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Abstract

Introduction: Cell surface thiol isomerase enzymes, principally protein disulphide isomerase (PDI), have emerged as important regulators of platelet function and tissue factor activation via their action on allosteric disulphide bonds. Allosteric disulphides are present in other haemostasis-related proteins, and we have therefore investigated whether thiol isomerase inhibition has any influence on two endothelial activities relevant to haemostatic regulation, namely activation of protein C and activation of plasminogen, with subsequent fibrinolysis.

Materials and Methods: The study was performed using the human microvascular endothelial cell line HMEC-1. Thiol isomerase gene expression was measured by RT-PCR and activation of protein C and plasminogen by cell-based assays using chromogenic substrates S2366 and S2251, respectively. Cell mediated fibrinolysis was measured by monitoring absorbance at 405 nm following fibrin clot formation on the surface of HMEC-1 monolayers.

Results and Conclusions: A variety of thiol isomerase enzymes, including PDI, were expressed by HMEC-1 cells and thiol reductase activity detectable on the cell surface was inhibited by both RL90 anti-PDI antibody and by the PDI inhibitor quercetin-3-rutinoside (rutin). In cell-based assays, activation of plasminogen, but not of protein C, was inhibited by RL90 antibody and, to a lesser extent, by rutin. Fibrin clot lysis occurring on a HMEC-1 monolayer was also significantly slowed by RL90 antibody and by rutin, but RL90-mediated inhibition was abolished in the presence of exogenous tissue plasminogen activator (tPA). We conclude that thiol isomerases, including PDI, are involved in fibrinolytic regulation at the endothelial surface, although not via a direct action on tPA. These findings broaden understanding of haemostatic regulation by PDI, and may aid in development of novel anti-thrombotic therapeutic strategies targeted via the fibrinolysis system.

Keywords

Thiol isomerase; Protein disulphide isomerase; protein C; plasminogen; fibrinolysis; endothelium.

Introduction

"Allosteric disulphide bonds" are involved in the functional regulation of a number of haemostatic proteins. The most closely studied examples are those in tissue factor and in integrin β3. Both proteins contain at least one disulphide bond with the –RHStaple configuration characteristic of allosteric disulphides, and redox alteration of the thiol/disulphide status of these bonds regulates the haemostatic function of the protein [1]. Proteomic studies have identified the presence of similar –RHStaple disulphides in a number of other haemostatically relevant proteins located on the surface of platelets (glycoprotein 1bα) and on cells of the vascular wall (thrombomodulin, uPA receptor), as well as in blood plasma (fibrinogen, plasminogen, tPA, uPA) [2]. Redox regulation of allosteric disulphides may therefore play a wider role in haemostatic control than previously realised.

Protein disulphide isomerase (PDI), a 57kD member of the thioredoxin superfamily of oxidoreductases, functions primarily within the endoplasmic reticulum as a protein folding catalyst and chaperone, by virtue of its ability to bring about the formation, reduction or isomerisation of disulphide bonds [3]. PDI also resides outside the endoplasmic reticulum, at a variety of sites which include the exofacial surface of the plasma membrane [4], where it is available for regulation of allosteric disulphide bonds in proteins of the haemostasis system. Activated platelets release PDI and a pool of PDI is found at the platelet cell surface [5]. More recently it has become clear that a range of thiol isomerase enzymes (including PDI, ERp57 and ERp5) become available at the platelet surface following activation [6]. Experiments using mouse models of thrombus formation have highlighted a critical role for PDI [7-9], or its close homologue ERp57 (10), in platelet accumulation and fibrin deposition [11] . PDI associates with tissue factor and targets the allosteric cys186-cys209 disulphide bond [12]. An alternative mechanism for the action of PDI on tissue factor is via modulated exposure of phosphatidylserine (PS) on the cell surface, since PDI has been shown to increase PS internalisation by affecting both flippase and floppase enzymes [13].

Although the functional role of cell surface PDI on platelets and tissue factor is established, its relevance to endothelial regulation of haemostasis is less well studied. This is an important question to address because of the crucial antithrombotic role played by healthy endothelium. PDI is present inside endothelial cells and is up-regulated under conditions of hypoxia [14-16]. Endothelial cells in culture also express PDI on their exofacial surface, and this PDI regulates the adhesive properties of both thrombospondin [17] and of its binding partner integrin αV β3 [18]. In contrast, experiments performed using mouse models of thrombus formation indicate that, *in vivo*, PDI is not expressed on the surface of unperturbed vessel wall, but that it accumulates rapidly following vascular injury by binding to β3 integrins [7,8,19].

In this study we have used the human endothelial cell line HMEC-1 [20,21] as a model in which to investigate the possible role of cell surface thiol isomerase enzymes, including PDI, in two important aspects of endothelial regulation of haemostasis, namely protein C activation and plasminogen activation with subsequent fibrin lysis. Our initial aim was to identify thiol isomerase enzyme expression by HMEC-1 endothelial cells and to determine whether enzyme activity

is detectable on their surface. We then investigated whether thiol isomerase inhibition was associated with alteration of protein C and plasminogen activation on the surface of these cells.

Materials and Methods

Materials

Human protein C (Cambridge Biosciences), human tissue plasminogen activator (tPA) (Calbiochem), RL90 anti-PDI antibody and IgG2a isotype control (Thermo Scientific), anti- tPA tissue plasminogen activator antibody, Clone T1 (Thermo Scientific and Abcam) and chromogenic substrates S2366 and S2251 (Chromogenix) were obtained from the sources shown. All other reagents were purchased from Sigma-Aldrich. Assays were performed in HEPES buffered saline (HBS) containing NaCl 140 mmol/L, KCl 2.7 mmol/L, glucose 5 mmol/L and HEPES 10 mmol/L, pH 7.3. The PDI inhibitor molecule quercetin-3-rutinoside (rutin) was prepared by dissolving to a concentration of 200 mM in dimethyl sulphoxide, followed by dilution in HBS to a final concentration in experiments of 100 μM. Dulbeccos Modified Eagle Medium and other cell culture reagents were purchased from Invitrogen, as were all molecular biology materials.

Methods

Cell culture

Human microvascular endothelial cells (HMEC-1) were kindly provided by Dr Ian Locke, University of Westminster. HMEC-1 cells were cultured in Dulbeccos Modified Eagle Medium (DMEM) containing fetal calf serum (10%), penicillin (100 U/mL), streptomycin 0.1 mg/mL) and glutamine (2 mmol/L). For assays of protein C activation, plasminogen activation and fibrin lysis cells were plated on colourless cell culture treated 96 well plates and used at 80-90% confluency. For assays of PDI activity cells were plated on cell-culture treated black 96 well plates.

RT-PCR to determine thiol isomerase gene expression in HMEC-1 cells

HMEC-1 cells were cultured to 90% confluency in 6 well dishes. Total RNA was extracted using TriZol reagent (Invitrogen) and cDNA was synthesised using the MMLV reverse transcriptase and random primers (negative controls included RNA without MMLV to check for genomic DNA contamination). Primer sequences for the PDI, PDIp, EndoPDI, ERp5 and ERp57 are described in Table 1. PCR reactions contained 0.5 pmol/L forward and reverse primers, Taq polymerase 0.02 U/µl, dNTPs 0.1 mmol/L, $MgCl_2$ 1.5 mmol/L in 10 mmol/L Tris-HCl pH9.0 and cycles for all amplifications were 95^{o}C for 5 minutes followed by 40 cycles: (94^{o}C for 20 sec, 55^{o}C for 20 sec, and 72^{o}C for 30 sec) except for *P4HB (*PDI) where the extension step was increased to 1 minute in each cycle. PCR products were separated on 2%

agarose gel stained with ethidium bromide with a 100bp ladder (Norgene 100-5000bp).

Gene name	Strand	Primers sequence 5'→ 3'	Product size (bp)
β-actin	Forward	AGCCATGTACGTAGCCATCC	220
	Reverse	CTCTCAGCTGTGGTGGTGAA	
PDIA6 (ERp5)	Forward	GGTCTGGTGAGCTGTACCTTC	369
	Reverse	ACTCAGCGCAGCATCTACAA	
PDIA3 (ERp57)	Forward	CACGGACGACAACTTCGAGA	340
	Reverse	TTCCTCAGTCCTGAGAGGCA	
TXNDC5 (EndoPDI)	Forward	TACACGGCCGACATGTTCAC	412
	Reverse	CGTGCAGCTCAAAGTTGCTT	
P4HB (PDI)	Forward	TGCCCCTTGTCATCGAGTTC	620
	Reverse	GAAGCTGTGCACTTTGACGG	
PDIA2 (PDIp)	Forward	CTTCTGGCCCCCTTATCTGC	396
	Reverse	ACTCCGTCACACCAAACTCC	

Table 1: Primer sequences and expected product sizes for human thiol isomerases.

Measurement of cell surface disulphide reductase activity of PDI:

Disulphide reductase activity was measured by fluorescence assay using the pseudosubstrate dieosin glutathione disulphide (Di-E-GSSG), as previously described [22]. The inhibitory effect of RL-90 antibody (100 μg/mL) was measured by comparison against isotype control, following pre-incubation with HMEC-1 cells for 30 minutes at 37^{o}C. In a similar way, the effect of rutin (100 μM) was measured by comparison against vehicle control.

HMEC-1 viability following pre-incubation of cells with thiol-isomerase inhibitor molecules for 30 minutes was determined by MTT assay [23].

Measurement of protein C activation and plasminogen activation on HMEC-1 monolayers:

The effect of PDI inhibition on (a) activation of protein C and (b) activation of plasminogen, was measured using chromogenic cell-based assays, essentially as described by Sandusky *et al.* [24] and Wileman *et al.* [25], respectively.

For measurement of protein C activation, HMEC-1 cells were washed once with HBS and then incubated for 60 minutes with 25 μg/mL human protein C and 0.5 units /mL human thrombin in HBS containing 5 mmol/L $CaCl_2$. Aliquots (75 μL) of this mixture were then transferred to microplate wells containing 50 μL of hirudin (10 units/mL), mixed and incubated for a further 5 minutes, after which chromogenic substrate S2366 (final concentration 0.5 mmol/L) was added and absorbance at 405 nm monitored over a period of 120 minutes. All incubations were carried out at 37^{o}C. To determine the effect of PDI inhibition, RL-90 anti-PDI antibody or isotype control (both 100 μg/mL) were included in the initial incubation mixture between HMEC-1 cells, protein C and thrombin.

For measurement of plasminogen activation, HMEC-1 cells were washed once with HBS and then incubated at 37^{o}C for 30 minutes with either RL-90 anti-PDI antibody or isotype control (both 100 μg/mL). Similar incubations were performed using rutin (100 μM) or vehicle control. Human plasminogen (2 μmol/L) was then added and, after a further 10 minutes incubation, chromogenic substrate S2251 (final concentration 0.5 mmol/L) was added and absorbance at 405 nm monitored over a period of 120 minutes. All incubations were carried out at 37^{o}C.

To further investigate the role of PDI in plasminogen activation, experiments were performed on washed HMEC-1 cells with addition of either bovine PDI or ovalbumin (as an inert protein control), at final concentrations of 1.75 μM.

Measurement of fibrin lysis on HMEC-1 monolayers

Cell-mediated lysis of fibrin clots was measured using a modification of the method described by Wileman *et al*. [25]. Briefly, HMEC-1 monolayers grown in 96 well microplates were washed once with HBS prior to addition of plasminogen (0.28 μmol/L), $CaCl_2$ (5.3 mmol/L), human thrombin (0.4 units/mL) and human fibrinogen (1 mg/mL). After mixing the absorbance at 405 nm was monitored for a period of 4.5 hours.

The role of tissue plasminogen activator (tPA) in this experimental system was investigated by performing experiments following pre-incubation of washed HMEC-1 cells for 30 minutes with anti-tPA antibodies or isotype control (both 100 μg/mL), prior to addition of the other reagents. All incubations were carried out at 37^{o}C.

To determine the effect on fibrin lysis of PDI inhibition, HMEC-1 cells were pre-incubated for 30 minutes with RL-90 anti-PDI antibody or isotype control (both 100 μg/mL). Similar experiments were performed using rutin (100 μM) or vehicle control.

To investigate in more detail the effects of RL90 antibody on the fibrinolytic process, further experiments were performed using an identical protocol, but this time with the addition of tissue plasminogen activator (0.05 ng/mL).

Statistical methods

Data from experiments to investigate the effect of thiol isomerase inhibition on protein C and plasminogen activation were obtained as sequences of absorbance readings over the first 120 minutes of each experiment. To measure fibrin lysis, sequential absorbance values were measured between 0.5 hours (completion of thrombin-induced fibrin formation) and 4.5 hours (completion of fibrinolysis process). In all cases, data were fitted to a sigmoidal model and comparison of curves performed by F test using Graphpad Prism software. A p value of <0.05 was taken as evidence of statistical significance. Thiol reductase activity was compared to the appropriate control using student T-test.

Results

Thiol isomerase expression in HMEC-1 cells

The HMEC-1 cells were found to express ERp5, ERp57, EndoPDI and P4HB (PDI) by RT-PCR. PDIA2, which has been previously described in placenta was not detectable in the HMEC-1 cells (Fig 1).

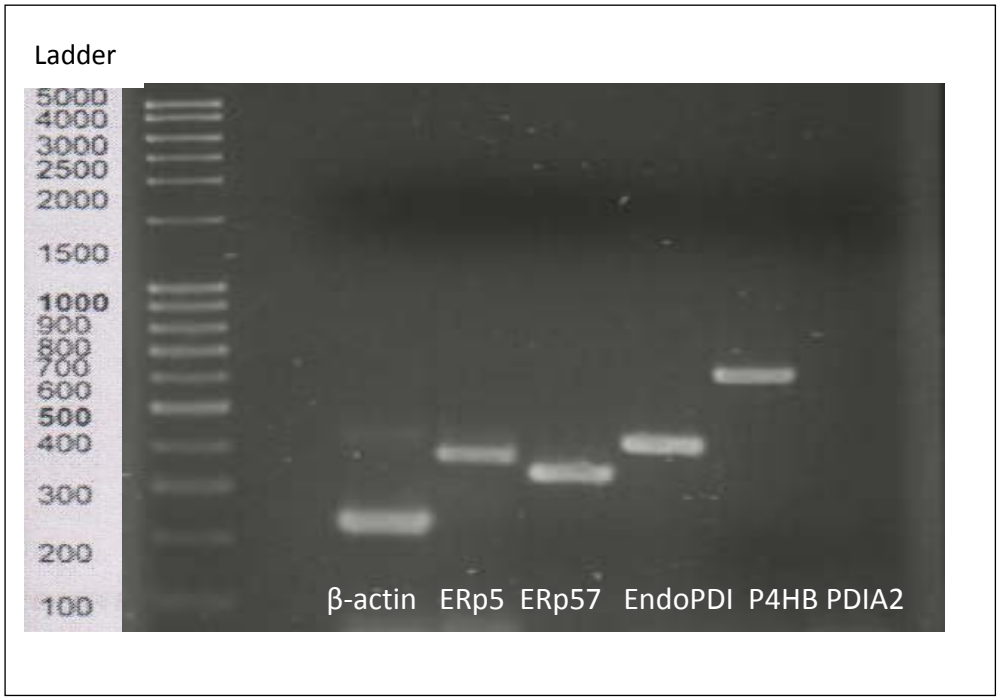

Figure 1: Thiol isomerase expression in HMEC-1 cells. RT-PCR Gene expression of β-actin (220bp), ERp5 (369bp), ERp57 (340bp), EndoPDI (412bp), P4HB (620bp), no PDIA2 product was detected. Products were separated on 2% agarose gel stained with ethidium bromide.

Thiol isomerase activity in HMEC-1 cells

A small but consistent disulphide reductase activity was detected on the surface of the HMEC-1 cells and this was significantly inhibited by both RL90 anti-PDI antibody (100 μg/mL) and by rutin (100 μM) (Fig 2). The thiol isomerase inhibitors caused no significant reduction in cell viability (as measured by MTT assay) compared with the appropriate controls (data not shown), therefore the inhibition was not due to increased cell death.

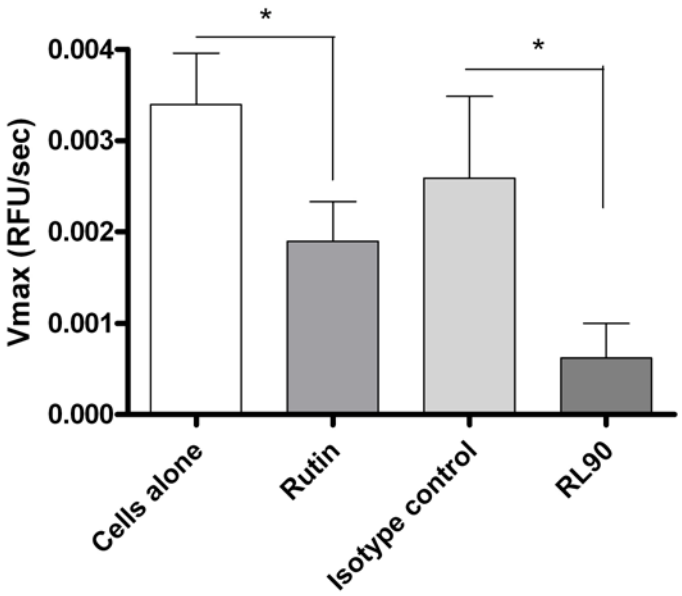

Figure 2. Inhibition of disulphide reductase activity on HMEC-1 cells by RL90 anti-PDI antibody (100 μg/mL) and by rutin (100 μM). Enzyme activity was measured by fluorescence assay using the pseudosubstrate dieosin glutathione disulphide. Data represent mean and SEM values from 5-10 experiments, *p<0.05 by student T-test.

Effects of PDI inhibition on protein C activation and plasminogen activation on HMEC-1 monolayers

In the absence of cells there was almost no colour generation from chromogenic substrates S2366 (activated protein C) and S2251 (plasmin), indicating minimal non-specific activation of either protein C or plasminogen under the experimental conditions employed. In contrast, in the presence of a HMEC-1 cell monolayer, there was a steady increase in absorbance at 405 nm over a period of 120 minutes. In experiments performed using S2366 (activated protein C), no difference was observed between results obtained in the presence of RL90 anti-PDI antibody and isotype control (Fig 3A), however using S2251 (plasmin) the increase in absorbance was significantly reduced by RL90 antibody compared with isotype control ($p < 0.001$) (Fig 3B). In a similar way, plasminogen activation, measured using S2251

(plasmin), showed a small but statistically significant inhibition in the presence of rutin (100 μM) ($p < 0.05$) (Fig 4A). Addition of exogenous bovine PDI (1.75 μM) caused a small but significant increase in plasminogen activation ($p<0.05$) (Fig 4B).

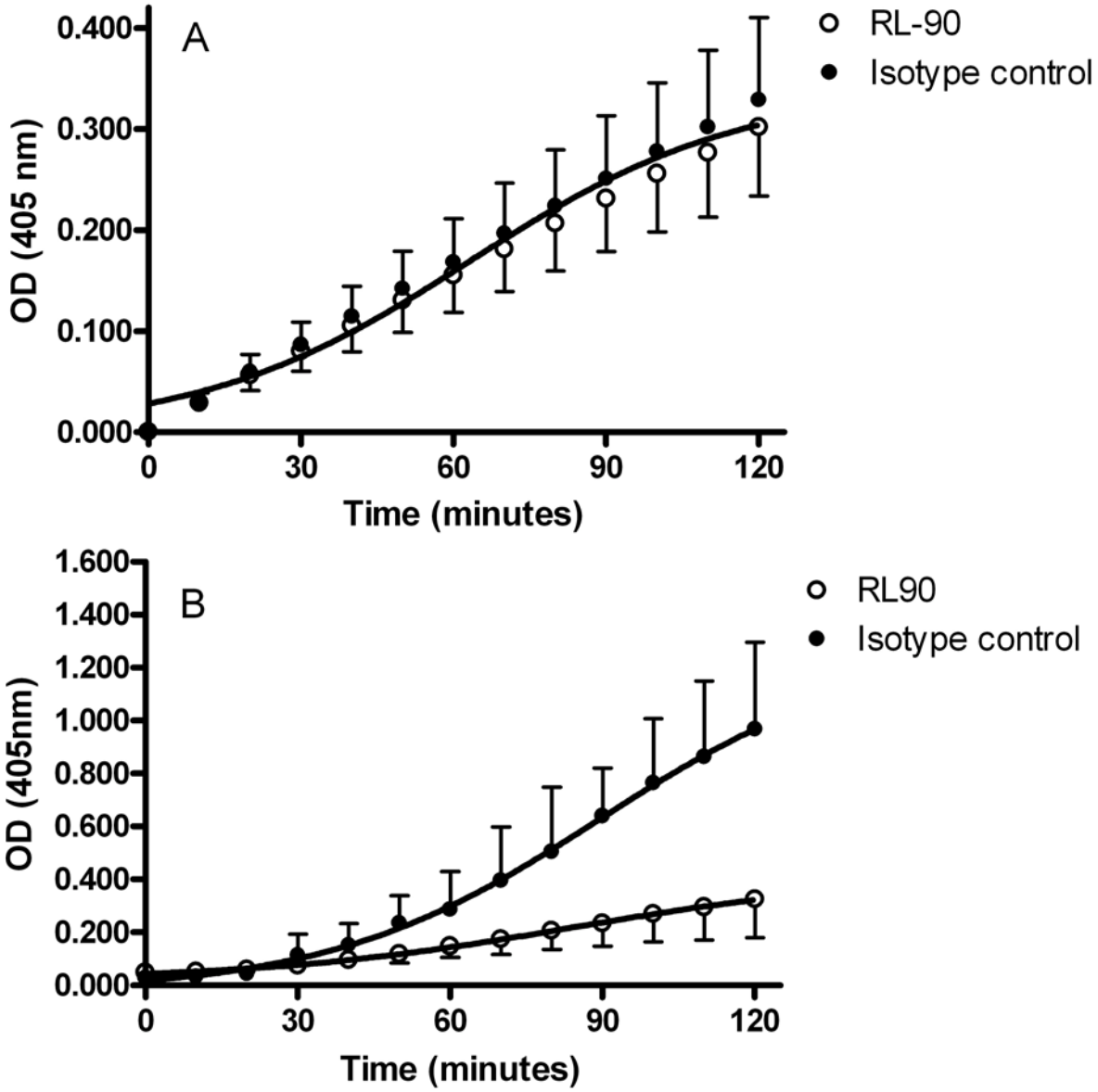

Figure 3. Protein C activation (**A**) and plasminogen activation (**B**) occurring on HMEC-1 endothelial cells in the presence of RL-90 anti-PDI antibody, or isotype control (both 100 μg/mL). Results display cell-mediated activity only (any non-specific activity has been subtracted). No significant difference was observed in protein C activation (RL90 and isotype control values fit to the same curve), however plasminogen activation is significantly inhibited by RL-90 ($p<0.001$ by F test). Data represent mean and SEM ($n = 6$).

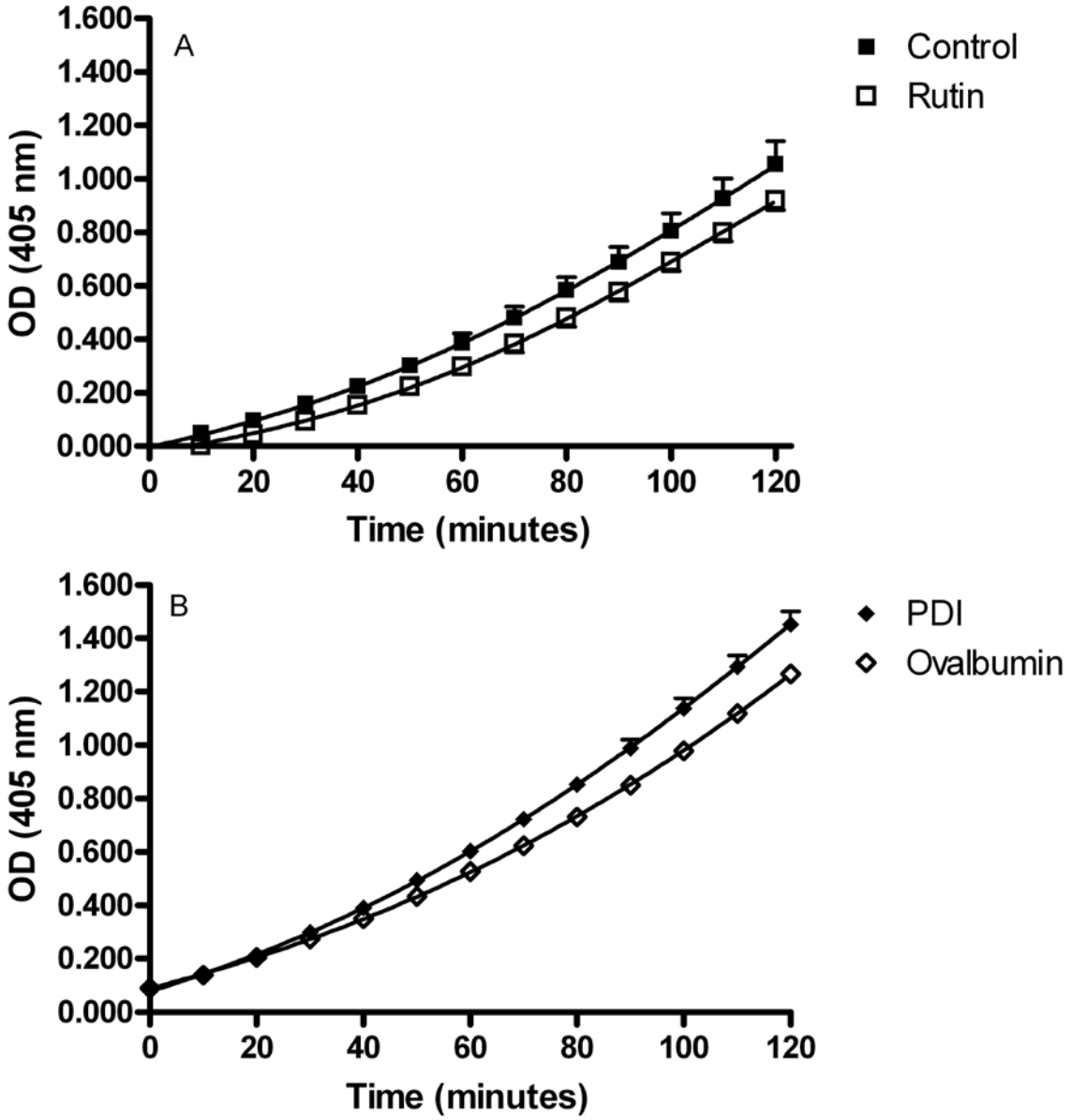

Figure 4: Plasminogen activation occurring on HMEC-1 endothelial cells in the presence of rutin (100 µM) or vehicle control (A) and the presence of exogenous bovine PDI or ovalbumin (both 1.75 µM) (B). Results display cell-mediated activity only (any non-specific activity has been subtracted). Data represent mean and SEM (n = 8). Treatments were significantly different to their relevant control in both (A) and (B) ($p<0.05$) when compared using sigmoid curve fit comparison and F test.

Effects of PDI inhibition on fibrin lysis on HMEC-1 monolayers

Conversion of fibrinogen to fibrin following addition of thrombin was reflected in a rise in absorbance during the first 30 minutes of the experiments. Following a plateau phase absorbance values fell as fibrin lysis occurred. Lysis curves showed a

significant shift to the right in the presence of anti-tPA antibodies (Fig 5A), confirming the role of tPA in this experimental model.

In the presence of both rutin (Fig. 5B) and RL90 antibody (Fig. 5C) there was a significant shift to the right in lysis curves, compared with relevant controls, indicating inhibition of fibrinolysis following blockade of thiol isomerise activity.

When RL90 experiments were repeated with the addition of human tPA to the system, there was no difference between fibrin lysis occurring in the presence of RL90 or isotype control (Fig 5D).

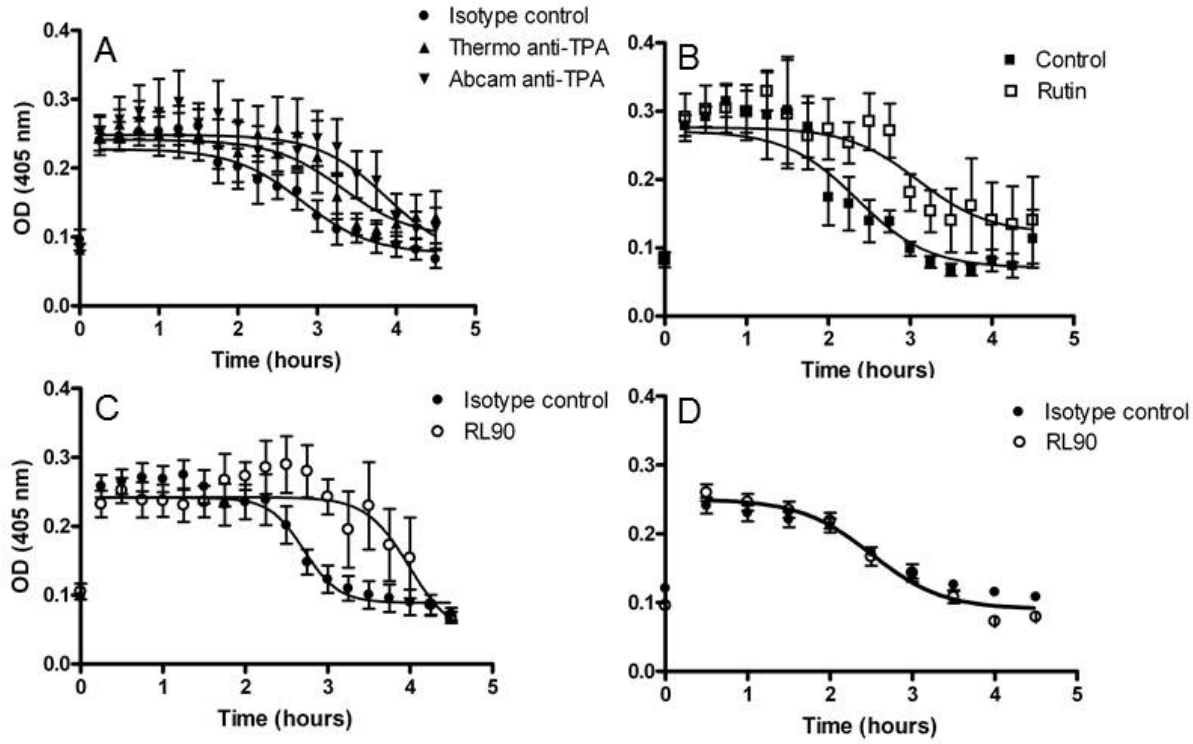

Figure 5. Fibrin lysis occurring on HMEC-1 endothelial cells in the presence of A: anti-tPA antibody from two sources, compared with isotype control (all 100 µg/ml); B: rutin (100µM) and vehicle control; C: RL-90 anti-PDI antibody or isotype control (both 100 µg/ml) in the absence of exogenous human tissue plasminogen activator; D: RL-90 anti-PDI antibody or isotype control (both 100 µg/ml) in the presence of exogenous human tissue plasminogen activator (0.05 ng/mL). In A-C curves were significantly different from their appropriate controls ($p<0.01$) when analysed using sigmoid curve fit comparison and F test but in D no significant difference was found and both RL90 and control data fit to a single curve. Data represent mean and SEM ($n = 8$).

Discussion

The major findings of this study are that inhibition of thiol isomerase activity on the endothelial surface resulted in diminished plasminogen activation and subsequent fibrin lysis. In contrast, there was no loss of protein C activation caused by thiol isomerase inhibition, indicating firstly that this aspect of endothelial haemostatic regulation is not thiol isomerase-dependent and secondly that the observations on plasminogen activation were not simply due to a non-specific effect.

The fibrinolytic mechanism plays a key antithrombotic role via clearance of fibrin from the vascular system. Understanding its detailed mechanism is therefore important in preventing and treating cardiovascular disease. Fibrin is degraded by the serine protease plasmin, derived from the inactive proenzyme plasminogen which is found circulating in blood plasma. Two main types of plasminogen activator are recognised: tissue-type (tPA) and urokinase type (uPA) plasminogen activator, with tPA generally considered the more important initiator of fibrin clearance in the circulation. In our experiments, we have not distinguished between the two, however we found fibrin lysis was significantly inhibited by anti-tPA antibodies, indicating that tPA plays a major role in this experimental model.

Vascular fibrinolysis is a highly regulated process requiring appropriate interactions between the various components (plasminogen, tPA, inhibitor molecules). These interactions are optimised by binding of the key proteins to relevant surfaces, either the surface of a fibrin clot or the surface of endothelial cells lining the blood vessel [26]. Recent research has identified the annexin A2 heterotetramer (AIIt), comprising two molecules of annexin A2 and two of the protein S100A10, as the key endothelial receptor bringing together both plasminogen and tPA to optimise plasmin generation at the surface of the vascular wall [27]. Annexin A2 binds to anionic phospholipids, chiefly phosphatidylserine (PS) [28], and thus anchors the S100A10 subunits to the membrane, where they act to co-localise plasminogen and tPA.

In our experiments measuring fibrin lysis (in the absence of added tPA), the rate of fibrin lysis was significantly diminished in the presence of thiol isomerase inhibitor molecules (RL90 and rutin), indicating that fibrinolytic regulation at the cell surface is, at least to some extent, dependent upon intact thiol isomerise activity. In contrast, when tPA was added to the system the inhibitory effect of RL90 was lost, suggesting that the effect of thiol isomerase inhibition was not targeted at the tPA molecule itself, but rather at the mechanism by which endothelium either releases plasminogen activator or else binds to tPA and /or other fibrinolytic components, in order to optimise the catalytic efficiency of fibrinolysis on the cell surface. Thiol isomerases may alter allosteric disulphides in a component of the AIIt heterotetramer to which tPA binds on the endothelial surface. Alternatively, PDI-mediated alteration of PS exposure [13] may destabilise the anchoring of AIIt by annexin A2, and thus diminish endothelial surface plasminogen activation.

Another possible interpretation of our results may involve thiol isomerase-mediated redox alteration of the plasmin molecule itself, which is known to undergo reduction and proteolysis to the fibrinolytically-inactive product “angiostatin”. This is less likely to explain our results however, since plasmin reduction is mediated by phosphoglycerate kinase [29], not by PDI [30], and occurs on the surface of vascular

smooth muscle, but not on endothelial cells [31]. An alternative mechanism proposes reduction of plasmin by the AIIt receptor itself, in a system involving electron transfer from NADPH via thioredoxin [32].

Thiol isomerase enzymes are sensitive to redox conditions and for isomerases located on the surface of blood cells and endothelium, the thiol/disulphide balance of redox couples within blood plasma, principally reduced and oxidised glutathione and cysteine, will strongly influence enzyme activity [33]. The extracellular redox environment is recognised as an important regulator of both platelet function [34,35] and endothelial biology [36] and changes in plasma redox state occur in conditions characterised by increased cardiovascular risk [37]. Our findings suggest that alteration in fibrinolysis may be a further aspect of deranged vascular biology in these conditions.

We detected mRNA expression of a variety of thiol isomerase enzymes in the HMEC-1 cell line and this highlights the likelihood that PDI is not the sole member of the thiol isomerase family to be involved in redox regulation of proteins at the endothelial surface. In our experiments, plasminogen activation and fibrin lysis were inhibited by RL90 antibody, which is known to cross react with ERp57 [10], but also (to a lesser degree) by rutin, which is a more specific inhibitor of PDI [38]. Addition of exogenous PDI produced a statistically significant increase in the rate of plasminogen activation. The fact that the effects on plasminogen activation of either rutin or exogenous PDI were smaller than those of RL90 is consistent with a model in which PDI does not act alone, but rather contributes to the overall action of a wider range of thiol isomerase enzymes.

Taken together, our results therefore identify thiol isomerase activity, including PDI, as an important factor influencing fibrinolysis on the endothelial surface, adding another dimension to the growing importance of this enzyme in the control of haemostasis and vascular inflammation [39]. A number of small molecule inhibitors of PDI have recently been identified by high-throughput screening and are being proposed as a new class of antithrombotic drugs [40]. This prospect is enhanced by evidence that the safety profile of PDI inhibitors may be better than that of existing antithrombotics since PDI appears to be essential for thrombus formation but not for normal haemostasis [41].

Conclusions

On the endothelial surface thiol isomerase enzymes, including PDI, appear to be required for plasminogen activation and fibrin lysis, although not via a direct action on t-PA. In contrast, protein C activation is not thiol isomerase-dependent. These findings broaden the scope of thiol isomerase involvement in haemostatic regulation and increase the knowledge base when considering the use of PDI inhibitors in anti-thrombotic therapy.

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