Universidade Federal do Maranhão
Centro de Ciências Biológicas e da Saúde
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São Luís - MA
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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Maranhão como requisito para obtenção do título de Doutora em Ciências da Saúde

Orientador: Prof. Dr. Antonio Marcus de Andrade Paes
Co- orientador: Prof. Dr. Andrés Trostchansky

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Sumário

Lista de Siglas e Abreviaturas

AA	Ácido arquidônico
ACD	Ácido citrato dextrose
AcMo	Anticorpo monoclonal
ADP	Difosfato de adenosina
AINE	Anti-inflamatório não esteroidal
APACT	Análise automatizada de agregação e coagulação de plaquetas (do inglês *Automated Platelet Aggregation and Coagulation Tracer*)
ATP	Trifosfato de adenosina
COX	Cicloxigenase
DAG	Diacilglicerol
DNA	Ácido desoxirribonucleico (do inglês *Deoxyribonucleic Acid*)
ERP	Extrato rico em polifenois
FvW	Fator de Von Willebrand
FITC	Isotiocianato de fluoresceína
GP	Glicoproteina
IP3	Inositol 1,4,5 trifosfato
LC-ESI-MS/MS	Cromatografia Líquida acoplada a Fonte de Ionização por Electrospray acoplada à Espectrometria de Massas tandem Espectrometria de Massas, (do inglês *Liquid Chromatography with Electrospray Ionization with Mass Spectrometry tandem Mass Spectrometry*)
LP	Lavado de plaqueta
PAF	Fator de agregação plaquetário (do inglês *Platelet Aggregation Fator*)
PDI	Proteína dissulfeto isomerase
PDGF	Fator de crescimento derivado de plaquetas (do inglês *Platelet Derived Growth Factors*)
PGH2	Prostaglandina H2

PKC	Proteína quinase C
PLC	Fosfolipase C
PPP	Plasma pobre em plaquetas
PRP	Plasma rico em plaquetas
RE	Retículo endoplasmático
RPM	Rotações por minuto
ST	Sangue Total
UV	Ultravioleta
TCLE	Termo de Consentimento Livre e Esclarecido
TRB	Trombina
TXA_2	Tromboxano A_2

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Resumo

As plaquetas, células sanguíneas envolvidas na manutenção da hemostase, exercem uma função essencial no desenvolvimento de eventos isquêmicos agudos coronarianos, cerebrovasculares e estão criticamente envolvidas no processo de formação da trombose. Em resposta à lesão vascular, às alterações no fluxo sanguíneo ou a estímulos químicos, as plaquetas desencadeiam três mecanismos funcionais: adesão, ativação e agregação. Após a captura da plaqueta, uma rápida estabilização da adesão é necessária para que ocorra a formação do trombo. A ativação plaquetária resulta de alterações conformacionais dependentes da proteína dissulfeto isomerase (PDI), de tal modo, que recentemente foi proposto o seu uso como alvo molecular na atividade antiagregante plaquetária. O uso de espécies vegetais ricas em compostos fenólicos como fonte de substâncias bioativas apresenta-se como uma estratégia promissora para o desenvolvimento de novas alternativas terapêuticas das doenças tromboembólicas. Anteriormente, temos mostrado que as folhas de *Syzygium cumini* (L.) Skeels contém múltiplos polifenóis, que apoiam a sua utilização para fins antiplaquetários. Portanto, este estudo procurou avaliar os efeitos do extrato rico em polifenóis (ERP) da folha de *S. cumini* sobre a ativação e agregação plaquetária, bem como, sobre a atividade redutase da PDI. Para tanto, plasma rico em plaquetas de voluntários saudáveis foi incubado com ERP (10 - 1000µg/mL), durante 25 min, antes da ativação com ADP, trombina ou PMA. Para analisar o efeito de ERP sobre a ativação da integrina αIIbβ3, os protocolos de citometria de fluxo foram conduzidos em plaquetas lavadas pré-tratadas com ERP (10 -1000 µg/mL) e ativadas com trombina antes da marcação com anticorpo PAC-1. Finalmente, os efeitos de ERP (0,1-100 µg/mL) na atividade redutase da PDI foram avaliados na ausência ou presença de padrões polifenólicos de ácido gálico, miricetina e quercetina. ERP inibiu a agregação plaquetária dependente da dose apesar do agonista utilizado, embora uma menor concentração de agonistas potencializasse os efeitos inibitórios de ERP até uma inibição máxima de 77% a 2,5 µM de ADP. De modo semelhante, o ERP reduziu a dose proporcionalmente a proporção de moléculas de αIIbβ3 ativadas por plaquetas até um terço do controle a 1000µg/mL. Estes efeitos correlacionaram-se com a forte ação inibitória de ERP na atividade da PDI, um efeito sinergicamente aumentado na presença de padrões. Portanto, nossos dados mostram que o ERP reduz a agregação e ativação plaquetária, provavelmente através da inibição da PDI, fortalecendo sua proeminente atividade antiplaquetária.

Palavras chave: Agregação plaquetária, proteína dissulfeto isomerase, jambolão, miricetina, agentes antitrombóticos.

Abstract

Platelets, the blood cells involved in maintaining hemostasis, play a key role in the development of acute ischemic coronary, cerebrovascular events and are critically involved in the thrombosis process. In response to vascular injury, changes in blood flow or chemical stimuli, platelets trigger three functional mechanisms: adhesion, activation and aggregation. After platelet capture, a rapid stabilization of adhesion is required for thrombus formation to occur. Platelet activation results from conformational changes dependent of the protein disulfide isomerase (PDI), so that it has recently been proposed as a molecular target in platelet antiaggregant activity. The use of plant species rich in phenolic compounds as a source of bioactive substances is a promising strategy for the development of new therapeutic alternatives for thromboembolic diseases. Previously, we have shown that *Syzygium cumini* (L.) Skeels leaf contains multiple polyphenols, which support its use for antiplatelet purposes. Therefore, this study sought to evaluate the effects of polyphenol-rich extract (PESc) from *S. cumini* leaf on platelet activation and aggregation, as well as on PDI reductase activity. Platelet-rich plasma from healthy volunteers (n=5) was incubated with PESc (10-1000 µg/mL), for 25 min, before activation with ADP, thrombin or PMA. To analyze PESc effect on integrin αIIbβ3 activation, flow citometry protocols were conducted in washed platelets pre-treated with PESc (10-1000µg/mL) and activated with thrombin before tagging with PAC–1 antibody. Finally, PESc (0.1-100 µg/mL) effects on PDI reductase activity were assessed in absence or presence of polyphenolic standards gallic acid, myricetin and quercetin. PESc dose-dependently inhibited platelet aggregation despite the agonist used, even though lower agonist concentration potentiated PESc inhibitory effects to a maximal 77% inhibition at 2.5 µM ADP. Similarly, PESc dose-dependently reduced the proportion of activated αIIbβ3 molecules per platelet up to one third of control at 1000 µg/mL. These effects correlated with the strong inhibitory action of PESc on PDI activity, an effect synergically augmented in presence of standards. Therefore, our data show that PESc reduces platelet aggregation and activation, probably through PDI inhibition, strengthening its prominent antiplatelet activity.

KEYWORDS: Platelet aggregation, protein disulfide isomerase, jabolan, myricetin, antithrombotic agents.

Apresentação

Os eventos trombóticos representam a principal causa de morte e invalidez em todo o mundo. O desequilíbrio nos mecanismos de regulação que controlam o crescimento e o tamanho do trombo é um dos fatores que favorecem o desenvolvimento de doenças relacionadas a estes eventos, sendo, o infarto do miocárdio e o acidente vascular cerebral apontados como as principais causas destas mortes.

Uma das estratégias para o tratamento dos eventos trombóticos é a interferência na função plaquetária, devido papel crucial, que as plaquetas desempenham na formação do trombo. Portanto, as pesquisas relacionadas a compostos que interfiram na agregação plaquetária são de grande relevância para o estudo de novos agentes antitrombóticos. Neste contexto, os compostos fenólicos têm sido estudados e apontados como potentes agentes antiplaquetários, pois podem inibir ou modular vias de sinalização na adesão, ativação e agregação.

Inserido em um grupo de pesquisas que trabalha na investigação de compostos e moléculas que tenham ação na atividade plaquetária, o presente trabalho descreve o estudo das atividades antiagregantes do extrato rico em polifenois (ERP) das folhas de *Syzygium cumini* (L) Skeels. Esta espécie vegetal é estudada por nosso grupo, em que, foram identificados compostos fenólicos, como: miricetina, ácido gálico e quercetina. Estes, apresentam atividades biológicas que envolvem capacidade antioxidante, anti-inflamatória e antitrombótica por inibição da agregação plaquetária, entre outras atividades descritas na literatura.

A presente tese de doutorado foi redigida de acordo com as normas vigentes no Programa de Pós Graduação em Ciências da Saúde da Universidade Federal do Maranhão e está contemplada em três sessões. A **primeira sessão** apresenta o referencial teórico, que aborda as funções e mecanismos plaquetários; a atividade antiagregante de compostos fenólicos e o *Syzygium cumini* (L) Skeels como fonte potencial de compostos fenólicos. Consiste de uma revisão fundamentada em publicações, a qual contextualiza o estudo desenvolvido durante o doutorado. A **segunda sessão** da tese apresenta o manuscrito “Antiplatelet activity of polyphenol-rich extract from *Syzygium cumini*

leaf is potentially mediated by protein disulfide isomerase inhibition". O artigo descreve os efeitos do ERP sobre a agregação plaquetária em plasma rico em plaquetas (PRP) ativadas com difosfato de adenosina (ADP), trombina (THB) e Phorbol 12-miristato 13-acetato (PMA), ainda sugere o mecanismo pelo qual o extrato age como regulador da agregação, através da inibibição da isomerase de dissulfetos protéicos (PDI). Esse artigo foi submetido à Thrombosis Research (Fator de impacto = 2,320; qualis B1 em Medicina I). Esta sessão foi escrita de acordo com os padrões exigidos pelo periódico. A **terceira sessão** compreende as considerações finais e perspectivas futuras à respeito dos resultados apresentados.

Nos **Anexos**, consta trabalho em que realizamos e analisamos experimentos que se encontram no manuscrito publicado na Journal of Thrombosis and Haemostasis (fator de Impacto: 5,72; qualis A1 em Medicina I) sob título: "Novel antiplatelet role for a protein disulfide isomerase-targeted peptide: Evidence of covalent binding to C-terminal CGHC redox motif", no qual demonstra a atividade antiplaquetária do peptídio CxxC através da ligação à Cys400 no domínio a' da PDI e o aponta como um modelo para o desenvolvimento de agentes antitrombóticos.

Referencial teórico

1. Introdução

O tromboembolismo consiste na obstrução da circulação de artérias ou veias pela instalação de coágulos, com redução ou cessação do fluxo sanguíneo na área afetada. Este processo patológico resulta da ativação e propagação inapropriada da resposta hemostática normal do organismo (Alvares, Pádua et al. 2003). O trombo arterial é rico em plaquetas que cresce de maneira desordenada, dificultando o fluxo sanguíneo, podendo provocar a oclusão da artéria que resulta em aterosclerose, necroses isquêmicas e infarto do miocárdio (Veiga, Santos et al. 2013). O trombo venoso se desenvolve em condições de fluxo lento, onde está favorecida a estase e se apresenta com maior composição de fibrina e hemácias. Ao atingirem vasos de menor calibre, causam obstrução, promovendo embolia, principalmente pulmonar.

As plaquetas, células sanguíneas envolvidas na manutenção da hemostase, exercem uma função essencial no desenvolvimento de eventos isquêmicos agudos coronarianos e cerebrovasculares, pois estão criticamente envolvidas no processo de formação da trombose (Gawaz 2006). Em resposta à lesão vascular, às alterações no fluxo sanguíneo ou a estímulos químicos, as plaquetas desencadeiam três mecanismos funcionais: adesão, ativação e agregação. Após a captura da plaqueta, uma rápida estabilização da adesão é necessária para que ocorra a formação do trombo (Varga-Szabo, Pleines et al. 2008).

Na Europa, o tromboembolismo é considerado um problema de saúde pública, sendo responsável por cerca de 370.000 óbitos anuais e requer um custo direto com cuidados hospitalares acima dos três milhões de euros por ano. Nos Estados Unidos, estima-se em dois milhões o número de casos de trombose venosa profunda, para uma mortalidade média de trezentos mil indivíduos (Okuhara, Navarro et al. 2014). No Brasil, entre janeiro de 2008 e março de 2016, o número de internações por embolias e tromboses registradas pelo Sistema Único de Saúde (SUS) foi de 701.906, a um custo total de R$ 515.188.559,35, um gasto médio de R$ 733,98 por paciente (Brasil 2016). Em uma análise mais ampla, as

doenças do aparelho circulatório representam 19,52% das causas de óbito, um dos maiores índices de mortalidade do país, sendo 2,46% do total de mortalidade diretamente decorrente de distúrbios tromboembólicos (Brasil 2016). Portanto, o *design* de drogas que atuem sobre esses processos ou ajudem a preveni-los é de grande importância, não somente sob aspectos sanitários, mas também, econômicos.

Atualmente, muitas classes de drogas têm sido utilizadas no tratamento de doenças tromboembólicas, como: anticoagulantes, anti-inflamatórios não esteroidais (AINES), bloqueadores dos receptores de difosfato de adenosina (ADP), inibidores enzimáticos e inibidores da integrina αIIbβ3. Porém, vários desses medicamentos possuem efeitos colaterais graves, necessitam de cuidados hospitalares para sua administração, desenvolvem resistência ou inibem poucas vias de ativação plaquetária (Maffei, Lastória et al. 2008).

A extensão e a gravidade das doenças tromboembólicas associadas às limitações da terapêutica vigente, tem estimulado a busca por novas moléculas que atuem sobre receptores plaquetários e enzimas relacionadas. Neste contexto, compostos polifenólicos tem se destacado como importantes inibidores ou moduladores da agregação plaquetária, através de mecanismos que atingem desde a inibição da ativação das plaquetas, à ligação e bloqueio aos receptores biológicos na superfície da plaqueta. Isto, tem tornado espécies vegetais ricas em polifenóis fontes promissoras de substâncias bioativas úteis ao desenvolvimento de novas terapias antiagregantes plaquetárias (Galleano, Calabro et al. 2012).

2. Estrutura e função das plaquetas

As plaquetas são fragmentos citoplasmáticos de megacariócitos, anucleadas, de forma discóide e diâmetro entre 2 a 3µm. Seu tempo médio de vida está em torno de 10 dias, após os quais são removidas pelas células reticuloendoteliais do baço e do fígado. (Castro, Ferreira et al. 2006). Apresentam características funcionais de células completas, como presença de moléculas contráteis (trombostenina, actina e miosina), mitocôndrias e sistemas enzimáticos no citoplasma, além de resíduos do complexo golgiense e retículo endoplasmático, os quais sintetizam enzimas e armazenam íons cálcio (Saluk, Bijak et al. 2013). Essas características são fundamentais para que as plaquetas desempenhem seu papel na hemostasia primária, que requer três eventos: adesão, ativação e agregação plaquetária, envolvendo para isso diversos receptores em cada um desses eventos (McNicol and Gerrard 1997).

2.1 Adesão plaquetária

Adesão plaquetária é o processo pelo qual as plaquetas aderem ao endotélio vascular após injúria. É um processo complexo que requer a interação coordenada dos receptores presentes na superfície das plaquetas e macromoléculas adesivas da matriz extracelular. As plaquetas são recrutadas da circulação para a matriz subendotelial exposta ao ocorrer lesão de um vaso sanguíneo (Jurk and Kehrel 2005).

Na superfície da membrana plaquetária existem diversas glicoproteínas que favorecem a adesão entre elas ou regiões lesionadas da parede vascular. São ricas em glicoproteínas (GP) que servem como alvos moleculares para: (a) reconhecimento do endotélio vascular ativado ou subendotélio lesado, iniciando a resposta plaquetária de manutenção da integridade vascular via GP Ia e GP IIa; (b) ancoramento das plaquetas na região do dano via interação do complexo GP Ib-IX-V com constituintes subendoteliais, fibras colágenas e fator de Von

Willebrand (FvW) (Andrews and Berndt 2005, Heemskerk, Kuijpers et al. 2005). Esta adesão ao vaso está diretamente ligada à tensão de cisalhamento. Quando em altas forças de cisalhamento, tal como é encontrado em artérias e arteríolas, o recrutamento inicial das plaquetas ao subendotélio exposto é mediado pela interação do FvW com a subunidade GPIbα do complexo glicoproteico GPIb-V-IX. A ligação entre esse receptor plaquetário e o FvW, imobilizado sobre a superfície ativada das plaquetas, também é essencial para a captura das plaquetas em fluxo. Em baixa turbulência, como aquelas características da circulação venosa, as plaquetas podem se ligar diretamente ao colágeno exposto (Kauskot and Hoylaerts 2012).

De acordo com, Flaumenhaft, Croce et al. (1999), a adesão das plaquetas ao endotélio vascular promove: 1) a liberação do conteúdo dos grânulos plaquetários, como: (a) grânulos densos, que contém Ca^{2+}, ADP, trifosfato de adenosina (ATP) e Serotonina (5-HT); (b) grânulos α, que contém FvW, fibronectina, trombomodulina, fator de crescimento derivado de plaquetas (PDGF) e fator 4 plaquetário (PF-4); 2) geração de agonistas da agregação, tais como: colágeno, trombina e tromboxano A_2 (TxA_2) (Essex 2004). Estes eventos em conjunto, atuam na sinalização, recrutamento de plaquetas inativas na circunvizinhança do sítio lesado e amplificam o estímulo para ativação e agregação plaquetária (Gurbel, Erlinge et al. 2012).

2.2 Ativação plaquetária

A ativação das plaquetas é um processo consequente tanto das interações de adesão, quanto da ligação de diversos agonistas plaquetários a receptores específicos de superfície (Wallace and Smyth 2013).

As plaquetas ativadas pelo ADP resultam da sua ligação com seus receptores: o P2Y1 e P2Y12, sendo que, a porção citoplasmática desses receptores interage com as proteínas-G. Quando o ADP estimula o receptor P2Y1 através da proteína G subclasse q, os segundos mensageiros inositol-1,4,5-

trifosfato (IP3) e diacilglicerol (DAG) são formados e modulam vias de ativação (Ueno, Kodali et al. 2011). O IP3 se liga a receptores do sistema tubular denso do retículo endoplasmático, principal local de estoque de Ca^{2+}, fazendo com que as concentrações citoplasmáticas do cátion aumentem. Esse aumento, promove o rearranjo do citoesqueleto, que resulta em mudança da forma da plaqueta, de discoide para irregular e ao prolongamento de múltiplas projeções citoplasmáticas. A mobilização do Ca^{+2} intracelular também é mediada pela via do ácido araquidônico (AA). Este é liberado dos fosfolipídeos de membrana pela ação da fosfolipase A_2 (PLA_2). A enzima ciclooxigenase (COX) metaboliza o AA e gera a prostaglandina H_2 (PGH_2) (Dennis, Cao et al. 2011). Nas plaquetas, a isoforma predominante da enzima COX é a COX-1, e o principal metabólito formado é TXA_2, sintetizado pela ação da enzima tromboxano sintase (TxS) sobre PGH_2 (Figura 1A). A TxA_2 ao interagir com seu receptor TP na superfície plaquetária acoplado à proteína Gq, ativa a fosfolipase C (PLC) e mobiliza o Ca^{2+} intracelular. O aumento das concentrações intracelulares de Ca^{2+} causa ativação plaquetária, mudança de forma da plaqueta e liberação de seu conteúdo granular (Simmons, Botting et al. 2004, Nakahata 2008). DAG causa a ativação da proteína quinase C (PKC), o que contribui para a liberação do conteúdo dos grânulos de secreção contendo os agonistas (Angiolillo, Ueno et al. 2010, Zaid, Senhaji et al. 2015).

Segundo, (Broos, De Meyer et al. 2012), a ligação do ADP ao receptor P2Y12 através da proteína G subclasse i, inibe a formação de cAMP. ADP e TxA_2 são responsáveis, para além do recrutamento da circulação de plaquetas, da promoção da alteração da forma das plaquetas e da secreção de grânulos citoplasmáticos.

A trombina ativa as plaquetas por ligação ao PAR-1 (ativado por concentrações muito pequenas de trombina) e ao PAR-4 que requer concentrações muito maiores de trombina do que o receptor PAR-1 (Kauskot and Hoylaerts 2012). As vias de sinalização, nas quais os PARs estão acoplados às proteínas Gq ativa a PLC, e Gi, que inibe a adenilato ciclase, resultam no aumento do Ca^{2+} intracelular e redução do cAMP, respectivamente. As subunidades G12/G13 se ligam aos RhoGEFs (fatores de troca do nucleotídeo guanina-Rho, os

quais ativam pequenas proteínas G), promovendo respostas do citoesqueleto dependente de Rho, as quais estão envolvidas na mudança de forma das plaquetas (Figura 1B) (Offermanns 2006, Davì and Patrono 2007).

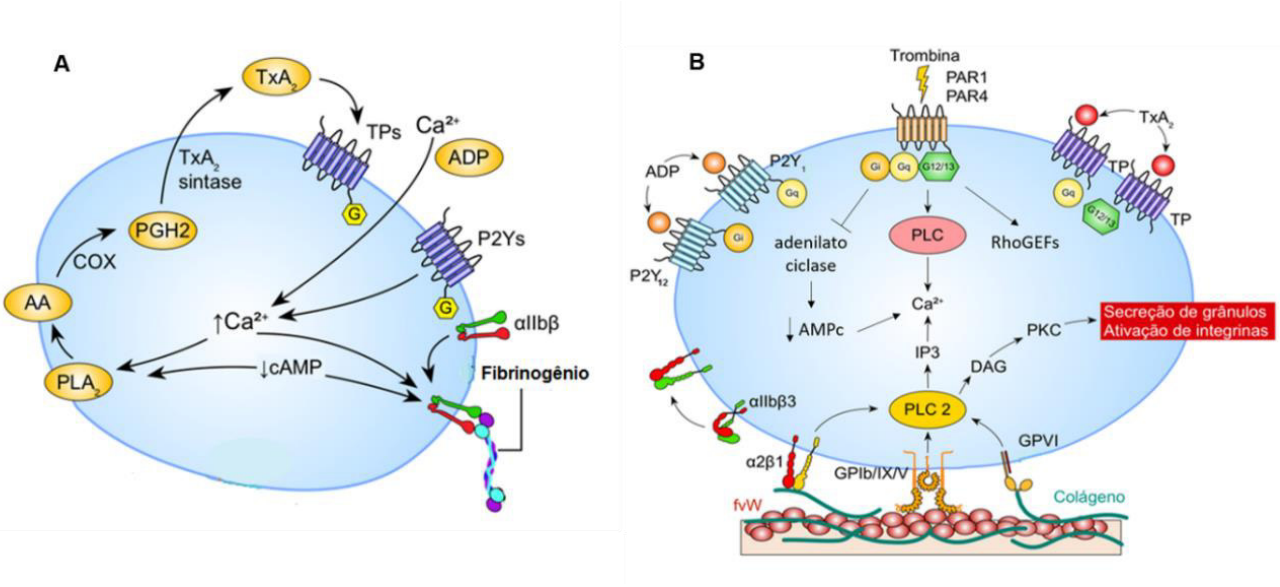

Figura 1: Vias de sinalização plaquetária: (A) Síntese de TXA2 liberado dos fosfolipídeos de membrana pela ação da fosfolipase A2 (PLA2). A enzima ciclooxigenase (COX) metaboliza o AA e gera a prostaglandina H2 (PGH2) que é metabolizada à TXA2 pela ação da enzima troboxano sintase. **(B)** A ligação do colagénio a GPVI ou a integrina α2β1 ou a ligação de vWF a Gp1b-IX-V conduz à ativação da fosfolipase C2 (PLC2). PLC2 cliva fosfatidilinositol (4, 5) -bisfosfato (PIP2) para gerar inositol (1,4,5) -trisfosfato (IP3) e diacilglicerol (DAG). IP3 e DAG são responsáveis pela mobilização de Ca^{+2} a partir de armazenamentos intracelulares levando à à ativação de isoformas de proteína quinase C (PKC). Os fatores libertados potenciam a sinalização de plaquetas através da ativação de receptores acoplados a proteína G / via de fosfolipase C. A ligação do fibrinogénio à integrina ativada inicia o espalhamento das plaquetas por ativação da via fosfoinosítido 3-quinase (PI3K) adaptado (Randriamboavonjy 2015).

Os mecanismos moleculares ativados pela ligação dos agonistas plaquetários aos seus receptores conduzem à ativação da integrina αIIbβ3, a qual está presente na superfície plaquetária e representa a via comum final do processo de ativação, culminando no processo de agregação plaquetária (Kauskot and Hoylaerts 2012).

2.3 Estrutura, ativação da integrina αIIbβIII e agregação plaquetária

De modo geral, as integrinas são expressas na superfície das células em um estado inativo, no qual não podem se ligar a seus ligantes. Quando uma integrina se liga ou se dissocia de seu ligante, ocorrem mudanças conformacionais que afetam tanto a extremidade intracelular quanto a extremidade extracelular da molécula (Alberts et al., 2010). Essa característica é importante para sua biologia funcional, principalmente tratando-se de integrinas da superfície de plaquetas (Hynes, 2002).

A αIIbβIII é uma integrina exclusiva da superfície plaquetária, com densidade de aproximadamente 80.000 cópias por plaqueta em repouso. Porém, durante a fase de secreção este número é elevado em 25% a 50% mediante a translocação dos grânulos α plaquetários para a superfície (Takagi, Petre et al. 2002, Xiong, Stehle et al. 2003). É um receptor para fibrinogênio, fibronectina, vibronectina e FvW com importante papel tanto para adesão quanto para agregação plaquetária, processo para o qual é indispensável. Apresenta-se como um heterodímero constituído de subunidades α e β. Cada subunidade consiste em uma longa porção extracelular, um domínio transmembrana e uma pequena porção voltada para o citoplasma. A região extracelular da subunidade α possui um domínio β-propeller (N-terminal), um domínio “thigh” e dois domínios “calf” (Zhu, Zhu et al. 2010). A subunidade β é constituída por *i)* o domínio βI, no interior do qual há uma região dependente de cátions divalentes (Ca^{2+}), incluindo um motivo MIDAS (*metal ion-dependent adhesion site*) diretamente envolvido na ativação da integrina; *ii)* um domínio PSI (plexin, semaforina, integrina); e *iii*) um domínio híbrido, todos implicados na ativação da integrina (figura 2) (Takagi, Petre et al. 2002; Lau, Kim et al. 2009). Além destes domínios, há também quatro regiões ricas em cisteínas, altamente conservadas, chamadas de domínios I-EGF (*integrin-epidermal growth fator*) e por um domínio βTD (*β-tail domain*) proximal à membrana (Takagi, Petre et al. 2002, Lau, Kim et al. 2009). Na membrana plaquetária, essas duas subunidades estão associadas, de modo que, o domínio β-propeller e o domínio A formam a região da “cabeça” da integrina, que é seguida por duas “caudas” compostas pelos outros domínios de cada subunidade. Tanto

a subunidade αIIb quanto a β3 possuem pontes dissulfeto na sua estrutura. αIIb contém 18 e β3 contém 53 resíduos de cisteínas, dos quais 33 estão nos domínios I-EGF da porção extracelular da molécula (Haubner, Finsinger et al. 1997).

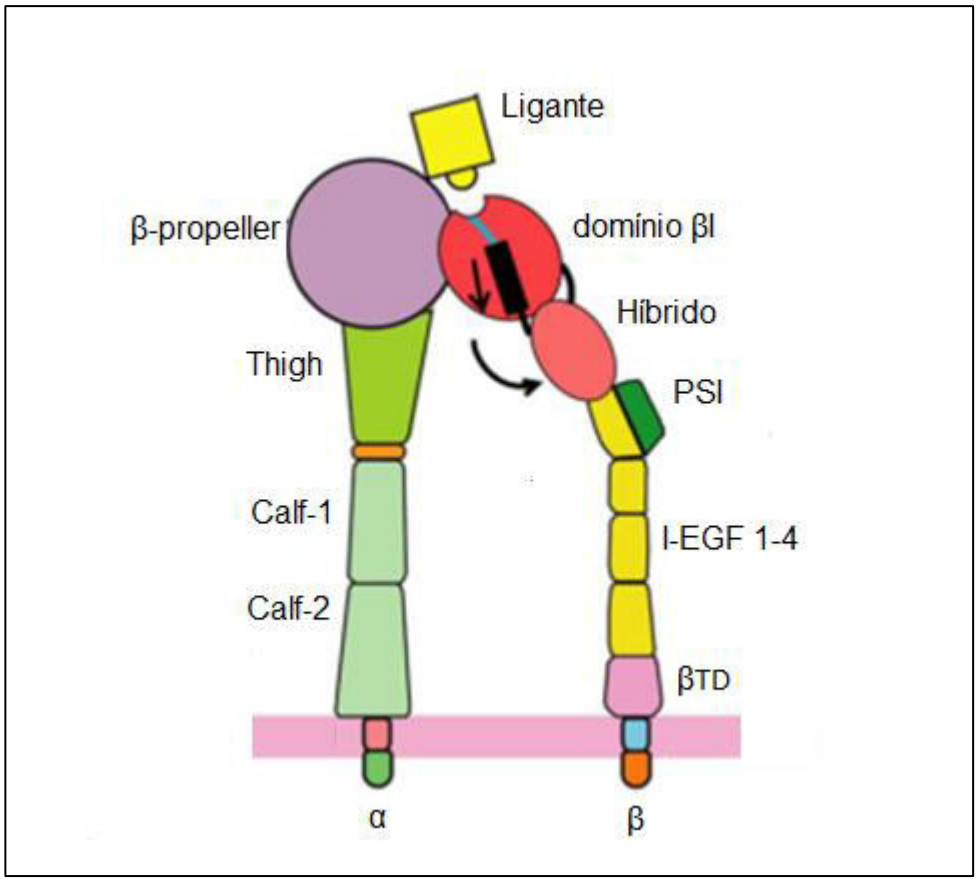

Figura 2: Estrutura da integrina αIIbβIII. Adaptado de (Zhu, Zhu et al. 2010).

Durante sua ativação, a integrina αIIbβ3 sofre três alterações na estrutura conformacional do seu domínio extracelular (Takagi, Petre et al. 2002). As três conformações observadas correspondem à: a) uma conformação dobrada, de baixa afinidade; b) uma conformação estendida com a região da cabeça ainda dobrada, correspondendo a um estado de afinidade intermediária; e c) uma conformação totalmente estendida correspondendo ao estado de alta afinidade pelo ligante, especialmente o fibrinogênio plasmático (Takagi, Petre et al. 2002, Margadant, Monsuur et al. 2011). Os eventos que levam à ativação da integrinasão

iniciados quando as plaquetas são ativadas com um ou mais agonistas e resulta na sinalização *inside-out* (de dentro para fora), em que, ocorre a migração de substâncias do ER (retículo endoplasmático) para a superfície plaquetária, auxiliando na ativação da integrina αIIbβ3 de superfície (Takagi, Petre et al. 2002, Hansen and Winther 2009).

Nesta sinalização *inside-out*, o estímulo por agonistas desencadeia ativação da PLC e a formação de IP3 e DAG. O IP3 induz a liberação das reservas de Ca^{2+} do retículo endoplasmático (ER) por meio de receptores de IP3 (IP3-R) e subsequente ativação da entrada de Ca^{2+} através das proteínas Orai1, mediada pela molécula de interação estromal-1 (STIM1) (Shattil, Kim et al. 2010). STIM1 atua como sensor dos estoques intracelulares de Ca^{2+}, enquanto as proteínas Orai1, presentes na membrana plasmática, representam as subunidades que formam os canais de $Ca+^{2}$ (Watson, Auger et al. 2005), DAG e Ca^{2+} ativam a PKC e o fator de troca de nucleotídeos de guanina I regulado por DAG e Ca^{2+} (CalDAG-GEFI), levando a ativação e translocação da proteína Rap1 para a membrana plasmática. A molécula efetora RIAM interage com ambos Rap1-GTP e talina-1 (talin-1), uma molécula de ancoramento intracelular, expondo o sítio de ligação à integrina na talina-1. A interação entre a talina-1 e a integrina resulta na mudança conformacional do domínio extracelular, de um estado de baixa afinidade para um estado de alta afinidade e na ligação ao ligante da matriz extracelular (Nieswandt, Varga-Szabo et al. 2009). Esse passo final requer também a ligação da proteína kindlin-3 à cauda β da integrina, não se sabe se simultaneamente ou sequencialmente à ligação da talina-1 (Figura 3) (Nieswandt, Varga-Szabo et al. 2009).

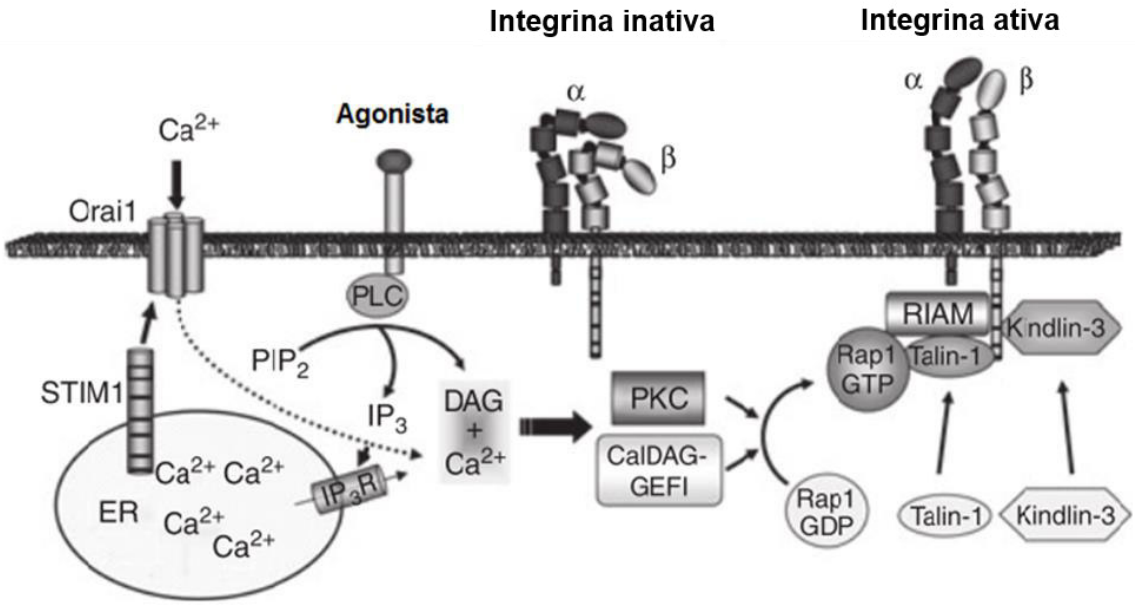

Figura 3. Mecanismo de ativação *inside-out*: A estimulação agonista desencadeia a ativação do PLC e a formação de IP3 e DAG. IP3 induz a libertação de armazenamento de cálcio através dos receptores IP3 (IP3-R) na membrana do retículo endoplasmático (ER) e subsequente ativação mediada por STIM1 da entrada de Ca^{2+} através de Orai1. DAG e Ca^{2+} activam PKC e CalDAG-GEFI, conduzindo à ativação e translocação de Rap1. A molécula efetora Rap1 e RIAM interagem com Rap1-GTP e talin-1. Esta etapa final requer também a ligação da kindlin-3 funcional a cauda do domínio b da integrina. Adaptado de (Nieswandt, Varga-Szabo et al. 2009).

A integrina ativada se liga ao fibrinogênio, desencadeando a sinalização *outside-in* (de fora para dentro) na subunidade β, os domínos conectam-se a filamentos de actina do citoesqueleto por meio de proteínas intracelulares como: talina, vinculina e α-actinina (Haubner, Finsinger, Kessler, 1997) e desta forma, as plaquetas reorganizam seu citoesqueleto, ativando as cascatas de quinases sinalizadoras e aumentando a degranulação. A ligação do fibrinogênio à integrina αIIbβ3 ativada garante a interação plaqueta-plaqueta e resulta na formação irreversível de agregados plaquetários (Millard, Odde et al. 2011).

Através de estudos estruturais da integrina tem-se proposto que a clivagem de pontes dissulfeto na αIIbβ3 pode ser a responsável pela conversão da integrina para um estado de alta afinidade ao fibrinogênio. A isomerase de dissulfetos protéicos (PDI) é sugerida como facilitadora, senão, a causadora da alteração

conformacional na estrutura da αIIbβ3 através de trocas tiol-dissulfeto (Manickam, Sun et al. 2008, Essex 2009).

2.4 PDI e seu papel na agregação plaquetária

A PDI foi a primeira proteína catalizadora de enovelamento proteico descoberta, sendo caracterizada como uma chaperona do retículo endoplasmático (Goldberger, Epstein et al. 1964). Membro da superfamília das tiorredoxinas, a PDI possui como principal característica a presença de um motivo catalítico ditiólico CxxC (Kozlov, Määttänen et al. 2010). A proteína está organizada em 5 domínios (a, b, a', b' e c), com peso molecular aproximado de 55 kDa. As cisteínas reativas estão localizadas na seqüência Trp-Cys-Gly-His-Cys (W**C**GH**C**) dos domínios a e a', sendo que os domínios b e b' não possuem os motivos redoxes (Figura 4A) (Pirneskoski, Klappa et al. 2004). Embora a PDI possua uma sequência de retenção (KDEL) presente na porção C-terminal da proteína para ancoragem no lúmen do RE, , uma quantidade significativa destas proteínas foram encontradas fora do RE demonstrando a sua capacidade de desprendimento e migração para o núcleo, citoplasma, superfície celular e meio extracelular (Wilkinson and Gilbert 2004).

A reatividade da PDI é atribuída à proximidade espacial dos tióis das duas cisteínas dos seus sítios ativos a e a´. Estes tióis tem a capacidade de interconversão entre a forma tiol e dissulfeto de acordo com o estado redox ou pH do meio (Gitler, Mogyoros et al. 1994). As cisteínas do sítio ativo atuam tanto como aceptores de elétrons, quando estão na forma dissulfeto (S-S, oxidada), quanto como doadores de elétrons, quando na forma ditiólica (-SH, reduzida) (Figura 4B) (Essex 2009).

Quando as PDIs se encontram em estado oxidado, suas pontes dissulfeto podem ser desfeitas pelo ataque de um ânion tiolato (S^-) do substrato, de modo a induzir a formação de uma ponte dissulfeto mista PDI-substrato, catalisando assim, a oxidação do sítio ativo do substrato quando essa ponte mista é desfeita e

tornando a PDI reduzida. O mecanismo oposto também é possível, onde a PDI em estado reduzido perde um próton, formando um íon tiolato que ataca a ponte dissulfeto do substrato, formando uma ponte dissulfeto mista PDI-substrato que ao ser desfeita por rearranjo, torna a PDI oxidada e o substrato reduzido (Essex, Li et al. 2001, Essex and Li 2003, Essex 2004). As PDIs também catalisam o rearranjo de ligações dissulfeto (isomerização) (Figura 4B) (Essex, Li et al. 2001, Essex 2009, de A. Paes, Veríssimo-Filho et al. 2011) em substratos que possuam essas pontes erroneamente inseridas ou com uma conformação instável, que resulta na exposição de resíduos hidrofóbicos, facilitando a ligação do substrato com o *pocket* hidrofóbico no domínio b' da PDI. As reações de isomerização se dão através de uma reação de redução seguida de uma reação de oxidação no mesmo substrato, em pares redox diferentes, conferindo a este, maior estabilidade estrutural (Walker and Gilbert 1997).

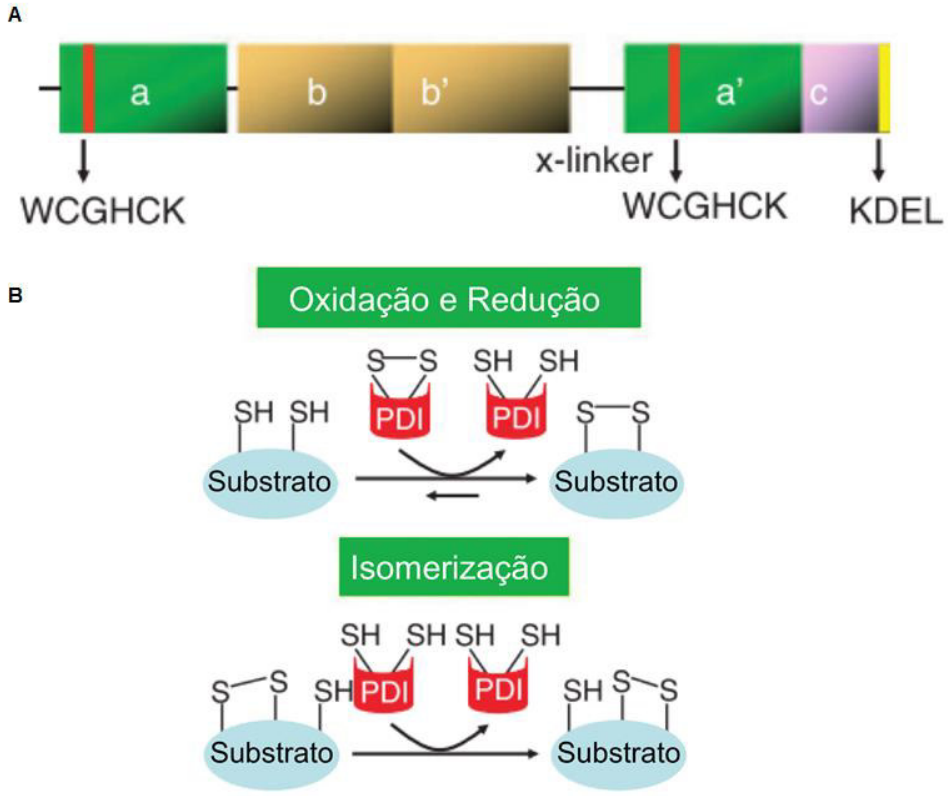

Figura 4: Função e a atividade catalítica da proteína disulfeto isomerase (PDI). (A) Ilustração esquemática de PDI humana. Os domínios a e a'; b e b'. Duas sequências CGHC ativas. Sinal de retenção no RE do C-terminal (KDEL). (B) PDI oxida, reduz e isomeriza ligações dissulfeto do substrato. Adaptado de (Cho 2013).

Nas plaquetas, a PDI desempenha papel crucial para ativação da integrina αIIbβ3 (Essex and Li 2003, Essex 2008, Furie 2009, Furie and Flaumenhaft 2014). Parte de uma conformação de repouso com baixa afinidade ao fibrinogênio para uma conformação intermediária (pré-ativada), catalisada pelo sistema glutationa (GSH/GSSG). Na sua conformação inicial, a integrina apresenta uma ligação dissulfeto que é sensível à clivagem redox pela GSH para formar tióis adicionais. Os tióis recém-gerados sofrem reação de isomerização catalisada pela PDI que resulta no estado de alta afinidade para ligação com o fibrinogênio e formação do agregado de plaquetas (Figura 5) (Essex, 2008).

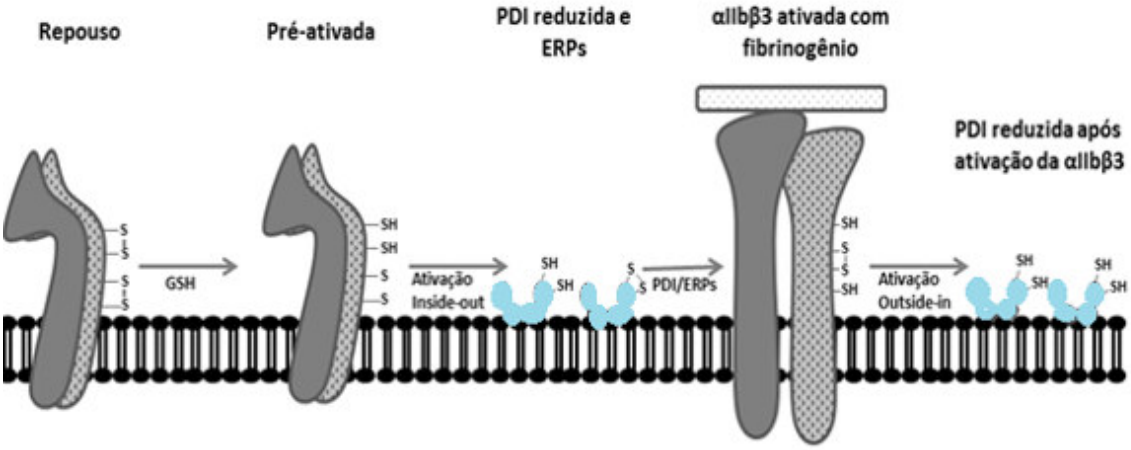

Figura 5: Mecanismo da ativação da integrina αIIbβ3 pela PDI. Mudança na estrutura conformacional da αIIbβ3 passando de um estado de baixa afinidade (repouso) para um estado intermediário que é preparado pela GSH através da geração de tióis adicionais. Estes tióis recentemente gerados participarão de nova reação que resulta no estado de alta afinidade pelo fibrinogênio e que é mediada pela PDI (em azul). Adaptado de (Essex 2008).

Linhas de pesquisa têm demonstrado que o bloqueio da atividade da PDI inibe a agregação plaquetária, embora essa inibição esteja mais direcionada a uma segunda fase do processo de agregação (Essex and Li 1999, Essex, Li et al. 2001, Bennett 2005, Essex 2009, Sousa, Gaspar et al. 2017). Experimentos em plasma citratado e utilizando como indutores epinefrina e ADP demonstraram a ocorrência de uma resposta bifásica de agregação (Essex and Li 1999). Com a adição de

bacitracina, um conhecido inibidor da PDI, o principal efeito foi a forte inibição da segunda fase de agregação, sugerindo que a fase primária é independente da PDI e que o bloqueio da fase secundária pela bacitracina compromete as alterações conformacionais facilitadas pela PDI (Essex and Li 1999).

Desta forma, a inibição da PDI tem sido apontada como um novo alvo para terapia antitrombótica, pois é distinta de proteínas da cascata de coagulação, receptores plaquetários ou proteínas de sinalização plaquetária, alvos das terapias atuais (Flaumenhaft 2013). O flavonóide quercetina foi capaz de inibir a agregação plaquetária, através da inibição da enzima, sem interferir em outras tiol isomerases (Jasuja, Passam et al. 2012). O efeito inibitório foi observado tanto em ensaios *in vitro* de agregação com plaquetas humanas lavadas ativadas com ADP e colágeno, como também, em PRP obtido de ratos após a infusão do flavonóide ativado com agonista do receptor de THB. Este trabalho propôs o flavonoide como uma nova classe de agentes antitrombóticos por inibir a PDI (Jasuja, Passam et al. 2012).

O uso de espécies vegetais ricas em compostos fenólicos como fonte de substâncias bioativas apresenta-se como uma estratégia promissora para o desenvolvimento de novas alternativas terapêuticas das doenças tromboembólicas. Além disso, os diversos mecanismos de ação na inibição da agregação plaquetária variam entre estes compostos, o que permite sugerir que tais substâncias possam ser potentes fármacos antiagregantes plaquetários.

3 Estrutura e função dos polifenois

Compostos fenólicos são pigmentos naturais amplamente distribuídos no reino vegetal, tendo sido identificados mais de 8000 destes compostos (Dreosti 2000). São componentes importantes, embora não energéticos, da dieta humana, sendo encontrados amplamente distribuídos em frutas, vegetais e seus derivados (SGARBIERI and PACHECO 1999, Aherne and O'Brien 2002).

Os compostos fenólicos são divididos em dois grupos: os flavonóides e os não flavonóides (Hodek, Trefil et al. 2002). Os flavonóides apresentam estrutura química constituída de 15 carbonos, que compõem um esqueleto de difenil propano com dois anéis benzênicos (A e B) ligados a um anel pirano (C) (Manach, Scalbert et al. 2004). De acordo com o estado de oxidação da cadeia heterocíclica do anel C, têm-se diferentes classes de flavonóides: antocianidinas, flavonóis, isoflavonas, flavononas, flavonas e catequinas (Cheynier 2005). Tais substâncias, apresentam-se tanto na forma livre (aglicona ou genina), quanto na forma ligada a um açúcar (glicona) (Paixão, Perestrelo et al. 2007). Por sua vez, os compostos fenólicos não flavonoídicos são classificados como: derivados das estruturas químicas C6-C1 específicas dos ácidos hidroxi- benzóico, gálico e elágico; derivados das estruturas químicas C6-C3 específicas dos ácidos caféico e p-cumárico hidroxi-cinamatos, além dos derivados das estruturas químicas C6-C2-C6 específicas do trans-resveratrol, cis-resveratrol e trans-resveratrol-glicosídio (Manach, Scalbert et al. 2004).

Vários efeitos biológicos têm sido atribuídos aos compostos fenólicos em virtude da sua capacidade de inibir a peroxidação de lipídeos, por radicais livres ou sistemas enzimáticos como as ciclooxigenases e lipoxigenases. Esses efeitos são atribuídos a sua propriedade de sequestrar radicais livres e de quelar cátions divalentes (Brody 1998). Estudos tem demonstrado que o risco de doenças cardíacas pode ser reduzido através do consumo de fenóis da dieta (Galleano, Calabro et al. 2012), pois inibem o desenvolvimento das doenças vasculares, reduzem a pressão arterial em indivíduos hipertensos e tem ações benéficas na obesidade devido à sua capacidade de regular a oxidação de ácidos graxos e melhorar a funcionalidade dos adipócitos (Kleemann, Verschuren et al. 2011, Oh, Endale et al. 2012). Vale salientar que além das atividades citadas sobre o sistema cardiovascular, vários compostos fenólicos tem sido descritos como potenciais inibidores da agregação plaquetária.

3.1 Mecanismos antiagregantes de compostos fenólicos

3.1.1 Aumento nas concentrações de AMPc e monofosfato cíclico de guanosina (GMPc) por flavonóides inbe a ativação plaquetária

Os efeitos inibitórios na agregação de plaquetas por nucleotídios cíclicos como o AMPc são devido à fosforilação de proteínas chave pela proteína quinase dependente de AMPc (PKA). Uma das etapas de inibição pelo AMPc inclui a inibição da ativação da PLC. Antagonistas plaquetários como a prostaciclina (PGI_2), prostaglandina E_1 (PGE_1) e prostaglandina D_2 (PGD_2), que aumentam os níveis de AMPc intracelulares, são os mais potentes inibidores da ativação plaquetária. Essas substâncias se ligam a receptores acoplados à proteína G estimulatória (Gs), estimula a adenilato ciclase e leva ao aumento do AMPc intracelular (Feijge, Ansink et al. 2004).Os níveis de GMPc na plaqueta são controlados pela ação da guanilato ciclase que é ativada por óxido nítrico (NO). No entanto, os níveis celulares de nucleotídeos cíclicos não são apenas regulados pelas enzimas que os sintetizam (adenilato e guanilato ciclases), mas também pelas enzimas que os degradam, representadas pela família das fosfodiesterases (PDEs). Existe uma importante relação entre as vias de sinalização dos dois nucleotídeos cíclicos, uma vez que, níveis elevados de GMPc exercem efeito inibitório sobre PDE3, aumentando, consequentemente, os níveis de AMPc e inibindo a ativação das plaquetas (Oh, Endale et al. 2012).

Em estudo que investigou a influência da quercetina nos níveis de AMPc em plaquetas lavadas estimuladas pelo colágeno, evidenciaram um aumento relevante destes níveis não somente pela ativação da adenilato-ciclase, mas, também pela inibição das PDEs (Dell'Agli, Maschi et al. 2008). Em um estudo *in vitro* foram avaliados os efeitos do resveratrol na produção de NO em plaquetas, os dados sugeriram aumento nesta produção, que por sua vez induziu um aumento nos níveis de GMPc com conseqüente redução na ativação de plaquetas (Gresele, Pignatelli et al. 2008). Freedman e colaboradores, ao realizarem ensaios de agregação com

plaquetas estimuladas com ADP e incubadas com suco de uva roxa, composto por: cianidina, quercetina e proantocianidina, evidenciou aumento na produção de NO e redução da agregação de 89,3% para 50,5% (Freedman, Parker et al. 2001).

3.1.2 Compostos fenólicos alteram a mobilização de Ca^{+2}

Todos os agonistas excitatórios da plaqueta, exceto a epinefrina, induzem o aumento das concentrações citosólicas de Ca^{2+}, tanto a partir da sua mobilização dos estoques internos (sistema tubular denso) quanto a partir do aumento do influxo de Ca^{2+} do meio extracelular.

Estudos de Guerrero, Lozano et al. (2005) sobre os efeitos dos flavonóides apigenina, genisteína, luteolina e quercetina no antagonismo de receptores de TxA_2 (TP), verificaram que em plaquetas humanas estimuladas com U46619 (agonista de TP) houve comprometimento na mobilização de Ca^{2+}. Além disso, foi mostrado que a quercetina inibiu fosfodiesterase (PDE) de nucleótidios cíclicos, o que resultou no aumento dos níveis de monofosfato cíclico de adenosina (AMPc) em plaquetas, levando à diminuição tanto na concentração intracelular de Ca^{2+}, quanto na ativação de plaquetas (Lanza, Beretz et al. 1987). Com efeito, em plasma rico em plaquetas (PRP), quercetina, kampferol e fisetina demonstraram antagonizar o receptor TxA_2 (Guerrero, Lozano et al. 2005). Em plaquetas humanas, foi demonstrado que o trans-resveratrol, a apigenina e a genisteína inibiu agregação de plaquetas estimuladas pela trombina através da inibição de canais de Ca^{2+} (Dobrydneva, Williams et al. 1999).

3.1.3 Flavonóides inibem a agregação plaquetária por inibição da formação de TxA_2 e da atividade de COX-1

O TxA_2 é bastante instável e é rapidamente hidrolisado no quase inativo, estável e mais mensurável metabólito tromboxano B_2 (TxB_2) (Nakahata 2008). A determinação da produção de TxB_2 em plasma rico em plaquetas é um método específico e mais comum para a avaliação da atividade de COX-1 (Gilmer, Murphy et al. 2003). Extraído de *Mitrella kentii,* o flavonóide crisina, foi testado por Saadawi e colaboradores, mostrando forte atividade inibidora na produção de TxB_2, refletindo a inibição da atividade de COX-1 (Saadawi, Jalil et al. 2012).

As formações de TxB_2 foram também inibidas por Fisetina, kamferol, quercetina em plaquetas estimuladas com AA. Os resultados demonstraram que o principal efeito antiplaquetário dos flavonóides testados pode ser devido tanto à inibição da formação de TxA_2, quanto ao antagonismo do receptor de tromboxano (Tzeng, Ko et al. 1991).

Apigenina e quercetina bloquearam o sítio ativo de COX-1, evidenciado em estudo de docagem molecular e interferiram com a conversão do AA a PGH_2 (Wu, Dastmalchi et al. 2012). Ainda, apigenina foi o mais potente antagonista de agregação plaquetária por ligar-se ao receptor de TxA_2 (Bojić, Debeljak et al. 2011). Flavonóides isolados a partir de *Sophora japonica* tiveram seus efeitos avaliados sobre a agregação plaquetária em PRP de rato, biochanina A e genisteína mostraram efeitos inibidores potentes na produção de TxA_2 em agregação induzida por AA (Kim and Yun-Choi 2008).

Resveratrol e quercetina inibiram a agregação de plaquetas induzida pelo AA, nos estudos de modelagem molecular em que mostraram encaixe dos polifenóis na estrutura cristalográfica da COX-1, sugerindo assim a inibição da agregação por inibição da COX-1 (Crescente, Jessen et al. 2009).

3.3.4 Ação de flavonóides sobre a integrina αIIbβIII

Ao estudar os efeitos das catequinas do chá verde (GTC) na agregação de plaquetas *in vitro*, o extrato de flavonóides do chá verde (catequina, epicatequina, epigalocatequina, galocatequina, galato de epicatequina galato e galocatequina) reduziu a agregação plaquetária e a expressão da integrina αIIbβ3 em plaquetas estimuladas pelo ADP, colágeno e trombina (Kang, Chung et al. 2001).

Os efeitos de uma bebida "rica" em flavonóides do cacau (procianidinas) foram observados na diminuição da expressão da integrina αIIbβ3 na agregação estimulada por epinefrina e ADP (Wolfender, Violette et al. 2010). Além disso, foi demonstrado que a Delfinidina-3-glicosídeo (DP-3-G) diminuiu a expressão da integrina αIIbβ3 ativada sobre as plaquetas, bem como, observou inibição da agregação plaquetária em ensaios com os agonistas ADP e TRAP (agonista da trombina) (Yang, Shi et al. 2012).Nos últimos anos, a espécie *Syzygium cumini*, tem recebido considerável atenção para os seus potenciais benefícios em várias doenças, especialmente, porque diversas partes da planta apresentam grandes teores de polifenóis.

4 Syzygium cumini: Potencial Fonte de compostos fenólicos antiagregante plaquetários

Syzygium cumini (L.) Skeels (família Myrtaceae) é uma espécie originária do subcontinente indiano, mas difundida por vários países, inclusive no Brasil (Samadder, Chakraborty et al. 2011). Apresenta sinonímia variada: *Syzygium jambolanum* (Lam.) DC, *Eugenia jambolana* Lam., *Eugenia cumini* (L.) Druce e *Myrtus cumini* L., sendo conhecida popularmente como jambolão (Lorenzi and de Abreu Matos 2002, Baliga, Bhat et al. 2011). *Syzygium cumini* (L.) Skeels (*S. cumini*)

é tradicionalmente utilizada na medicina indiana para tratamento de bronquite, asma, úlcera e disenterias (Nambiar 1996).

No Brasil, o gênero Syzygium, encontra-se distribuído por todas as regiões e apresenta um elevado potencial terapêutico, pois, suas espécies, especialmente o *S. cumini* é empregado pela população como adjuvantes no tratamento de diabetes tipo II (Oliveira et al, 2005). Além do controle do diabetes, diferentes partes da espécie são utilizadas na medicina tradicional. As folhas são utilizadas em gastropatias e leucorréia; as cascas são utilizadas como adstringente, antihelmíntico; os frutos são utilizados no tratamento de faringites e esplenopatias (Pepato et al, 2001).A composição fitoquímica de *S. cumini* compreende compostos como taninos hidrolisáveis, flavonoides, antocianinas, terpenos e ácidos alifáticos (Mahmoud, Marzouk et al. 2001, Shafi, Rosamma et al. 2002, Li, Zhang et al. 2009, Baliga, Bhat et al. 2011). As diferentes partes de *S. cumini* são ricas em polifenóis. O fruto e flores apresentam em sua composição antocianinas como cianidina, delfinidina, peonidina, pelargonidina, petunidina e malvidina (Sagrawat, Mann et al. 2006, De Brito, De Araujo et al. 2007), os quais detem reconhecida atividade antioxidante e anticarcinogênica (Wang and Stoner 2008, Cui, Zhang et al. 2013). A semente contém rutina e quercetina, compostos envolvidos na regulação do metabolismo glicídico e lipídico (Sharma, Viswanath et al. 2008). Enquanto as folhas possuem compostos com atividade anti-inflamatória, cardioprotetora e antioxidante como kanferol, quercetina, miricetina e seus glicosídeos (Mahmoud, Marzouk et al. 2001, Xiao, Peng et al. 2011).

Embora amplamente estudado por suas atividades anti-hiperglicemiantes (Bopp, De Bona, Belle, Moresco, & Moretto, 2009), por possuírem vasta composição de polifenois e por estes estarem ligados à atividade antiagregante plaquetária, o *S. cumini* tem despertado interesse de alguns pesquisadores sobre sua atividade em mecanismos da função plaquetária. Recentemente, os efeitos do extrato de S. cumini sobre plasma rico em plaquetas (PRP) de pacientes diabéticos mostraram uma redução na agregação das plaquetas ativadas por colágeno (Raffaelli, Borroni et al. 2015). O extrato das folhas desta mesma espécie quando posto na presença de PRP, também de pacientes diabéticos, diminuiu a atividade

da adenosina desaminase (ADA), uma moduladora da função plaquetária (De Bona, Bellé et al. 2010).

Em estudo desenvolvido por nosso grupo de pesquisa foi caracterizado o perfil fitoquímico do extrato rico em polifenois das folhas do *S. cumini* cultivado no norte da América do Sul, através de cromatografia líquida de alta eficiência acoplada à espectrometria de massas (CLAE-EM/EM), foi identificado por tentativa, a presença de três compostos: ácido gálico, miricetina e quercetina, promissores agentes antiagregantes plaquetários (Chagas, 2017. Dados não publicados).

Objetivos

5. Objetivos

5.1 Objetivo Geral

Investigar a atividade antiagregante plaquetária do Extrato Rico em Polifenóis das folhas de *Syzygium cumini* (L.) Skeels via inibição da isomerase de dissulfetos protéicos.

5.2 Objetivos Específicos

- Avaliar o efeito do ERP sobre plasma rico em plaquetas estimuladas com ADP, trombina e PMA.

- Verifcar o efeito do extrato sobre a ativação da integrina αIIbβIII em plaquetas lavadas estimuladas com trombina.

- Determinar a ação de padrões de Ácido Gálico, Miricetina e Quercetina sobre a atividade redutase da PDI.

- Verificar o efeito do ERP sobre a atividade redutase da PDI.

- Propor mecanismo de ação antiagregante plaquetário do extrato.

Manuscrito

Original Research

Antiplatelet activity of polyphenol-rich extract from *Syzygium cumini* leaf is potentially mediated by protein disulfide isomerase inhibition.

Authorship

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ABSTRACT

Introduction: Protein disulfide isomerase (PDI) has been proposed as an antithrombotic target, for which polyphenolic compounds have emerged as potential inhibitors. Previously, we have shown that *Syzygium cumini* (L.) Skeels leaf contains multiple polyphenols, which support its use for antiplatelet purposes. Therefore, this study sought to evaluate the effects of polyphenol-rich extract (PESc) from *S. cumini* leaf on platelet activation and aggregation, as well as on PDI reductase activity. **Material and Methods:** Platelet-rich plasma from healthy volunteers (n=5) was incubated with PESc (10-1000 µg/mL), for 25 min, before activation with ADP, thrombin or PMA. To analyze PESc effect on integrin αIIbβIII activation, flow cytometry studies were conducted in washed platelets pre-treated with PESc (10-1000µg/mL) and activated with thrombin before tagging with PAC–1 antibody. Finally, PESc (0.1-100 µg/mL) effects on PDI reductase activity were assessed in absence or presence of polyphenolic standards gallic acid, myricetin and quercetin. **Results and Conclusions:** PESc dose-dependently inhibited platelet aggregation despite the agonist used, even though lower agonist concentration potentiated PESc inhibitory effects to a maximal 77% inhibition at 2.5 µM ADP. Similarly, PESc dose-dependently reduced the proportion of activated αIIbβIII molecules per platelet up to one third of control at 1000 µg/mL. These effects correlated with the strong inhibitory action of PESc on PDI activity, an effect synergically augmented in presence of standards. Therefore, our data show that PESc reduces platelet aggregation and activation, probably through PDI inhibition, strengthening its prominent antiplatelet activity.

INTRODUCTION

Ischemic heart disease and stroke collectively account for one in each four deaths worldwide, mostly associated to thromboembolic disturbances (Lozano et al., 2012). In turn, thrombus formation inside blood vessels results, at least partially, from platelet activation and aggregation; a process triggered by multiple agonists, e.g. adenosine diphosphate (ADP), thrombin, and collagen, but whose downstream signaling pathways involve in all cases the activation of the platelet surface integrin αIIbβIII (Banno and Ginsberg, 2008; Ghoshal and Bhattacharyya, 2014). Integrin αIIbβIII becomes activated when a variety of stimuli, both inside-out and/or outside-in, lead to the isomerization of disulfide bonds between critical thiols on extracellular β domain (Essex, 2008).

Protein Disulfide Isomerase (PDI) is the leading member of the so-called PDI family, a set of thioredoxin-like thiol isomerases originally described in the endoplasmic reticulum, but later found in virtually all cell compartments, including platelet surface (Ellgaard and Ruddock, 2005; Essex et al., 1995). By its modulatory role on integrin αIIbβIII activation, PDI has emerged as a prominent target for antithrombotic therapy (Flaumenhaft, 2013; Flaumenhaft et al., 2015). In a recent report, we described the antiplatelet role of a synthetic peptide whose action is ascribed to its covalent binding to Cys_{400} residue on PDI a' domain redox-active site (Sousa et al., 2017). Besides, some low molecular weight molecules, both natural and synthetic, have also been shown to promote antiplatelet effects through PDI inhibition. A series of compounds denominated bepristats were found to reversibly block the hydrophobic substrate-binding site at PDI b' domain (Bekendam et al., 2016), a mechanism also described for the flavonoid quercetin-3-rutinoside (Lin et al., 2015). Accordingly, many other flavonoids and related compounds have been shown to promote antiplatelet

78 properties through diverse mechanisms (Wright et al., 2010; Wright et al., 2013a).

79 *Syzygium cumini* (L.) Skeels (Fam.: Myrtaceae; Syn.: *Syzygium*
80 *jambolanum* (Lam.) DC, *Eugenia jambolana* Lam., *Eugenia cumini* (L.) Druce) is
81 a worldwide cultivated medicinal plant, popularly known as jamun, black plum,
82 jambolan and jambolão (Ayyanar and Subash-Babu, 2012). Because of its high
83 polyphenolic content, *S. cumini* has been proposed as a prominent source of
84 bioactive compounds against cardiometabolic disorders (Chagas et al., 2015).
85 We have previously shown that this species' leaf contains a wide diversity of
86 polyphenolic compounds, mostly hydrolysable tannins and flavonoids, specially
87 myricetin derivatives, which were implicated in the improvement of metabolic
88 outcomes in obese rats (Sanches et al., 2016). Noteworthy, *S. cumini* has also
89 been shown to inhibit hyperactivation of platelets from diabetic patients (De Bona
90 et al., 2010; Raffaelli et al., 2015).

91 Therefore, in the present study, we hypothesized that a polyphenol-rich
92 extract (PESc) from *S. cumini* leaf would present potential antiplatelet properties.
93 Of importance, PESc phytochemical analysis by HPLC-MS/MS allowed the
94 identification of five main compounds: gallic acid, myricetin, myricetin-3-α-
95 arabinopyranoside, myricetin deoxyhexoside and quercetin (Chagas et al, 2017;
96 under review). Moreover, given the already described antiplatelet properties of
97 quercetin-3-rutinoside through PDI inhibition, we also sought to characterize
98 PESc effects on PDI function *in vitro* as a feasible mechanism of action. Data
99 herein presented endorse our hypothesis, as well as describe PESc anti-PDI
100 effect, corroborating the use of *S. cumini* leaf constituents as potential
101 antithrombotic compounds.

MATERIAL AND METHODS

Botanical material

S. cumini leaves were collected from different trees at the Federal University of Maranhão campus (São Luís, Maranhão, Brazil) and were identified and cataloged in the Herbarium MAR of the Department of Biology of the same institution under nº 4573.

Extract preparation

The extract was prepared according to Sharma et al. (2008), with modifications. Fresh leaves were dried at 38°C and pulverized into powdered dry leaves (150g), and then macerated in 70% ethanol (1:6, w/v) under constant stirring for 3 days (solvent renovation every 24h) at 25°C. This material was filtered with filter paper and the residual powder submitted to the same process twice, totaling three extractions. The extracts were pooled, centrifuged at 3500 rpm for 10 minutes at 25 °C. The supernatant was concentrated in a rotavaporator to obtain the crude hydroalcoholic extract (HE). HE was partitioned with chloroform (1:1 v/v, 3x) and the organic phase was washed with ethyl acetate (1:1 v/v, 3x). The ethyl acetate fraction was concentrated (38 °C) and lyophilized, yielding the polyphenol-rich extract (PESc).

Platelet-rich plasma and washed platelets preparation

Healthy volunteers who did not use antiplatelet drugs and had previously signed informed consent had their blood samples collected in tubes containing citric acid dextrose (CAD, 9:1, v/v). Platelet-rich plasma (PRP) and washed platelets (WP) were prepared as previously described (Sousa et al., 2017). Briefly, the blood was centrifuged at 250 x g for 10 minutes at 22°C to obtain

PRP, which was centrifuged again (800 x g, 10 min, 22°C). The platelet pellet was diluted in Ca^{2+}-free Tyrode buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.4) containing CAD (Tyrode:ACD 9:1 v/v) and centrifuged again. Finally, WP were diluted in Tyrode buffer, the final concentration adjusted to 2-3 x 10^8 platelets/mL and used within 4 hours.

Platelet aggregation

PRP aggregation assays were performed on a four-channel APACT-4 aggregometer (Helena Biosciences, Gateshead, England) as described (Sousa et al., 2017). PRP samples (2-3 x 10^8 platelets/mL) were incubated 25 minutes at 37°C with 10, 30, 100, 300 or 1000 µg/mL of PESc prior to the addition of ADP (2.5 and 5 µM, Sigma Chemical Co, Saint Louis, USA), thrombin (THB, 0.01 and 0.02 U/mL, Sigma Chemical Co., St. Louis, USA) or phorbol-12-myristate-13-acetate (PMA, 100 nM, Sigma Chemical Co., St. Louis, USA). Aggregation was recorded for 5 minutes with PRP from at least 3 different donors.

Flow cytometry

WP (2-3 x 10^8 platelets/mL) were incubated for 25 min at 37º C with PESc at 10, 100 and 1000 µg/mL. Then WP were incubated for 10 minutes with THB (0.02 U/mL). 2 mM C^{a2+} was added 2 min berofe activation. Controls corresponded to non-activated platelets which were used to characterize the basal platelet activation. PESc 1000 µg/mL was used to determine possible auto-fluorescence. Next, platelet activation was measured by incubation of the PAC-1 FITC antibody (PAC-1 clone, BD Biosciences, Franklin Lakes, USA) for 10 minutes in dark and fluorescence read out on a FACS Calibur (BD Biosciences, Franklin Lakes, USA). Fluorescence data were acquired from a total count of 2.5

151 x 10^4 cells per sample and analyzed using FlowJo VX software (Tree Star, USA).

152 **PDI reductase activity assay**

153 The insulin precipitation assay was performed to verify the inhibitory effect
154 of PESc on PDI reductase activity by analysis of insulin β-chain precipitation at
155 650 nm (de et al., 2011). Initially, PDI reductase activity was evaluated in the
156 presence of the phenolic standards: gallic acid (GLA), myricetin (MYR) and
157 quercetin (QCT) at concentrations of 0.1, 1 and 3 µg/mL. Then, the effect of PESc
158 (0.1, 0.5, 1, 3, 10, 30, and 100 µg/mL) on enzyme reductase activity was tested.
159 Finally, to determine a possible synergistic activity between the pure compounds
160 and PESc, 0.1 µg/mL of each standard was added to 10 µg/mL PESc. In all
161 experiments, PDI was incubated with PESc and/or standards for 1 hour at 37°C
162 to ensure interaction between the enzyme and the compounds. At the end of the
163 incubation period, DTT (1 mM) and insulin (1 mg/mL) were added in 100 mM
164 sodium phosphate buffer at pH 6.5, and the reaction read at 650 nm for 1 hour
165 (Gonzalez-Perilli et al., 2017).

166 **Statistical analysis**

167 Quantitative results were expressed as mean ± SEM of 3 to 6 independent
168 experiments per protocol. Statistical analysis was performed by One way ANOVA
169 and Newman Keuls posttest, considered significant when $p < 0.05$ (GraphPad
170 Prism 6.0).

RESULTS AND DISCUSSION

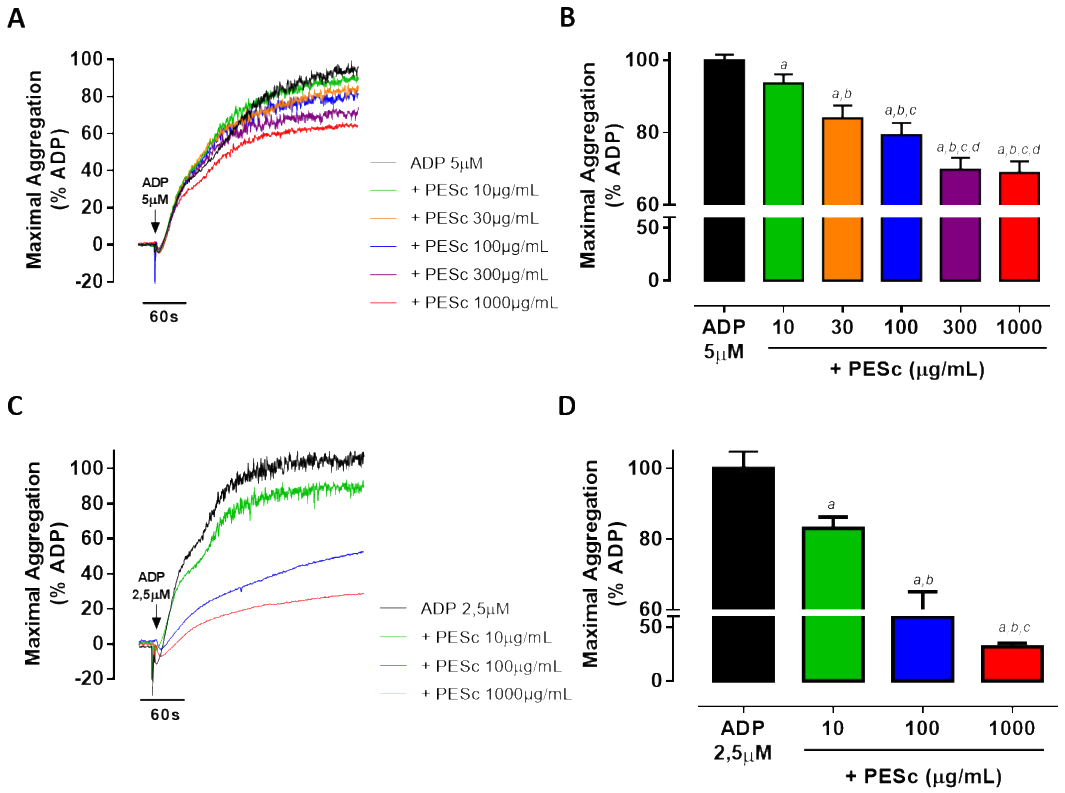
PESc decreases ADP-, THB- and PMA-induced platelet aggregation

PESc incubation at increasing concentrations decreased 5μM ADP-induced platelet aggregation in a concentration-dependent manner, with maximal aggregation being reduced up to 31.2 ± 3.1% at the maximal concentration used (1000 μg/mL PESc; Figures 1A and 1B). When ADP concentration was lowered to 2.5 μM, PESc action was potentiated and maximal aggregation significantly lowered to 83.0 ± 3.2 %, 59.0 ± 5.6 % and 32.0 ± 3.1 %, at the concentrations of 10, 100 and 1000 μg/mL, respectively, (Figures 1C and D). As well, PESc also decreased 0.02 U/mL THB-triggered PRP aggregation (Figures 2A and B), suggesting that the inhibitory process is independent of the agonist used. Again, by halving the agonist's concentration, PESc inhibitory effect was potentiated, as aggregation inhibition in the presence of 1000 μg/mL PESc was augmented from 35.0 ± 1.8 % to 55.3 ± 3.2 % in comparison to the one exerted in the absence of the extract (Figure 2).

ADP triggers platelet aggregation by binding to $P2Y_1$ e $P2Y_{12}$ receptors. $P2Y_1$ activates, via Gq protein, β isoforms of phospholipase C (PLC) and leads to hydrolysis of 4,5-biphosphate phosphatidyl inositol generating inositol-triphosphate (IP3) and diacylglycerol (DAG), increasing cytoplasmic Ca^{2+} levels and activating protein kinase C (PKC), respectively (Newton, 1997; Salzman and Ware, 1989; Savage et al., 2001). On the other hand, $P2Y_{12}$ inhibits, via Gi protein, the protein adenylate cyclase to reduce cyclic adenosine monophosphate (cAMP) levels in the intracellular space (Davì and Patrono, 2007; Offermanns, 2006). Likewise, THB stimulates its PAR1 and PAR4 receptors, which will also trigger PKC activation (Offermanns, 2006; Zaid et al.,

196 2015). Interestingly, these receptors may present differential sensibility
197 depending on agonist concentration. For instance, PAR1 mediates platelet
198 responsiveness to low concentrations of THB, whereas PAR4 is activated at
199 higher concentrations (Offermanns, 2006). Similar behavior has been described
200 by us (Sousa et al., 2017) and others (Kim et al., 2013) for ADP and THB actions,
201 which are probably involved in the amplified inhibitory action of PESc when facing
202 lower concentrations of these agonists.

203



204

205 **Figure 1. PESc decreases platelet aggregation induced by ADP in platelet rich plasma.** All
206 protocols were performed on platelet rich plasma (PRP, 2-3x10^8 platelets/ml, 37°C) incubated for
207 25 minutes with PESc prior to addition of the agonist. A and B: Curves representative of the
208 maximum aggregation of platelets activated with ADP (5μM) in absence or presence of PESc in
209 the indicated concentrations and with the following statistical significance: [a]p<0.05 *vs* ADP;
210 [b]p<0.05 *vs* PESc 10μg/mL; [c]p<0.05 *vs* PESc 30μg/mL; [d]p<0.05 *vs* PESc 100μg/mL; [e]p<0.05 *vs*
211 PESc 300g/mL. C and D: Curves representative of the maximum aggregation of platelets
212 activated with ADP (2.5 μM) in absence or presence of PESc at the indicated concentrations and
213 with the following statistical significance. [a]p<0.05 *vs* ADP; [b]p<0.05 *vs* ERP 10μg/mL; [c]p<0.05 *vs*
214 100μg/mL PESc. All vertical bars represent mean ± SEM of the maximum percent aggregation in
215 at least 3 independent experiments. Arrows indicate when agonists have been added.

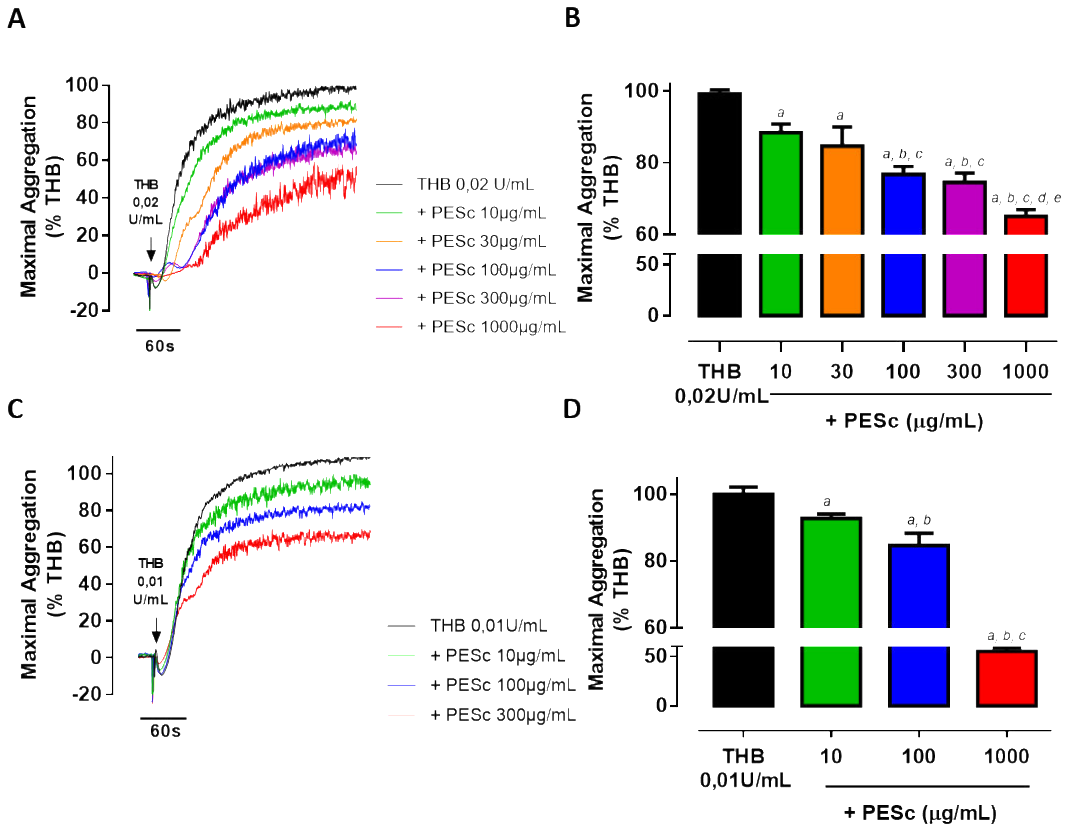
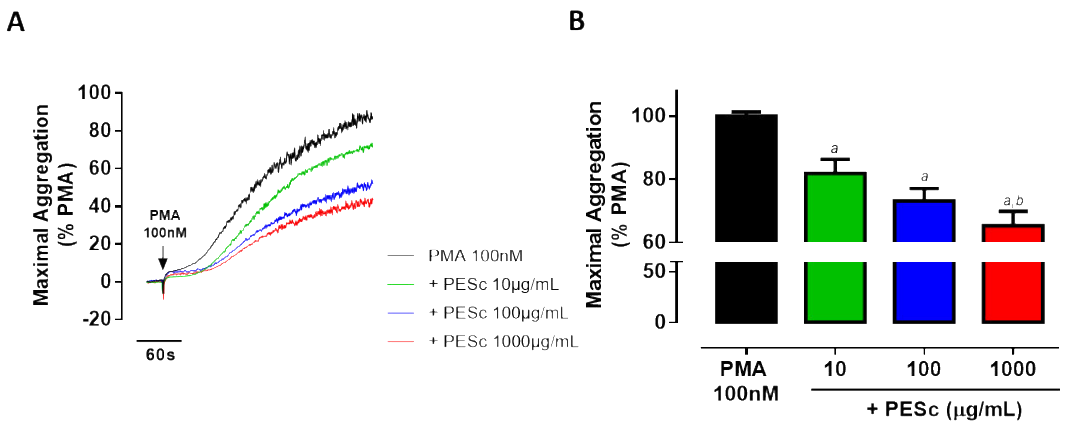


Figure 2. PESc reduces platelet aggregation induced by THB in platelet rich plasma. All protocols were performed on platelet rich plasma (PRP, 2-3x10^8 platelets / ml, 37°C) incubated for 25 minutes with PESc prior to addition of the agonist. A and B: Curves representative of the maximum aggregation of activated platelets with THB (0.02U/mL) in absence or presence of PESc at the indicated concentrations and with the following statistical significance: [a]p<0.05 *vs* THB; [b]p<0.05 *vs* PESc 10µg/mL; [c]p<0.05 *vs* PESc 30µg/mL; [d]p<0.05 *vs* PESc 100µg/mL; [e]p<0.05 *vs* PESc 300g/mL. C and D: Curves representative of the maximum aggregation of platelets activated with THB (0.01U / mL) in absence or presence of PESc at the indicated concentrations and with the following statistical significance. [a]p<0.05 *vs* THB; [b]p<0.05 *vs* PESc 10µg/mL; [c]p<0.05 vs 100µg/mL PESc. All vertical bars represent mean ± SEM of the maximum percent aggregation in at least 3 independent experiments. Arrows indicate when agonists have been added.

Assessment of a *S. cumini* extract on collagen-activated platelets from diabetic patients resulted in diminished aggregation and augmented membrane fluidity, properties ascribed to the extract antioxidant capacity (Raffaelli et al., 2015). Previously, it had been shown that a leaf aqueous extract inhibited adenosine deaminase (ADA) activity in platelets from similar diabetic patients, an effect potentially relevant to platelet aggregation (De Bona et al., 2010). Extracts from other polyphenol-rich plant species, such as: *Operculina macrocarpa*

(Pierdoná et al., 2014), *Vitis vinifera* and *Aronia melanocarpa* (Bijak et al., 2013) also displayed anti-platelet properties ascribed to their phenolic content. Corroborating our findings, the mixture made of *O. macrocarpa* roots inhibited platelet aggregation evoked by different agonists in a similar way (Pierdoná et al., 2014). The broadness of these effects led us to hypothesize that PESc constituents would target a mediator shared by distinct platelet-activating pathways.

Taking this into account, we next evaluate whether PESc was able to impair PMA-induced platelet aggregation, since it is a cell-permeant PKC activator (MELLOR and PARKER, 1998; Shibasaki, 2000). As shown in Figure 3, PESc was able to decrease PMA-activated maximal aggregation in a dose-dependent manner (Figure 3). Under our experimental conditions a maximal inhibition of 67.0 % ± 2.8 % at 1000 µg/mL PESc. A previous study has shown that a mixture of quercetin and catechin, but not each one individually, inhibited PKC activity with consequent decrease of activation. PKC inhibition decreased the phosphorylation of platelet NADPH oxidase (NOX) $p47^{phox}$ subunit , reducing superoxide (O_2^{-}) generation and leading to increased NO bioavailability (Pignatelli et al., 2006).



255

256 **Figure 3. PESc decreases platelet aggregation induced by PMA in platelet rich**
257 **plasma.** All protocols were performed on platelet rich plasma (PRP, 2-3x10^8 platelets/ml,
258 37ºC) incubated for 25 minutes with PESc prior to addition of the agonist. A and B:
259 Curves representative of the maximum aggregation of platelets activated with PMA
260 (100nM) in absence or presence of PESc at the indicated concentrations and with the
261 following statistical significance: [a]p<0.05 *vs* PMA; [b]p <0.05 *vs* ERP 10 µg/mL; [c]p<0.05 *vs*
262 100µg/mL PESc. All vertical bars represent mean ± SEM of the maximum percent
263 aggregation in at least 3 independent experiments. Arrows indicate when agonists have
264 been added.

265

266 **PESc inhibits THB-induced αIIbβ3 integrin activation**

267 Even though, NOX inhibitors have also been shown to inhibit platelet
268 aggregation by down-regulatign integrin αIIbβ3 expression in a NO-independent
269 way (Begonja et al., 2005). This set of data reinforced our hypothesis of a common
270 target or signaling branch for PESc anti-platelet actions, leading us to evaluate its
271 effect on the activation of the αIIbβ3 integrin. This platelet-exclusive integrin is the
272 ultimate converging target of platelet-activating pathways triggered by diverse
273 agonists as ADP, THB, thromboxane A_2 (TxA_2), collagen, epinephrine and platelet
274 activating factor through inside-out mechanisms mostly mediated by PKC (Banno
275 and Ginsberg, 2008; Ghoshal and Bhattacharyya, 2014)

276 Incubation of WP with PESc (10, 100 and 1000 µg/mL) reduced PAC-1
277 positive platelets in 4.0 ± 0.6, 10.0 ± 0.4 and 19.0 ± 0.8 %, respectively (Figures
278 4A – D). Nevertheless, this effect was even more evident when analyzing the

average fluorescence emitted by these activated platelets. Figure 4E shows that PESc dose-dependently decreased the number of activated integrin αIIbβ3 molecules per platelet, inhibiting integrin activation in more than 60%. Our results are in accordance to reports in the literature showing that a green tea flavonoid-rich extract reduced platelet aggregation and integrin αIIbβ3 activation upon stimulus with ADP, THB or collagen (Kang et al., 2001). Similar results were showed for a procyanidins-containing cocoa beverage (Wolfender et al., 2010). Moreover, a quercetin-rich extract of *Anonia melanocarpa* also decreased αIIbβ3 integrin activation by H_2O_2 (Olas et al., 2010).

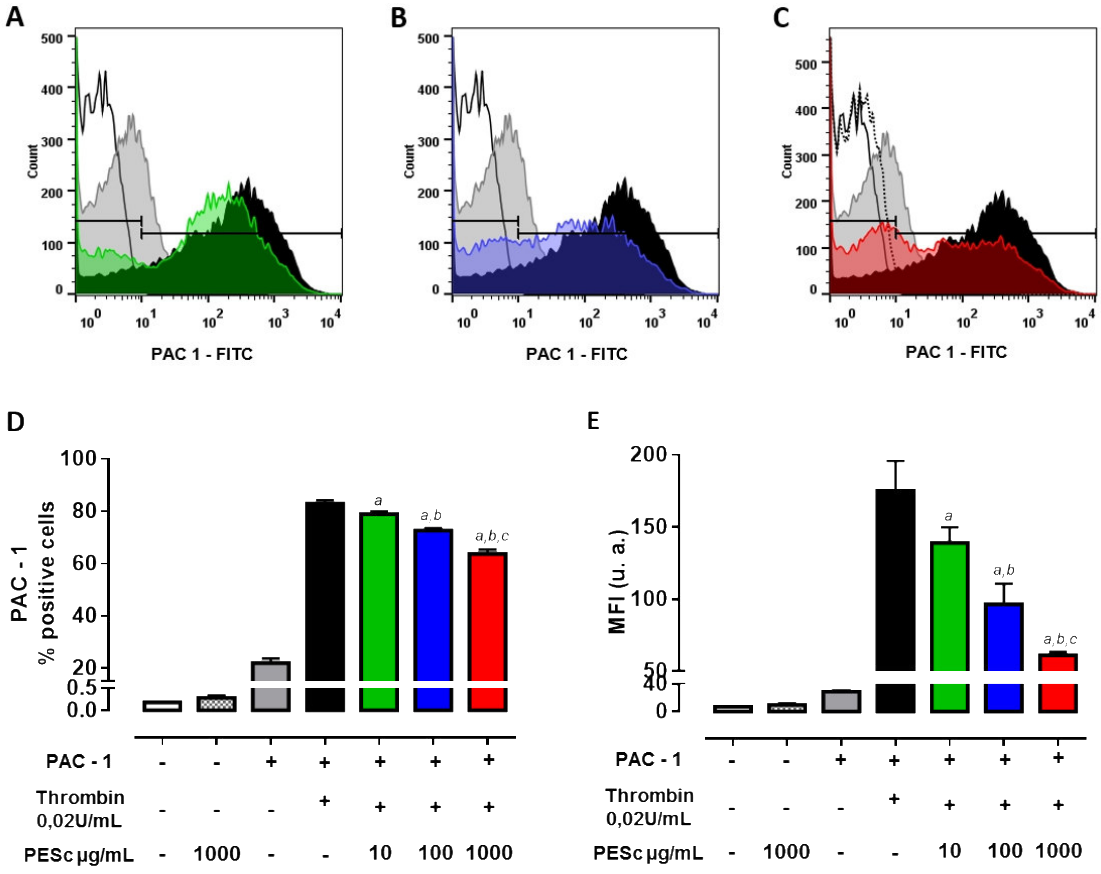


Figure 4. PESc decreases the activation of integrin αIIbβ3 in THB-activated platelets. **W**ashed platelets (2-3x10^8 cells / ml) were incubated with PESc (10, 100 and 1000 µg / ml, 25 min, 37°C) prior to activation by thrombin (0.02 U/ml, 10 min, 37°C) and then exposed to PAC-1 FITC antibodies. Platelets at rest, without labeling and without incubation with the extracts were used as baseline. A: PESc 10µg/mL. B: PESc 100µg/mL. C: 100µg/mL are shown separated from each other, in their respective homes and activated states. [a]p <0.05 *vs* THB; Other letters p <0.05 *vs* all The histograms are representative of at least 3 independent experiments.

296 **PESc inhibits PDI reductase activity**

297 In circulating platelets, triggering inside-out signaling pathways ultimately
298 converts integrin αIIbβ3 from its resting to its active conformation by binding of
299 two cytosolic proteins, talin and kindling-3 (Moser et al., 2009). However, redox
300 mechanisms have been increasingly implicated in this process, particularly the
301 isomerization of very specific disulfide bonds on αIIbβ3 by members of the so-
302 called PDI family, from which PDIA1 is the most abundant and physiologically
303 relevant (Essex, 2008; Furie and Flaumenhaft, 2014). Notwithstanding, PDI's
304 modulating role still extends to Mn^{+2}-evoked outside-in pathway of integrin αIIbβ3
305 activation (Essex, 2008). Thus, given this tight association between integrin
306 αIIbβ3 and PDI, we next investigated if PESc inhibitory effect on integrin
307 activation would be related to a possible inhibition of PDI activity.

308 The well-characterized *in vitro* reaction between PDI and insulin, where
309 the former reduces a disulfide bond in the latter, causing insulin's β-chain
310 precipitation, was used to determine if PESc as well as standards of phenolic
311 compounds previously identified in our extract, were able to inhibit PDI activity.
312 Pre-incubation of GLA and MYR (0.1, 1 and 3 µg/mL) decreased PDI reductase
313 activity in a concentration-dependent manner, with inhibition varying from 34.0 ±
314 0.6 % to 71.5 ± 2.3 % and from 64.0 ± 1.2 % to 76.0 ± 1.6 %, respectively (Figure
315 5A). QCT also inhibited PDI being maximal at the lowest concentration used (0.1
316 µg/mL), exerting similar effects at all concentrations tested under our
317 experimental conditions. PESc significantly decreased PDI reductase activity
318 from 1 µg/mL (35.0 ± 1.9 %) to 67 ± 1.6 % at 100 µg/mL (Figure 5B).

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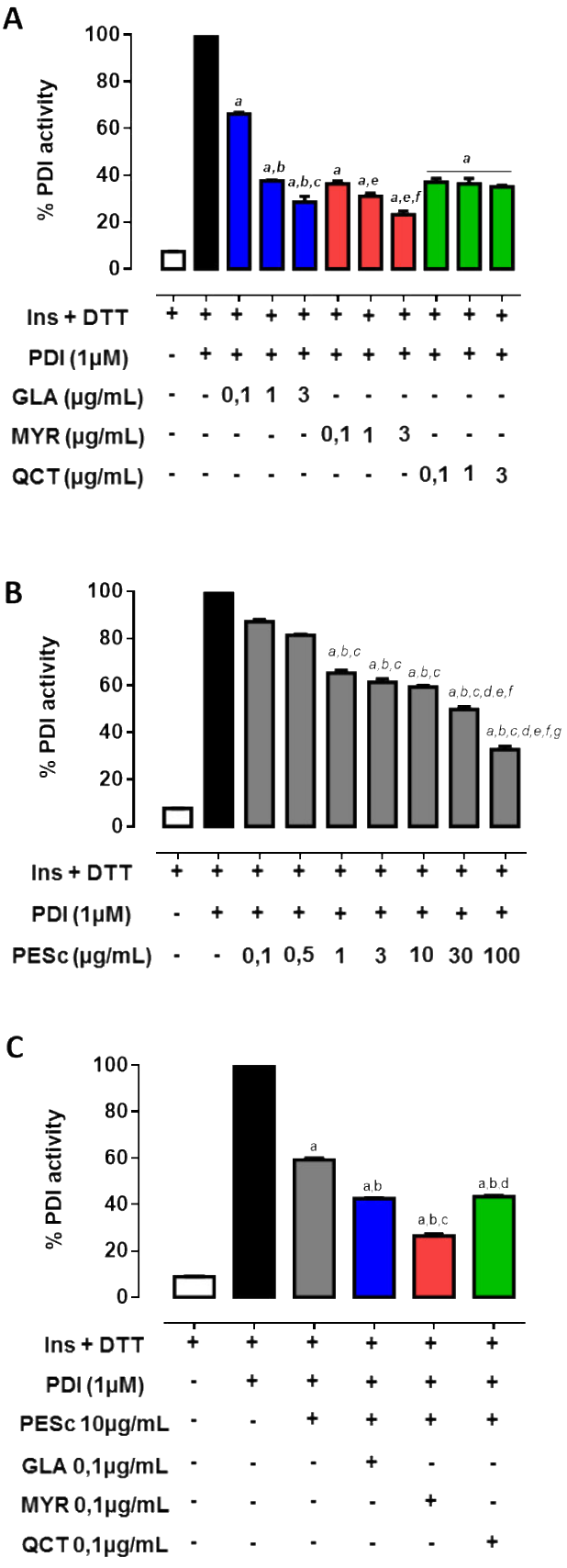

Figure 5. PDI activity is decreased in the presence of PESc. Decreased insulin by PDI-catalyzed DTT was used to evaluate the inhibitory effects of the extracts and the PDI activity was assessed in the absence or presence of PESc. A: Gallic acid (GLA), myricetin (MYR) and quercetin (QCT), B: pure PESc and C: PESc (10µg/mL) supplemented with 0.1µg/mL of each standard and a reducing agent (DTT) at 1mM concentration were also used. PDI and/or the isolated extracts were incubated for 25 min at the indicated concentrations in a phosphate buffer containing 1 mg/ml human insulin (Ins). The reaction was started by the addition of DTT, and precipitation of the insulin A chain was monitored at 650 nm. The mean activity of each condition was normalized to 100% of the PDI activity. All results shown represent an average of three to four independent experiments. ([a]$p<0.05$ *vs* 1µM PDI; other letters represent each condition $p<0.05$ *vs* all).

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Given that plant extracts are a mixture of compounds, their biological actions often come from synergism among such molecules, targeting different targets or acting by distinct mechanisms of action (Yang et al., 2014), as aforementioned for the associative action of the pair quercetin-catechin on PKC activity (Pignatelli et al., 2006). Likewise, we assessed the possible synergism between PESc and the same phenolic standards. Figure 5C shows the inhibition exerted by 10 µg/mL PESc on PDI activity, reducing the activity in 40.0 ± 1.2 %, an effect that was potentiated in presence of GLA, MYR and QCT. Since the inhibition observed was higher that the simple addition of the separated effects, our data suggest a larger synergism between PESc and myricetin, whose derivatives compose the major part of the extract.

The inhibition of PDI has arisen as a prominent mechanism to treat or prevent thromboembolic events, leading to the recent demonstration of low molecular weight molecules, both natural and synthetic, able to inhibit it (Flaumenhaft, 2013; Sousa et al., 2017; Wang and Essex, 2017). Particularly, the flavonoid rutin (quercetin-3-rutinoside) has emerged as an anti-thrombotic agent that specifically inhibits PDI (Jasuja et al., 2012), via reversible interaction with the substrate-binding site located in the b' domain of this enzyme (Lin et al., 2015). Similar mechanism has been shown for bepristats, a new class of platelet inhibitors (Bekendam et al., 2016). Besides quercetin, the myricetin derivatives identified in PESc might also been involved in the inhibitory activity herein described. To the best of our knowledge, this is the first time myricetin is shown to inhibit PDI reductase activity.

Myricetin differs from quercetin only on the hydroxylation degree of its B ring, being tri-hydroxylated at the positions 3', 4' and 5', whereas quercetin is di-

hydroxylated at 3' and 5' positions (Rice-Evans et al., 1996), a difference that does not interfere with their potencies as platelet inhibitors (Wright et al., 2013b). Therefore, it is reasonable that myricetin derivatives harboring 3-*O*-glycosylation inhibit PDI and platelet aggregation, similar to rutin (Jasuja et al., 2012). On the other hand, myricetin seems to be able to form thiol adducts through carbons 2' and 6' on ring B (Masuda et al., 2013), which also makes reasonable to speculate that myricetin inhibition of PDI would come from its binding to PDI reactive thiols, such as those present on CGHC active sites. This supposition is further supported by our recent description that CxxC, a synthetic dithiol dodecapeptide, forms a thiol adduct with Cys_{400} on PDI a' domain, which has been assigned as the mechanism responsible for CxxC antiplatelet properties (Sousa et al., 2017).

Our study, however, has some critical issues that deserve consideration. Firstly, it is possible that PESc antiplatelet effects simply come from its antioxidant capacity, even though this hypothesis is weakened by the fact that myricetin-3-α-arabinopyranoside is the most prevalent compound in PESc and substituents at C3 on ring C promote significant loss of antioxidant activity (Rice-Evans et al., 1996). Secondly, PESc effects could come from the direct action of its constituents on $O_2^{-\cdot}$ generation by platelet NOX, as already showed for quercetin and myricetin (Pignatelli et al., 2006; Wright et al., 2013a). Thirdly, PDI has been shown to closely associate and modulate distinct members of NOX family in the vascular system (de et al., 2011; Laurindo et al., 2008), disclosing an additional mechanism by which PESc would inhibit platelet function. Thus, future studies should further address molecular mechanisms underlying the actions of PESc constituents on PDI activity, as well as other possible mechanisms involved in the properties herein described.

In conclusion, this study shows that the PESc prepared from *S. cumini* leaf decreases platelet activation and aggregation evoked by agonists acting either extra or intracellularly. Furthermore, our data consistently support the inhibition of platelet PDI as the potential mechanism underlying such effects. Nevertheless, this study also accounts for the well-described cardiometabolic properties of *S. cumini*, supporting its use as a complementary and alternative medicine for treatment of patients presenting platelet hyperactivity associated to metabolic disturbances.

DISCLOSURE OF INTEREST

The authors declare no actual or potential conflict of interest

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Considerações Finais

Os resultados descritos neste trabalho, mostram como o extrato rico em ácido gálico, miricetina e quercetina de *S. cumini*, pode proporcionar a diminuição da agregação de plaquetas ativadas por ADP, trombina e PMA, através da inibição da atividade da PDI. Essa inibição pode resultar numa diminuição de ativação da integrina αIIbβ3 da superfície plaquetária.

Futuramente, serão testados os compostos AGL, MIR e QUER isolados do ERP de *S. cumini* em testes *in vitro* de agregação plaquetária e sobre a ativação da integrina. Serão testados também sobre a atividade da PDI e sobre a atividade da PKC.

Por meio dos resultados obtidos neste trabalho espera-se um maior enfoque para espécies vegetais ricas em compostos fenólicos inibidores da PDI, por já ter sido provado, a importância dessa inibição na fisiologia das plaquetas, a fim de, consolidar os inibidores dessa proteína como uma nova e promissora classe de agentes antitrombóticos.

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Anexos

APÊNDICE A – TERMO DE CONSENTIMENTO

UNIVERSIDADE FEDERAL DO MARANHÃO
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE
TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

1. Apresentação:

O Sr(a) está sendo convidado a participar do projeto de pesquisa "Investigação da atividade anti-agregante plaquetária de compostos fenólicos presentes nas folhas de *Syzygium cumini* (L.) Skeels via inibição da isomerase de dissulfetos protéicos (PDI)" na **qualidade de doador voluntário**. Neste projeto pretendemos validar a atividade antiagregante plaquetária de peptídeos sintéticos com vistas ao desenvolvimento de futuros medicamentos para o tratamento de distúrbios tromboembólicos. Este é um estudo inteiramente *in vitro*, ou seja, não lhe utilizaremos para a administração ou aplicação de qualquer substância. **O que lhe solicitamos** neste momento é a doação de **uma amostra de sangue** que será coletada por pessoa qualificada e com o uso de material estéril, **em um volume máximo de 50 mL**. Esta amostra será utilizada para o fim específico e exclusivo de separação de plaquetas, as quais serão utilizadas nos experimentos previstos pelo projeto.

Caso não se sinta à vontade para aceitar, por quaisquer motivos que sejam, o Sr(a) tem total liberdade para recusar este convite sem qualquer ônus ou prejuízo. Em aceitando, lhe agradecemos por contribuir para o desenvolvimento científico do nosso Estado.

2. Consentimento:

São Luís, _____ de _____ de _____.
Eu,_____
__, CPF/RG _____, aceito ser doador voluntário de amostra de sangue para o estudo acima descrito e que foi me foi apresentado e explicado pelos pesquisadores abaixo –assinados.

3. Pesquisadores responsáveis:

_____	_____
Samira Abdalla da Silva	Prof. Dr. Antonio Marcus de A. Paes
TEL: (98) 984272367	TEL: (98) 99226-0505

UNIVERSIDADE FEDERAL DO MARANHÃO

Fundação Instituída nos termos da Lei nº 5.152, de 21/10/1966 – São Luís - Maranhão.

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA

PARECER CONSUBSTANCIADO		
X PROJETO DE PESQUISA	Número do Protocolo:	23115-017432/2011-29
PROJETO DE INICIAÇÃO CIENTÍFICA	Data de entrada no CEP	12/12-2011
TRABALHO DE CONCLUSÃO DE CURSO	Data da assembléia	01/03/2012

I - Identificação:

Título do projeto:	**Investigação da atividade anti-agregante plaquetária in vitro de peptídeos inibidores da dissulfeto isomerase protéica**				
Identificação do Pesquisador Responsável:	**Prof. Dr. Antonio Marcus de Andrade Paes**				
Identificação da Equipe executor:	**Prof. Dr. Antonio Marcus de Andrade Paes, Profa. Dra. Ana Paula dos Santos, Elyjany Morais Lima, Lucas Martins França**				
Instituição onde será realizado:	**Departamento de Ciências Fisiológicas- CCBS/UFMA**				
Área temática:	**III**	Multicêntrico:	**Não**	Data de recebimento:	**01/03/2012**
Cooperação estrangeira:	**Não**	Patrocinador:	**Não**	Data de devolução	**27/02/12**

II - Objetivos:

Investigar os efeitos de peptídeos sintéticos ditiólicos PDI – miméticos sobre a agregação plaquetária in vitro e o mecanismo molecular dos mesmos.

III - Sumário do projeto:

O presente projeto busca avaliar a potencial atividade antiagregante plaquetária dos de peptídeos sintéticos ditiólicos PDI – miméticos, de modo a ser capaz de propor um mecanismo de ação que os eleve ao patamar de potenciais agentes farmacológicos antitrombogênicos. Visa desenhar e sintetizar novos peptídeos com base na estrutura tridimencional da PDI e submeter pedido de depósito de patente junto ao Instituto Nacional de Propriedade Intelectual (INPI).

IV - Comentários do relator:

Projeto não atende a descrição detalhada e ordenada do projeto de pesquisa, conforme prega a Resolução 196/96, pois não esclarece como ocorrerá o recrutamento dos voluntários e a coleta da amostra de sangue. Consta o Cv Lattes de toda a equipe executora.

V - Pendências:

Nenhuma

VI – Recomendações:

Nenhuma

VII - Parecer Consubstanciado do CEP

Foram apresentados os documentos enumerados em **Pendências**; desse modo, o **23115-017432/2011-29,** referente a pesquisa de Dissertação de Mestrado sob o título **Investigação da atividade anti-agregante plaquetária in vitro de peptídeos inibidores da dissulfeto isomerase protéica**. É considerado por este **CEP COMO APROVADO.**

Journal of Thrombosis and Haemostasis, **15**: 1–11 DOI: 10.1111/jth.13633

IN FOCUS

Novel antiplatelet role for a protein disulfide isomerase-targeted peptide: evidence of covalent binding to the C-terminal CGHC redox motif

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See also Wang L, Essex DW. A new strategy for development of antithrombotic agents: inhibition of the C-terminal active site of PDI. *J Thromb Haemost* 2017; DOI: 10.1111/jth.13634.

Essentials

- Inhibitors of protein disulfide isomerase (PDI) have been considered a new antithrombotic class.
- CxxC is a PDI-targeted peptide that has been previously shown to inhibit its reductase activity.
- CxxC binds to surface PDI and inhibits ADP- and thrombin-evoked platelet activation and aggregation.
- CxxC binds to Cys_{400} on CGHC redox motif of PDI *a*' domain, a site for PDI prothrombotic activity.

Summary. *Background:* Protein disulfide isomerase (PDI) plays a major role in platelet aggregation, and its inhibitors have emerged as novel antithrombotic drugs. In previous work, we designed a peptide based on a PDI redox motif (CGHC) that inhibited both PDI reductase activity and PDI-modulated superoxide generation by neutrophil Nox2. Thus, we hypothesized that this peptide would also inhibit platelet aggregation by association with surface PDI. *Methods:* Three peptides were used: CxxC, containing the PDI redox motif; Scr, presenting a scrambled sequence of the same residues and AxxA, with cysteines replaced by alanine. These peptides were tested under platelet aggregation and flow cytometry protocols to identify their possible antiplatelet activity. We labeled membrane free thiol and electrospray ionization liquid chromatography tandem mass spectrometry to test for an interaction. *Results:* CxxC decreased platelet aggregation in a dose-dependent manner, being more potent at lower agonist concentrations, whereas neither AxxA nor Scr peptides exerted any effect. CxxC decreased aIIbb3 activation, but had no effect on the other markers. CxxC also decreased cell surface PDI pulldown without interfering with the total thiol protein content. Finally, we detected the addition of one CxxC molecule to reduced PDI through binding to Cys400 through mass spectrometry. Interestingly, CxxC did not react with oxidized PDI. *Discussion:* CxxC has consistently shown its antiplatelet effects, both in PRP and washed platelets, corroborated by decreased aIIbb3 activation. The probable mechanism of action is through a mixed dissulphide bond with Cys400 of PDI, which has been shown to be essential for PDI's actions. *Conclusion:* In summary, our data support antiplatelet activity for CxxC through binding to Cys400 in the PDI a0 domain, which can be further exploited as a model for sitedriven antithrombotic agent development.

Keywords: antithrombotic agents; oxidation–reduction; peptides; platelet aggregation; protein disulfide isomerase.

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Introduction

Protein disulfide isomerase (PDI) is the prototypic member of an oxidoreductase family of enzymes, the PDI

family, whose primary function is to catalyze redox protein folding in the endoplasmic reticulum (ER) [1]. Based on a multidomain structure, the role of PDI in disulfide oxidation, reduction and isomerization reactions requires vicinal thiols in its catalytic motifs (CGHC), located in the *a* and *a′* domains [2], besides a substrate-binding hydrophobic pocket located in the *b′* domain [3]. Outside of the ER, PDI has also been found on the cell surfaces of several cell types, such as lymphocytes, neutrophils, and particularly platelets, where this enzyme is functionally active and has proved to be critical to thrombus formation [4–6].

PDI inhibitors, such as bacitracin, scrambled RNase, or anti-PDI antibodies, inhibit platelet activation and aggregation [5]. In addition, they inhibit the activation of $\alpha_{IIb}\beta_3$ [7], a polythiol integrin found exclusively on platelet surfaces, and that promotes platelet binding to soluble fibrinogen and von Willebrand factor [8]. Mn^{2+}-induced $\alpha_{IIb}\beta_3$ activation does not depend on intracellular signaling, and is also inhibited by anti-PDI antibodies, suggesting a close interaction between $\alpha_{IIb}\beta_3$ and platelet surface PDI [9]. Other membrane thiol proteins, such as glycoprotein Ibα [10] and collagen receptor $\alpha_2\beta_1$ [11], are also regulated by PDI. PDI inhibits ADP-induced platelet aggregation, although it does not interfere with the $P2Y_{12}$ ADP receptor, whose free thiols are targeted by clopidogrel [9,12]. Therefore, PDI is a converging hub for different signaling pathways of platelet aggregation, mainly by means of $\alpha_{IIb}\beta_3$ modulation via thiol–disulfide exchange [5,9,13].

Although it is ubiquitous, PDI has emerged as a novel antithrombotic target, particularly because it involves mechanisms different from those targeted by current therapies, namely coagulation cascade proteins, platelet receptors, and classic platelet signaling proteins [14,15]. Recently, several low molecular weight molecules, both natural and synthetic, have been reported to promote antiplatelet effects through selective PDI inhibition, e.g. quercetin-related flavonoids containing an O-glycosidic linkage in ring 3 [15], mastoparan, a wasp venom tetradecapeptide that inhibits platelet-derived tissue growth factor-β1 activation [16], and bepristat analogs 1a and 2a, which bind to the hydrophobic pocket at the *b′* domain of PDI [17].

We recently showed that a dodecapeptide mimicking the CGHC active site of PDI (CxxC) inhibited both PDI reductase activity and PDI-modulated superoxide generation by neutrophil Nox2 [18]. Thus, we hypothesized that CxxC would inhibit platelet aggregation by association with surface PDI. In the present report, we demonstrate that CxxC inhibits both ADP-induced and thrombin-induced platelet aggregation, decreases $\alpha_{IIb}\beta_3$ activation, and reduces PDI-associated thiol availability on platelet surfaces. Such effects are ascribed to covalent binding of CxxC to Cys400 in the PDI *a′* domain. These data support the vital role of the C-terminal CGHC redox motif for PDI's activity in platelet aggregation, and suggest that CxxC can be further exploited as a model for site-driven antithrombotic agent development.

Materials and methods

Peptide design and synthesis

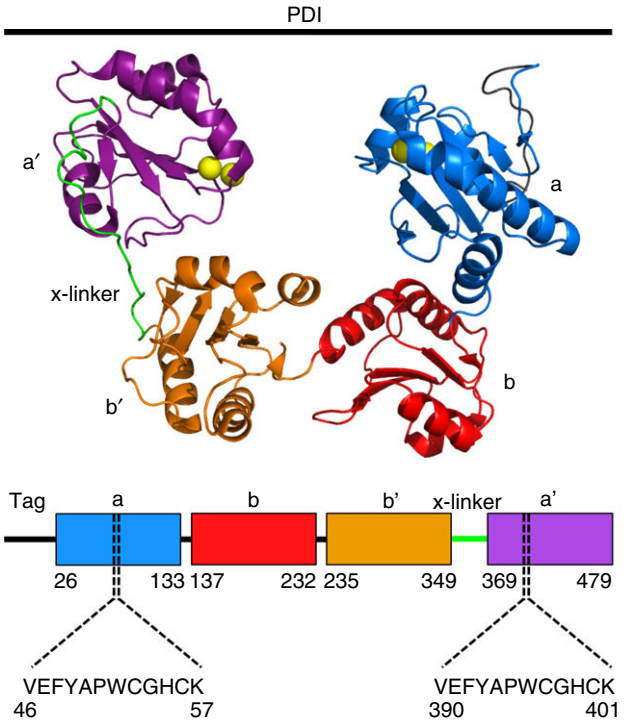
The peptides were designed on the basis of the linear sequence of PDI's active sites (accession number P21195). For design purposes, we took into consideration the redox motifs of PDI (CGHC) in both the *a* domain and the *a′* domain (Fig. 1). Thus, we designed the following peptides (USPTO pending patent PCT/BR2016/050170, 22 July 2016) carrying an acetyl group at the C-terminal residue, and an amide at the N-terminal residue acid (Fig. 1, bottom panel): (i) CxxC (VEFYAPWCGHCK), analogous to the sequences containing the redox motifs from Val46–Lys57 and Val390–Lys401; (ii) AxxA (VEFYAPWAGHAK), with the same sequence as CxxC, except that Cys residues are replaced with Ala residues; (iii) Scr (FCYPKACEWGHV), containing the same residues of CxxC in random sequence without forming vicinal thiols. Peptide synthesis was performed by EZ Biolab (Carmel, IN, USA), and peptides showed a purity of > 95%. For all experimental assays, the redox state of the peptides in aqueous solution was assessed with the DTNB reagent [19], and peptides were used in their reduced state.

Preparation of platelet-rich plasma (PRP) and washed platelets

Blood samples were obtained from self-declared healthy volunteers who had not used antiplatelet medications for at least 10 days prior to venipuncture. Informed consent was obtained and the protocols were approved by Comitê de Ética em Pesquisa of the Federal University of Maranhão, identified as 017432/2011-29. Blood samples were collected by venipuncture into acid–citrate–dextrose (ACD) tubes, and centrifuged at 200 × *g* for 10 min to obtain PRP. To obtain washed platelets (WPs), we used a previously described method [20]. Briefly, PRP was centrifuged at 800 × *g* for 10 min at room temperature, and the remaining pellet was resuspended in Ca^{2+}-free Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.4) containing ACD (Tyrode's/ACD 9 : 1 v/v). Platelets were once again centrifuged at 800 × *g* for 10 min and resuspended in Ca^{2+}-free Tyrode's buffer, and WPs were used within 5 h.

Platelet aggregation

The aggregation assays with PRP and WPs were performed in a four-channel AggRam aggregometer (Helena Biosciences, Gateshead, UK), as previously described [7].



Sequence	Name	Mass (Da)
Ac-VEFYAPWCGHCK-amide	CxxC	1480.63
Ac-VEFYAPWAGHAK-amide	AxxA	1416.56
Ac-FCYPKACEWGHV-amide	Scr	1480.69

Fig. 1. Schematic representation of protein disulfide isomerase (PDI) and the amino acid sequences of CxxC, AxxA and Scr peptides. A diagram showing the crystal structure of human PDI (accession number P21195) was used to design three dodecapeptides whose sequences were based on PDI's CGHC redox motifs. CxxC has the identical sequence contained in PDI, whereas AxxA has its cysteines substituted with alanines and Scr has a scrambled sequence with no vicinal thiols. The precise sequences and molecular weights for the peptides are shown in the bottom panel table and in Fig. S3.

Briefly, PRP ($2–3 \times 10^5$ platelets mL^{-1}) was incubated for 10 min at 37 °C with CxxC, Scr, or AxxA (3–30 μM). Then, PRP was activated with either ADP (2.5 μM or 5 μM; Sigma Chemical Co., Saint Louis, MO, USA) or thrombin (0.01 U mL^{-1} or 0.02 U mL^{-1}; Sigma Chemical Co.). For WPs, platelets ($2–3 \times 10^8$ platelets mL^{-1}) in Ca^{+2}-free Tyrode's buffer were preincubated with CxxC (1–10 μM), AxxA (10 μM) or Scr (10 μM) for 10 min prior to thrombin (0.02 U mL^{-1}) addition. When indicated, anti-PDI RL90 (2 μg mL^{-1}; Affinity Bioreagents, Golden, CO, USA) or anti-PDI BD34 (10 μg mL^{-1}; BD Biosciences, Franklin Lakes, NJ, USA) was added 5 min prior to aggregation with thrombin. In all WP experiments, Ca^{2+} (2 mM) was incubated for 2 min prior to activation.

Flow cytometry

WPs were incubated for 10 min at 37 °C with CxxC (10 μM), AxxA (10 μM), or Scr (10 μM). Then, they were incubated with thrombin (0.02 U mL^{-1}) for an additional 10 min. Non-activated platelets were used as the basal condition. For preliminary titration experiments, antibodies for platelet identification (CD41/61 PerCP; Novus Biologicals, Littleton, CO, USA), $\alpha_{IIb}\beta_3$ activation (PAC-1 fluorescein isothiocyanate [FITC], EUA; BD Biosciences), P-selectin (CD62-P FITC; Novus Biologicals) or granulophysin (CD63 PerCP; Novus Biologicals) expression on platelet surfaces were incubated for 10 min and flow cytometry performed with a FACS Calibur cytometer (BD Biosciences). Fluorescence data were acquired with a total of 2.5×10^3 cells per sample, and analyzed with FLOWJO VX software (Tree Star, Ashland, OR, USA).

Platelet labeling of cell surface free thiols

Free thiols present on resting platelet surfaces were labeled as described by Burgess *et al.* [10], with modifications. Aliquots containing 1.7×10^7 platelets were

incubated for 20 min at 37 °C with or without 25 μM CxxC, AxxA, or Scr, and then with added tris(2-carboxyethyl)phosphine (TCEP) (1 mM) for 10 min on some occasions. These were labeled with 3-*N*-maleimide-propionyl biocytin (MPB) (100 μM; Molecular Probes, Eugene, OR, USA) for 30 min at 25 °C under continuous agitation. Subsequently, 200 μM of reduced glutathione (Sigma Chemical Co.) was incubated for 30 min and 400 μM iodoacetamide (10 min, 25 °C; Sigma Chemical Co.) added for residual thiol group neutralization. Then, platelets were resuspended in lysis buffer (50 mM Tris-HCl, 0.5 M NaCl, 0.1% Triton X-100, and 5 mM EDTA, pH 8, plus protease inhibitors aprotinin, leupeptin, and phenylmethanesulfonyl fluoride), incubated for 50 min, and sonicated. Cell homogenates were left overnight with streptavidin beads at 4 °C for binding to MPB-labeled free thiols. The beads were washed twice, and then boiled in Laemmli buffer for 5 min. The streptavidin-bound proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and revealed by western blotting with anti-PDI (RL90; 1 : 1000; Abcam, Cambridge, MA, USA) and anti-biotin (1 : 2000; Calbiochem, La Jolla, CA, USA) antibodies. Detection was performed by the use of infrared with an Odyssey scanner (Li-Cor, Lincoln, NB, USA) or by the use of chemiluminescence.

Electrospray ionization (ESI) liquid chromatography (LC)–tandem mass spectrometry (MS/MS) studies and peptide mapping

Human recombinant PDI was obtained with a method described in a previous report from our group [18]. Reduced PDI was prepared by incubating the enzyme with dithiothreitol in a 10 : 1 ratio for 30 min at room temperature. Oxidized PDI was prepared in accordance with Brophy *et al.* [16] by incubating the enzyme overnight with 10 mM glutathione disulfide (GSSG). Excess GSSG was removed with a PD-10 Sephadex G-25 column (Sigma-Aldrich Brasil Ltda., São Paulo, Brazil) equilibrated with phosphate-buffered saline. Both reduced and oxidized PDI (1 μM) were incubated in the absence or presence of CxxC (6 μM) or Scr (6 μM) for 1 h at room temperature. In all cases, after the reaction mixture had been passed through centrifugal filter devices (Merck KGaA, Darmstadt, Germany), the protein was analyzed in a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 4500; ABSciex, Framingham, MA, USA). The protein was separated in a C4 column (5 μm, 150 × 1 mm; Grace Vydac, Hesperia, CA, USA), and eluted with solvent A (0.1% formic acid) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 0.1 mL min^{-1} by use of the following solvent gradient: 0–2 min, 5% solvent B; 2–10 min, 5–50% solvent B; 10–20 min, 50% solvent B; and 20–21 min, 5–50% solvent B. Re-equilibration to the initial condition was then performed for 15 min. The electrospray voltage was 5 kV, and the capillary temperature was 300 °C [21]. The protein mass spectrometry analysis was performed in positive ion mode; data were acquired and analyzed with ANALYST 1.6.1 software (ABSciex). The results were processed with PEAK VIEW software (ABSciex) to obtain the protein molecular weight.

To determine the site of covalent adduction, trypsinization of the enzyme followed by LC-MS/MS analysis was performed. Reduced PDI treated or not treated with the peptides was digested overnight in 50 mM pyrophosphate buffer (pH 7.4) with sequencing-grade trypsin, at an enzyme/substrate ratio of 1 : 50 (w/w). Peptides were separated in a reversed-phase column (5 μm, 2.1 × 150 mm, 300 Å; Grace Vydac), and eluted with solvent A (0.1% formic acid) and solvent B (0.08% formic acid in acetonitrile). Peptides were eluted at 40 °C at a flow rate of 0.25 mL min^{-1} with a linear gradient of solvent B (2–60% in 105 min). The electrospray voltage was 5 kV, and the capillary temperature was 260 °C. Peptide MS and MS/MS analyses with the QTRAP4500 were performed in positive ion mode with a mass range of 100–2000. Peptide analysis was performed and *y* and *b* series were obtained with PEAK VIEW software, and identification of the protein was performed by comparing the obtained peptides with data in MASCOT (Matrix Science, London, UK) [21].

Statistical analysis

The quantitative results were expressed as mean ± standard error of the mean of three independent experiments per protocol. One-way ANOVA was performed, with a Newman–Keuls *post hoc* test, and a *P*-value of < 0.05 was considered to be significant.

Results

CxxC decreases ADP-evoked and thrombin-evoked platelet aggregation

We first assessed the roles of CxxC, AxxA and Scr in platelet function. As shown in Fig. 2A–D, 30 μM CxxC decreased ADP-triggered platelet maximal aggregation in PRP by 70% and 28%, respectively, when 2.5 μM or 5.0 μM of ADP was used. Probably, 30 μM CxxC also inhibited thrombin-induced platelet aggregation (Fig. 2E–H), suggesting CxxC effects to be independent of the selected agonist. The antiplatelet effect of CxxC was then analyzed in WPs (Fig. 3). CxxC dose-dependently inhibited platelet aggregation (Figs 3A,B, S1 and S2), whereas no effect was observed for AxxA or Scr in any experiment (Figs 2, 3C,D and S2). Nevertheless, we coincubated CxxC with different anti-PDI antibodies, namely RL90 and BD34, to examine possible synergism between these compounds (Fig. 3E–H). None of the antibodies had their effects amplified by CxxC, whereas RL90

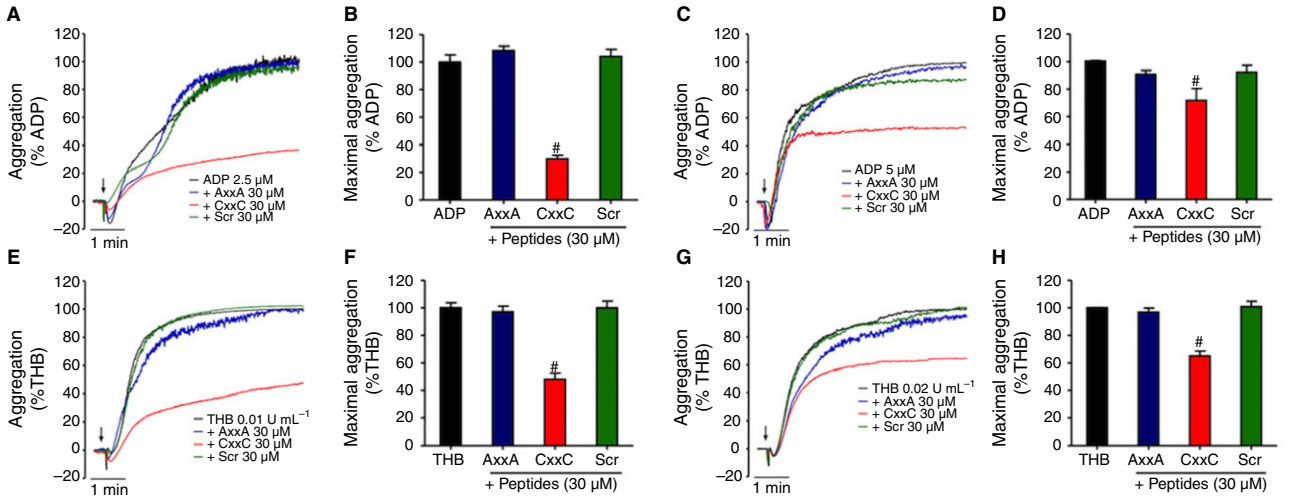


Fig. 2. CxxC inhibits both ADP and thrombin (THB)-induced platelet aggregation in platelet-rich plasma (PRP). All experiments were performed in PRP ($2–3 \times 10^8$ platelets mL^{-1}, 37 °C), and all peptides (30 μM) were incubated for 10 min prior to agonist addition. (A) Representative platelet aggregation induced by 2.5 μM ADP. (B) Maximal aggregation from experiments in (A). (C) Same as (A), but with 5 μM ADP. (D) Maximal aggregation from experiments in (C). (E) Representative platelet aggregation induced by 0.01 U mL^{-1} THB. (F) Maximal aggregation from experiments in (E). (G) Same as (E), but with 0.02 U mL^{-1} THB. (H) Maximal aggregation from experiments in (G). All significances were determined with *t*-tests. Data were compared by the use one-way ANOVA with a Newman–Keuls *post hoc* test. #$P < 0.05$ versus all. All curves are representative of least three distinct experiments. One hundred per cent is defined as maximum ADP or THB values in curves, and mean ADP or THB maximal aggregation in bar graphs. Arrows indicate agonist addition.

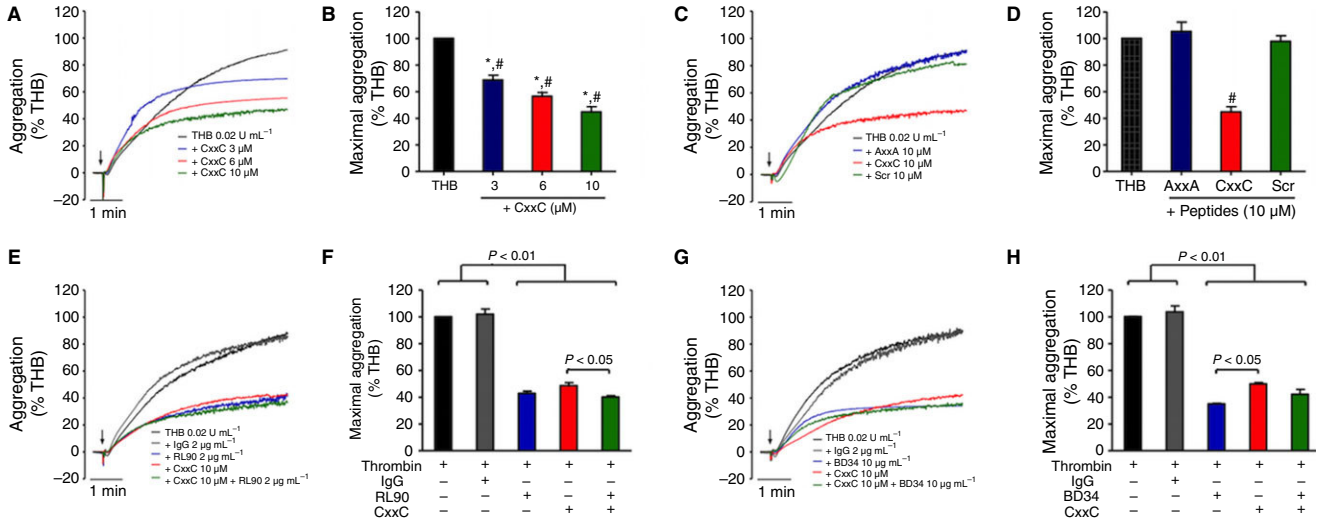


Fig. 3. CxxC effects on thrombin (THB)-evoked washed platelet (WP) aggregation are not enhanced by coincubation with anti-protein disulfide isomerase (PDI) antibodies. (A) WPs ($2–3 \times 10^8$ platelets mL^{-1}, 37 °C) were incubated with CxxC (3, 6 and 10 μM) for 10 min, and then activated with THB (0.02 U mL^{-1}). (B) Maximal aggregation from experiments performed in (A). *$P < 0.05$ versus THB; #$P < 0.05$ versus all. (C) WPs under the same conditions as in (A) were incubated with AxxA, Scr or CxxC (10 μM), and activated with THB (0.02 U mL^{-1}). (D) Maximal aggregation from experiments performed in (C). #$P < 0.05$ versus all. (E) Anti-PDI RL90 (2 μg mL^{-1}) and CxxC (10 μM) were incubated alone or coincubated, as described in Materials and methods. (F) Maximal aggregation from experiments in (E). (G) Same as (E), but with anti-PDI BD34 (10 μg mL^{-1}). (H) Maximal aggregation from experiments in (G). Data were compared by the use of one-way ANOVA with a Newman–Keuls *post hoc* test; $n = 3$ except for BD34 alone ($n = 2$). One hundred per cent is defined as maximum ADP or thrombin values in curves, and mean ADP or thrombin maximal aggregation in bar graphs. Arrows indicate agonist addition.

enhanced CxxC-induced platelet aggregation inhibition. Overall, these results suggest that CxxC strongly inhibits platelet aggregation without exerting additional effects on anti-PDI antibody inhibition.

CxxC diminishes $\alpha_{IIb}\beta_3$ activation on the platelet surface

The role of CxxC in platelet activation was investigated with flow cytometry, to measure the expression of

activated $\alpha_{IIb}\beta_3$ (Fig. 4A–D, PAC-1), P-selectin (Fig. 4E–H, CD62-P) and granulophysin (Fig. 4I–L, CD63) on resting and thrombin-activated platelets. All three markers statistically increased surface expression upon activation (Fig. 4D, H,L). Incubation of 10 μM CxxC prior to platelet activation decreased $\alpha_{IIb}\beta_3$ activation and mean fluorescence intensity (MFI) by approximately 30% and 66%, respectively (Fig. 4D,E), whereas no effect was observed on P-selectin and granulophysin (Fig. 4H,L). Both AxxA and Scr showed no effects on surface expression of any marker. This set of data reinforces the idea that CxxC reduced $\alpha_{IIb}\beta_3$ activation without interfering with granule secretion.

CxxC diminishes PDI free thiol availability on the platelet surface

To analyze the possible association of CxxC and its control peptides with PDI free thiols on the platelet surface, resting platelets were incubated with the membrane-impermeable biotinylated reagent MPB. Figure 5A shows that exposure of resting platelets to 1 mM TCEP, a membrane-impermeable thiol reductant, resulted in a nearly 2.5-fold increase in MPB labeling. The presence of the peptides did not significantly alter MPB binding to total surface free thiols (Fig. 5B). Assessment of MPB binding to surface PDI showed a four-fold increase in MPB labeling in the presence of TCEP (Fig. 5C). On the other hand, when CxxC (25 μM) was present, MPB binding to surface PDI was significantly diminished by ~ 30%, whereas AxxA and Scr had no effect (Fig. 5D). It is noteworthy that thiol reduction by TCEP did not affect the intracellular PDI content, which was assessed in the post-MPB supernatant (Fig. 5C,D, lower right panels). These data allow us to hypothesize that CxxC covalently binds to surface PDI without affecting total free thiol pulldown from the resting platelet membrane.

CxxC covalently binds to the C-terminal active site of PDI

The probable association of CxxC with PDI was addressed through its *in vitro* reaction with recombinant human PDI followed by MS analysis. The spectral data in Fig. 6A–C show a peak of 60 497 Da, which corresponds to the reduced form of the recombinant protein. In contrast, oxidized PDI (Fig. 6D–F) showed two peaks, in accordance with the presence of one (61 110 Da) or two (61 720 Da) PDI–GSSG adducts. When CxxC was present, reduced PDI spectra showed the appearance of a peak of 61 978 Da (Fig. 6B), in accordance with the

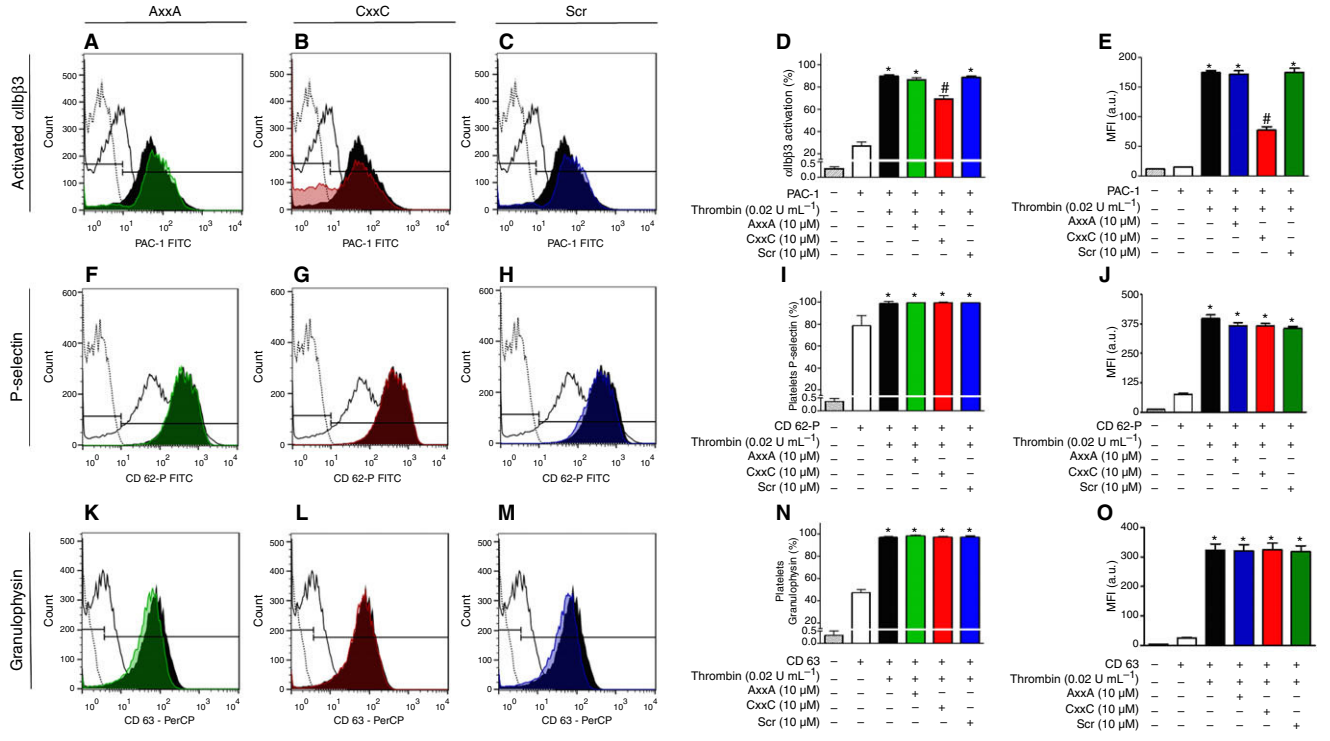


Fig. 4. CxxC decreases PAC-1 binding and fluorescence in washed platelets activated by thrombin, without interfering with granule exposure. Washed platelets (2–3 × 10^8 cells mL^{-1}) were incubated with the peptides (10 μM, 10 min, 37 °C) prior to thrombin activation (0.02 U mL^{-1}, 10 min, 37 °C), and then exposed to antibodies PAC-1 fluorescein isothiocyanate (FITC) (activated $\alpha_{IIb}\beta_3$ [A–E]), CD62-P FITC (P-selectin [F–J]), and CD63 PerCP (granulophysin [K–O]) as described in Materials and methods. Resting platelets without peptides (white bars and open lines) were used as the basal condition, and activated platelets (black lines and bars) were used for comparison. AxxA (green, panels A, E and I), Scr (blue [C, G and K]) and CxxC (red [B, F and J]) are shown separately from each other, in their respective resting and activated states. *$P < 0.05$ versus resting platelets, and #$P < 0.05$ versus (one-way ANOVA with a Newman–Keuls *post hoc* test, $n = 3$. MFI, mean fluorescence intensity.

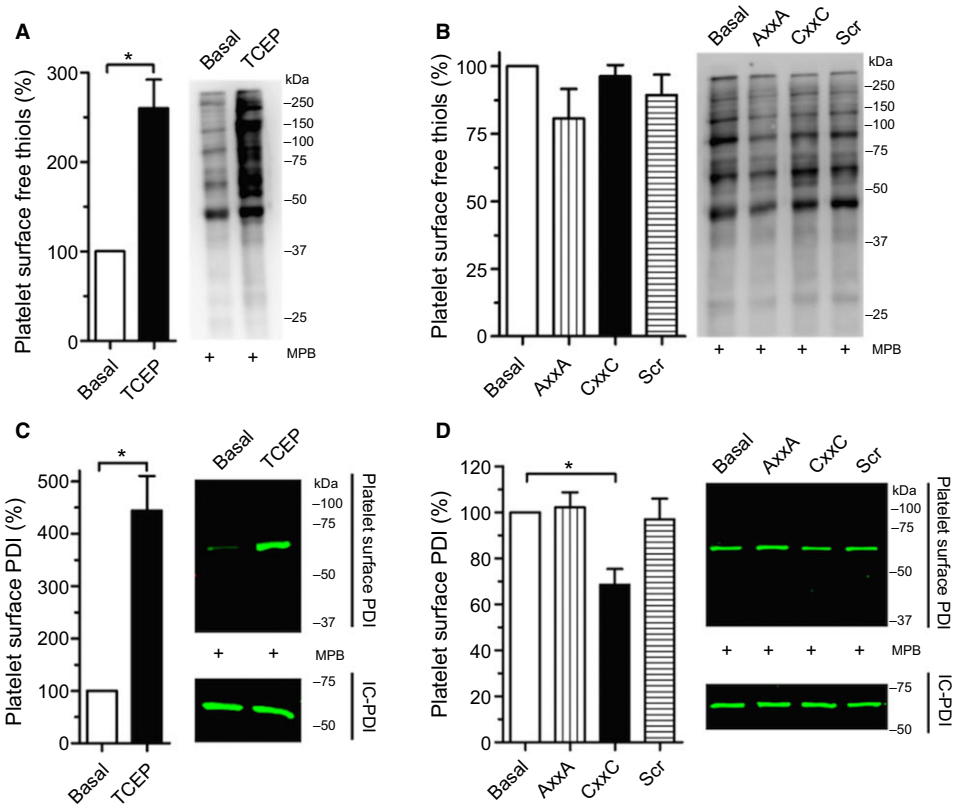


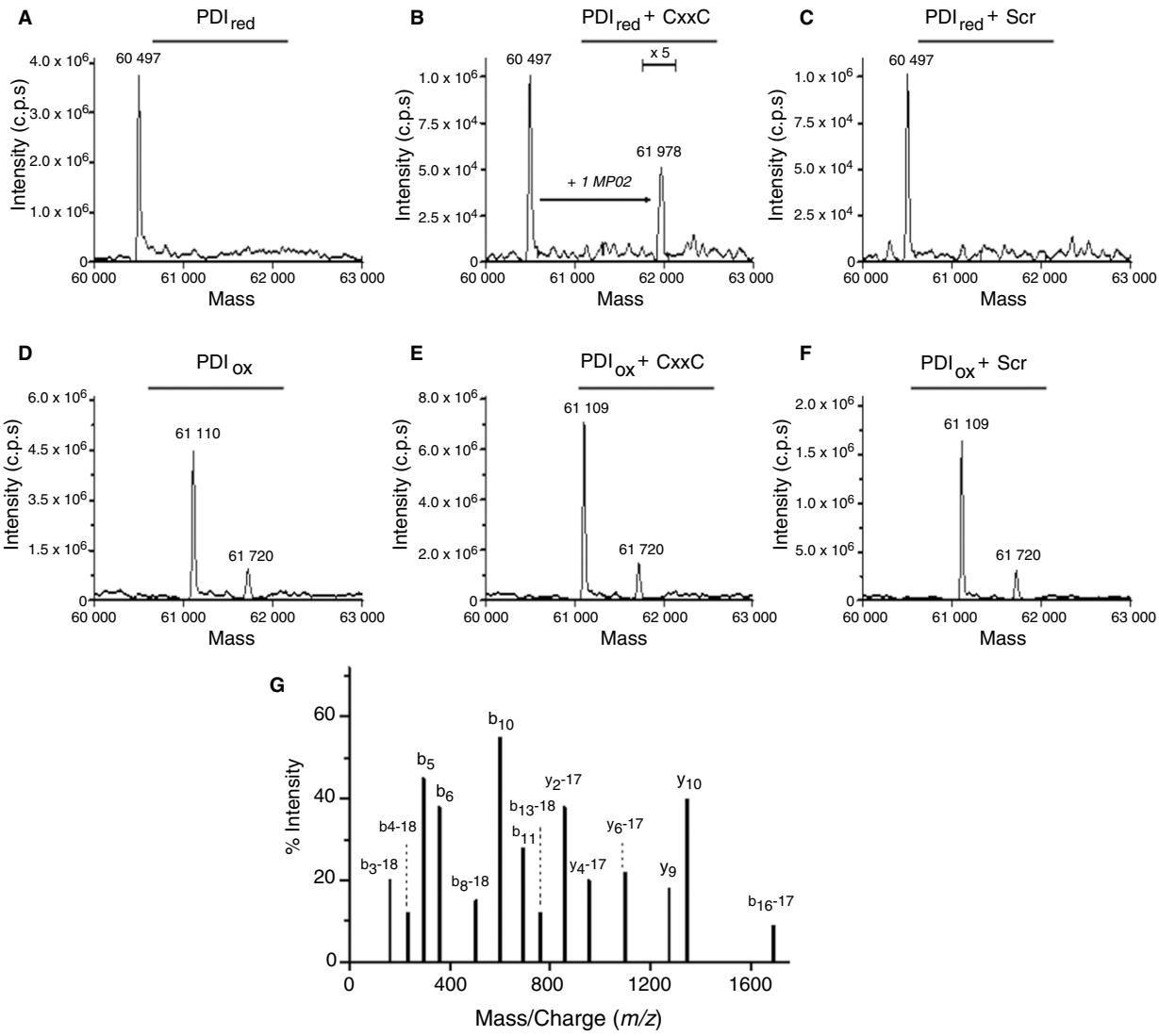
Fig. 5. CxxC decreases the number of free thiols in 3-*N*-maleimide-propionyl biocytin (MPB)-labeled protein disulfide isomerase (PDI) on the platelet surface membrane. (A) Extracellular thiol groups of washed platelets, incubated or not incubated with tris(2-carboxyethyl)phosphine (TCEP) (1 mM, 10 min) were labeled with MPB and precipitated with streptavidin magnetic beads, for the detection of membrane-exposed total free thiols by western blotting. (B) Resting platelets were incubated with or without CxxC, Scr or AxxA (25 μM, 20 min, 37 °C), and were subjected to the same procedures as described in (A). (C, D) The nitrocellulose membranes from (A) and (B) were blotted with anti-PDI antibody (RL90, 1 : 1000). The blots at the bottom of (C) and (D) are representative of the intracellular content of PDI (IC-PDI) that was detected in the supernatant of the cell lysate after precipitation. In all panels, the vertical bars represent densitometric analyses, expressed as mean ± standard error of the mean of the percentages relative to the basal condition, and were obtained from three to four independent experiments. *$P < 0.05$ versus basal condition analyzed with Student's *t*-test.

addition of 1481 Da, equivalent to the mass of CxxC (Fig. S3). No additional peak was found in the presence of Scr (Fig. 6C), either when the oxidized PDI was incubated with CxxC (Fig. 6E) or when it was incubated with Scr (Fig. 6F). Upon trypsinization, peptide mass mapping of reduced PDI by ESI LC-MS/MS in the absence or presence of CxxC showed coverage of almost 80% of the PDI primary sequence (Fig. S4). The fragment containing Lys386–Lys401, which includes both cysteines (Cys397 and Cys400) from the PDI a' domain, was the only one to show a different observed mass and a different retention time when it reacted with CxxC (Fig. 6D,E). The adducted fragment ($[M\text{-}17]^{+2}$ = 1686.93 Da), when compared with the unmodified one ($[M\text{-}17]^{+2}$ = 945.94 Da), showed a difference of 740.99 Da, which corresponds to the addition of the double-charged CxxC ([M+H] = 1481 Da) (Figs. 6C and S3). As shown in Fig. 6E, such a difference was found from a comparative analysis of the double-charged fragments y_2, y_4, y_6 and b_{16} from PDI–CxxC reactions. On the other hand, no difference was found in fragment b_{13}, the only one to contain Cys397 but not Cys400. Therefore, these results clearly demonstrate that CxxC covalently binds to Cys400 on the PDI a' domain, probably through a mixed disulfide bond.

Discussion

PDI has long been known to be of vital importance for platelet function and thrombus formation [4–6], and has recently emerged as a prominent target for antithrombotic therapy [14,15]. In a previous study, we demonstrated that CxxC, a dodecapeptide mimicking the PDI active redox site, inhibited PDI reductase activity [18]. Here, we expand CxxC's actions by demonstrating a novel antiplatelet role for this peptide, whose inhibitory effects on platelet activation and aggregation seem to be related to its covalent binding to Cys400 in PDI.

CxxC incubation promoted dose-dependent inhibition of human platelet aggregation, reaching its maximum



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Peptide sequence	Rt {min}	Ecpected mass {m/z}	Observed mass {m/z}	z detected
KNVFEFYAPWCGHC$_{400}$K	83.73	643.336	644.569	3
KNVFEFYAPWCGHC$_{400}$[CXXC]K	53.4	1137.171	1136.170	3

386 K N V F V E F Y A P W C G H C K 401

CxxC

	b_{16}	b_{13}	y_6	y_4	y_2
PDI	945.94	761.86	358.13	213.59	116.55
PDI + CxxC	1686.93	762.36	1096.64	954.09	857.05
PDI + Scr	946.43	762.36	358.64	213.56	116.54

Fig. 6. CxxC covalently binds to Cys400 at the C-terminal *a*′ active site. Reduced (A–C) or oxidized (D–F) recombinant protein disulfide isomerase (PDI) (1 μM) were analyzed by liquid chromatography–mass spectrometry (LC-MS) in the positive ion mode in the absence (A, D) or presence of 6 μM CxxC (B, E) or 6 μM Scr (C, F). Reduced PDI spectra showed a protein of 60 497 Da (A), which is similar to that observed in the presence of Scr (C), but in (B) a new peak of 61 978 Da (Δ1481 Da) was observed, corresponding to the addition of one molecule of CxxC. Oxidized PDI showed, in all three cases analyzed, the presence of ions corresponding to the formation of a disulfide bond with one (61 110 Da) or two (61 720 Da) glutathione molecules (D–F), without any other peaks appearing in the presence of CxxC (E). (G) Positive ion electrospray ionization LC-MS/MS analysis of the *y* and *b* series of the peptide Lys386–Lys401 are shown. The results shown correspond to the triple-charged peptide and the double-charged fragments. (H) Coverage of both series confirmed the structure of the peptide. The unmodified and modified peptide amino acid sequence in addition to the retention time (Rt) and expected mass are reported in the upper table. The second table shows the ions used to confirm the addition of CxxC to Cys400..

effect at 30 μM in PRP (Fig. S1) and at 10 μM in WPs (Fig. S2), with more prominent effects being seen at lower concentrations of agonists (Figs 2 and S2). This is consistent with a previous study showing that PDI-null platelets had 30–40% lower aggregation upon ADP or thrombin activation, an effect that was overcome at higher concentrations of agonists [22]. These authors also demonstrated that BD34, a monoclonal anti-PDI antibody, inhibited thrombin-triggered aggregation in wild-type but not in PDI-null platelets, suggesting that BD34 does not inhibit platelet thiol isomerases other than PDI [22]. In contrast, anti-PDI RL90 has been shown to also inhibit ERp57 activity [23]. Therefore, we coincubated CxxC with anti-PDI RL90 or BD34 to assess a possible associative effect (Fig. 3E–H). Interestingly, CxxC did not enhance the level of inhibition of any antibody used, whereas coincubation with CxxC and RL90 did promote additional inhibition of platelet aggregation as compared with CxxC alone, meaning that RL90 targets molecules that CxxC does not. Thus, these results allow us to hypothesize that CxxC reduces platelet aggregation by inhibiting PDI, possibly without affecting other thiol isomerases, as no additive effect was observed under similar experimental conditions when BD34 was coincubated with the peptide.

We then used flow cytometry to determine whether CxxC affected platelet membrane glycoproteins that, upon activation, either change conformation ($\alpha_{IIb}\beta_3$ integrin) or become exposed (P-selectin and granulophysin). CxxC reduced the binding of PAC-1 to activated platelets by 30%, whereas MFI was reduced by 66% (Fig. 4D,E), suggesting less intense activation. This is compatible with our data on platelet aggregation, and similar to the findings of another study showing decreased $\alpha_{IIb}\beta_3$ activation in PDI-null platelets [22]. It is of note that CxxC did not interfere with P-selectin or granulophysin membrane exposure (Fig. 4F–O). Our data on P-selectin are in agreement with those from other studies [7,17,22], despite a contrasting report describing decreased P-selectin exposure in PDI-null platelets [24]. However, the fact that CxxC did not inhibit granulophysin exposure was not expected, as some reports have shown that PDI inhibition decreases ATP secretion, suggesting that dense granule exocytosis is downstream of PDI [22,24]. It should be noted that, even though ATP is considered to represent an indirect measurement of dense granule exposure, there is no literature evaluating the effects of PDI inhibition on granulophysin exposure. However, we did not measure ATP secretion, and this should be considered a limitation of the present study. Therefore, this set of data shows that CxxC diminishes $\alpha_{IIb}\beta_3$ activation without interfering with platelet granule exposure, similarly to bepristats, a novel specific anti-PDI class of compounds [17].

We next investigated whether the effects exerted by CxxC on functional studies were attributable to an interaction with reactive thiols and/or PDI on the platelet surface. Labeling of free cysteines with MPB showed that the amount of surface thiols in resting platelets was 2.5-fold higher after incubation with TCEP, a strong membrane-impermeable thiol reductant (Fig. 5A). Given that CxxC was in its reduced state, one could hypothesize a thiol–disulfide exchange between this peptide and oxidized thiol proteins. However, there was no alteration in MPB binding to total surface thiols when platelets were incubated with any peptide, suggesting that CxxC is not an unspecific thiol reagent (Fig. 5B). Otherwise, considering the hypothesized association of CxxC with PDI, we revealed surface PDI in the MPB-labeled proteins. Similarly to other thiol-containing proteins, TCEP caused a four-fold increase in PDI labeling (Fig. 5C). However, CxxC incubation decreased MPB binding to free PDI thiols by almost 30% (Fig. 5D). Such a reduction is proportional to CxxC's effect on $\alpha_{IIb}\beta_3$ activation, but is lower than the observed inhibition of platelet aggregation. These results are corroborated by findings that successive thiol–disulfide exchanges between critical cysteines are crucial for platelet activation; that is, one PDI molecule may successively interact with multiple $\alpha_{IIb}\beta_3$ molecules [9,25]. The lack of additive inhibition of platelet aggregation with CxxC and anti-PDI BD34 coincubation, coupled with lack of effect on total thiol MPB binding upon incubation with CxxC, supports rather specific PDI inhibition, even though other platelet isomerases should be tested in future studies. Therefore, these observations reinforce our hypothesis of a direct thiol-mediated reaction between CxxC and PDI.

We then speculated whether CxxC would covalently bind to PDI cysteines. PDI inhibition by bacitracin involves the formation of mixed disulfide bonds with Cys314 and Cys345, located in the hydrophobic pocket of the b' domain and linker region x, respectively [26]. Thus, LC-MS/MS studies were performed to analyze the interaction of CxxC with PDI and tentatively identify the potential binding site. When reduced human recombinant PDI [18] was incubated with CxxC, there was a mass increase of 1481 Da, corresponding to the addition of one CxxC molecule (Figs 6 and S3). To identify the site of binding of CxxC to PDI, recombinant protein, reacted or not with CxxC, was subjected to trypsinization followed by peptide mass mapping. Mass differences were only found in the y and b series of the fragment containing Cys397 and Cys400 from the a' CGHC domain. According to ESI LC-MS/MS data, the PDI-derived fragment containing Cys400 showed a mass increase of 740.99 Da (1481 Da in double-charged fragments), as verified in series fragments b_{16}, y_6, y_4, and y_2, whereas no additional mass was found in fragment b_{13}, which contained only Cys397. These data allow us to conclude that CxxC forms a mixed disulfide bond with Cys400 of PDI, even though we did not retrieve the fragment containing Cys53 and Cys56 from the a domain (Fig. S4), which constitutes a limitation of this analysis. Nevertheless, our assumption that the effects of CxxC are attributable to its binding to

Cys400, rather than Cys56, is supported by the recent demonstration that only the C-terminal active site of PDI is important for its properties in platelet function [24].

A mixed disulfide may have two constituent thiols of different acidities. The cleavage of the mixed disulfide by a nucleophilic thiolate (RS^-) occurs favorably with release of the more acidic thiol, the less acidic group being retained in the new mixed disulfide [27]. Given that the N-terminal cysteines of PDI (Cys53 and Cys397) have a lower pK_a, i.e. are more reactive, mixed disulfide bonds with these cysteines are less stable [28]. Additionally, it has been shown that Cys56 and Cys400 are involved in the stabilization of mixed disulfide bonds [29,30], making it reasonable to speculate that Cys397 attacks CxxC, or vice versa, to form a mixed disulfide that is, rather, stabilized at Cys400.

In conclusion, the data presented herein demonstrate that CxxC markedly inhibits platelet aggregation and activation, without interfering with granule secretion. These properties are, at least in part, ascribed to the covalent binding of CxxC to Cys400 of PDI, providingg a potential mechanism for its antiplatelet activity. Furthermore, they corroborate a recent report in which reactive cysteines of the C-terminal CGHC motif of PDI were shown to be of vital importance for thrombosis [24]. In this regard, this work may be considered to be a proof of concept that Cys400 is a potential target for site-specific drug design and the development of new antithrombotic agents.

Addendum

H. R. Sousa, E. M. L. Sena, and R. S. Gaspar were responsible for aggregation protocols. H. R. Sousa, R. S. Gaspar, and A. P. S. Azevedo-Santos were responsible for cytometry studies. T. L. S. Araujo, R. S. Gaspar, and J. L. Fontelles were responsible for biology protocols. H. R. Sousa, M. Mastrogiovanni, S. A. Silva, and A. Trostchansky were responsible for spectrometry studies. S. A. Silva, A. P. S. Azevedo-Santos, D. M. Fries, and A. M. Paes were responsible for of protocol execution and data discussion. F. R. M. Laurindo and A. M. Paes were responsible for and study supervision. R. S. Gaspar, F. R. M Laurindo, A. Trostchansky, and A. M. Paes were responsible for discussion and manuscript drafting. All authors read and approved the manuscript's final format.

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Disclosure of Conflict of Interests

H. R. Sousa, R. S. Gaspar, E. M. L. Sena, S. A. da Silva, J. L. Fontelles, F. R. M. Laurindo, A. Trostchansky and A. M. Paes have a Brazilian patent (BR 10 2015 0180764, 23 July 2015) and a USPTO pending patent (PCT/BR2016/050170, 22 July 2016), issued to the Federal University of Maranhão. The other authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. CxxC dose-dependently inhibits thrombin-evoked platelet aggregation in platelet-rich plasma.
Figure S2. CxxC dose-dependently inhibits thrombin-evoked platelet aggregation in washed platelets.
Figure S3. MS/MS characterization of peptides.
Figure S4. Analysis of PDI trypsinization by HPLC/MS.

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