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Inhibition of thiol isomerase activity diminishes endothelial activation of plasminogen, but not of protein C

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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.

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

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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 °C for 5 minutes followed by 40 cycles: (94 °C for 20 sec, 55 °C for 20 sec, and 72 °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 100 bp ladder (Norgene 100-5000 bp).

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

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	

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 °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 °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 °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 °C.