta_3$ [28], and in addition catalyses transnitrosation of endothelial target molecules [29]. In contrast, experiments performed using mouse models of thrombus formation indicate that, *in vivo*, csPDI is not expressed on the endothelial surface of the unperturbed vessel wall, but that it accumulates rapidly following vascular injury [30,31].

The observed kinetics of csPDI accumulation under such circumstances suggest that it derives from both disrupted vascular cells and also from platelets adhering at the site of injury. csPDI has been detected on endothelial cells by flow cytometry [9] and in one study [28] the percentage of endothelial cells showing positivity for csPDI was much higher than our observed results. This discrepancy may arise from our use of primary cells in contrast to the EA.hy926 cell line (derived from fusion of endothelial and lung carcinoma cells) used in the earlier study.

PDI is present inside vascular smooth muscle cells and shows a shift to the membrane fraction (detectable by both western blotting and increased refolding activity) following cell stimulation with angiotensin II. Under these conditions membrane-associated PDI appears to co-localise with and regulate the activity of NADPH oxidase [32]. In similar fashion, activation of platelets is associated with increased secretion of both PDI [33] and a range of other thiol isomerases which go on to modulate various aspects of platelet function [34,35]. It should be noted, however, that csPDI is also present on the surface of resting platelets [33,36], so activation is not required for csPDI to be displayed. We found that cell activation with PMA and ionomycin brought about an increase in thiol isomerase activity on both platelets and smooth muscle cells, however this effect was not apparent in endothelial cells. PMA and ionomycin were deliberately chosen in order to stimulate generic intracellular signaling pathways (involving protein kinase C and intracellular calcium, respectively), so the variation in response between the cell types was not due to differential expression of particular agonist receptors. Interestingly, in hepatocytes the extracellular secretion of PDI was actually reduced by cellular stress in the form of tunicamycin treatment or heat shock [22], so it is clear that the effects on surface thiol isomerases of cell stress / stimulation are not uniform across cell types.

Our measurements of cell surface reductase activity were made using a 96 well plate adaptation of the original method described by Raturi and Mutus [18] which allowed the use of small sample volumes. Our results clearly showed that platelets possess a more active surface, in this respect, than endothelial and smooth muscle cells. In parallel, the delivery of NO_x into platelets was greater than into the other cell types, and the degree of thiol isomerase-dependence was greatest for platelets, as reflected in the loss of NO delivery brought about by the thiol isomerase inhibitor PAO. In these experiments, DAF-FM was used to detect the arrival of NO-related signalling inside cells. This fluorescent probe measures a range of NO / oxygen reaction products, therefore it is not possible to specify the identity of the NO derivative which is delivered. The NO donor used for these experiments was GSNO, so these results are relevant to the previously described clinical data indicating platelet-selective action of this agent [16].

Research into the mechanisms by which cells take up NO from impermeable donor compounds such as GSNO has highlighted two major pathways: NO release in the proximity of the plasma membrane by denitrosating enzymes (such as csPDI) (10-12), and transnitrosation to cysteine followed by import of intact S-nitrosocysteine via the L-AT family of amino aid transporters [37]. We have previously argued that the former mechanism is more important in platelets [15] and the results of the present study are consistent with this hypothesis. We suggest that the platelet selective actions of GSNO result from platelet csPDI metabolising GSNO more rapidly than the endothelial lining of the blood vessel wall, allowing anti-platelet action of NO to occur without significant NO delivery to vascular smooth muscle to bring about vasodilation. The emerging evidence of PDI involvement in various components of haemostasis / thrombosis suggests that differential csPDI availability

may have wider relevance for vascular biology which goes beyond nitric oxide delivery alone.

Acknowledgements

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Figure Captions

Figure 1. 96-well microplate fluorescence assay for PDI reductase activity. Di-E-GSSG (150nM) was incubated with serially diluted authentic PDI (0 to 160 nM) (**A**) and washed human platelets (0 to 16 x 10^7 per well) (**B**). Fluorescence was monitored over 120 min with excitation at 510 nm and emission at 550 nm, and the initial rate of change (relative fluorescence unit per min) was calculated. Samples were assayed in triplicate and data represent mean ± SEM from 3 separate experiments.

Figure 2. Inhibition of platelet csPDI reductase activity by PAO. Washed platelets were pre-incubated with PAO (12.5 to 100 μM) at 37^0C for 30 min before addition of Di-E-GSSG (150nM) and measurement of rate of change of fluorescence (Ex 510nm / Em 550nm). Values are expressed as percentage inhibition vs control, and data represent mean ± SEM from 3 separate experiments, each performed in triplicate.

Figure 3. Expression of csPDI on HCAEC, HCASMC and human platelets. (**A**) Washed platelets and vascular cells were characterized by forward (FSC) and side scatter (SSC). The gated cells (R1) were analyzed. (**B**) Surface staining of the cells with PE-labelled isotype-matched control antibody (IgG1) (red) and anti-PDI antibody (blue). (**C**) The anti-PDI antibody positive cells were quantified. Percentage positivity in HCAEC and HCASMC was significantly lower compared with platelets (** $P < 0.01$). Data represent means ± SEM from 3 separate experiments. Values are expressed as a percentage of positivity in 10,000 cells.

Figure 4. Comparison of csPDI reductase activity on HCAEC, HCASMC and human platelets. Vascular cells (10^5/ml) and platelets (10^8/ml) were incubated with Di-E-GSSG (150nM) and csPDI activity was measured by monitoring change in fluorescence (Ex 510 nm

/ Em 550 nm) for 30 min. Values are corrected for amount of cell protein to take account of differences in cell size and number. As an internal control, maximum available fluorescence was measured in parallel by incubating Di-E-GSSG with10 mM DTT, and results were then expressed as a percentage of this maximum value. Data represent mean ± SEM from 3 separate experiments, each performed in triplicate. ** P < 0.01.

Figure 5. Effects of cellular activation on csPDI reductase activity in HCAEC, HCASMC and human platelets. Following addition of PMA (**A**) and ionomycin (**B**) (both 1-100 μM), vascular cells (10^5/ml) and platelets (10^8/ml) were incubated with Di-E-GSSG (150nM) for 30 min to measure change of fluorescence (Ex 510 nm / Em 550 nm). Results are expressed as percentage increase compared with untreated control, and data represent mean ± SEM from 3 separate experiments, each performed in triplicate. Both PMA and ionomycin increased csPDI activity in HCASMC and platelets however no increase was observed in HCAEC. This difference was significant ($p < 0.01$) when analyzed by Two-way ANOVA followed by Bonferroni post-hoc correction for multiple comparisons.

Figure 6. Intracellular NO delivery from GSNO. (A) DAF-FM diacetate - loaded vascular cells (10^5/ml) and platelets (10^8/ml) were incubated with GSNO (10^{-7} – 10^{-4} M) at 37^0C for 15 min, and entry of NO-related signalling was measured by increase in fluorescence intensity (Ex 495 nm / Em 550 nm). To take account of differences in absolute fluorescence values between the different cell types, parallel measurements of maximum fluorescence (defined as response to 10^{-3} M GSNO) were made and responses reported as percentage of this maximum value. Intracellular NO delivery was greater in platelets, compared with both HCAEC and HCASMC ($P < 0.001$), whereas there was no significant different between HCAEC and HCASMC. Data were analyzed by Two-way ANOVA followed by Bonferroni

post-hoc correction for multiple comparisons. (**B**) To determine the PDI dependence of NO delivery in each cell type, fluorescence changes in response to 1 mM GSNO were measured following pre-incubation with PAO (100 uM) for 30 min. Values are expressed as percentage inhibition vs untreated controls. Data represent mean ± SEM from 3 separate experiments, each performed in triplicate. (* $p < 0.05$, ** $p < 0.01$ by One-way ANOVA followed by Bonferroni post-hoc correction for multiple comparisons).