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The protein disulphide isomerase inhibitor CxxCpep modulates oxidative burst and mitochondrial function in platelets

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Short communication: The protein disulphide isomerase inhibitor CxxCpep modulates oxidative burst and mitochondrial function in platelets.

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Authorship

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SUMMARY

Background: We have previously described CxxCpep, a peptide with anti-platelet properties that inhibits peri/epicellular protein disulphide isomerase (pecPDI) by forming a mixed disulfide bond with Cys400 within the pecPDI active site.

Objectives: Here we sought to determine if pecPDI targeted by CxxCpep is relevant to redox mechanisms downstream of the collagen receptor GPVI in platelets.

Methods and Results: Restriction of effects of CxxCpep to the platelet surface was confirmed by LC-MS/MS following cell fractionation. Platelet aggregation was measured in platelet-rich plasma (PRP) incubated with 30 μM CxxCpep or vehicle. CxxCpep inhibited collagen-induced platelet aggregation but exerted no effect in TRAP-6-stimulated platelets. PRP was incubated with DCFDA to measure oxidative burst upon platelet adhesion to collagen. Results showed that CxxCpep decreased oxidative burst in platelets adhered to immobilized collagen while the number of adherent cells was unaffected. Furthermore, flow cytometry studies using a FITC-maleimide showed that the GPVI agonist CRP stimulated an increase in free thiols on the platelet outer membrane, which was inhibited by CxxCpep. Finally, CxxCpep inhibited platelet mitochondrial respiration upon activation with collagen, but not with thrombin.

Conclusions: Our data suggest that pecPDI is a potential modulator of GPVI-mediated redox regulation mechanisms and that CxxCpep can be further exploited as a template for new antiplatelet compounds.

KEYWORDS: Platelets; Protein Disulphide Isomerase; Collagen; Platelet Inhibitor; Redox biology

1. INTRODUCTION

Thrombosis is a common underlying feature of cardiovascular diseases, such as acute myocardial infarction, ischemic heart disease, and ischemic stroke [1]. Indeed, thromboembolic conditions are estimated to account for one-quarter of deaths worldwide. Platelet activation is a critical step towards thrombus formation since it culminates in the exposure of the fibrinogen binding site of $\alpha_{IIb}\beta_3$, a polythiol integrin found exclusively on the platelet outer surface [2]. Integrin $\alpha_{IIb}\beta_3$ reactivity is modulated by peri/epicellular protein disulfide isomerase-A1 (PDIA1, herein referred as pecPDI) and its siblings ERp5 (PDIA6), ERp57 (PDIA3), and ERp72 (PDIA4), which support the isomerization of critical disulphide bonds on the β-domain of $\alpha_{IIb}\beta_3$ (recently reviewed in [3]).

Members of the PDI family have been recognized as important targets for the screening and development of novel antithrombotic agents [4-6]. The set of PDI inhibitors harbouring antiplatelet properties includes both natural (myricetin [7], quercetin-3-rutinoside, and isoquercetin [8]) and synthetic (bepristats [9] and HPW-RX40 [10]) compounds. Our group has previously characterized CxxCpep, a peptide inhibitor of pecPDI that is able to bind to Cys400 of PDI and decrease platelet function [11]. However, it is still unclear if CxxCpep is able to modulate intracellular redox signalling in platelets.

PDI has been shown to modulate the generation of reactive oxygen species (ROS) through interactions with key components of ROS-producing enzyme NADPH oxidase 1 (Nox-1) [12]. In platelets, Nox-1 activation is triggered by collagen binding to its receptor glycoprotein VI (GPVI) [13], while we have recently shown that PDI and Nox-1 translocate to a similar compartment upon activation with the GPVI agonist collagen-related peptide (CRP) [14]. Parallel to this, PDI has been recently suggested to facilitate mitochondrial respiration and ROS generation through its interaction with Drp1 in endothelial cells [15]. Therefore, PDI can modulate intracellular and peri/epicellular redox processes of

cardiovascular cells.

These observations, together with our interest in clarifying the effects of CxxCpep on platelet regulation mechanisms led us to investigate if this peptide inhibitor could inhibit redox processes in platelets. We hypothesized that CxxCpep would not be able to cross the platelet outer membrane due to its structure, thus restricting its effects to pecPDI, which could potentially block redox processes both inside and on the cell outer membrane. Therefore, we explored if CxxC could inhibit collagen-dependent redox processes, including redox shifts on the outer membrane and mitochondrial respiration of platelets.

2. MATERIALS AND METHODS

2.1. Washed platelets preparation

Platelet-Rich Plasma (PRP) and washed platelets (WP) were prepared as described previously in the presence of prostacyclin (PGI_2) (1 μg/mL) [16-18]. For aggregation experiments using PRP, PGI_2 was not added. Platelets were ressuspended in modified Tyrode's-HEPES buffer (20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 5 mMglucose, 134 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, and 1 mM MgCl2, pH 7.3). All laboratory reagents were from Sigma-Aldrich unless otherwise specified. All protocols were approved by the Facultad de Medicina (MSP N° 3573138) and the University of Reading Research Ethics Committees following the Helsinki declaration.

2.2. Mass spectrometry analysis of CxxCpep penetration into platelets.

CxxCpep was detected through HPLC coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). LC-ESI-MS/MS was performed in a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 4500, ABSciex, Framingham, MA).

The presence of CxxCpep (VEFYAPWAGHAK, synthesized by PeptideSynthetics, Fareham, UK) was evaluated in the extracellular and intracellular milieu of platelets as outlined in Figure 1A. Samples were diluted 1:8 v/v in 3% w/v trichloroacetic acid (TCA) for 30 minutes on ice to precipitate CxxCpep. This solution was centrifuged at 15,000 × *g*, at 4 °C or 37 °C for 10 minutes and the supernatant was separated, lyophilized in vacuum at 45 °C for 2 hours, and resuspended in an aqueous solution for HPLC-MS/MS analysis. Peptides were separated and identified as described previously [17]. The y and b peptide series were obtained with PEAK VIEW software (ABSciex, Framingham, MA).

2.3. Platelet aggregation

Plate-based platelet aggregation was performed as described [19]. Briefly, 30 μM CxxCpep was incubated with PRP for 10 min in a 96-well plate with increasing concentrations of thrombin receptor activator peptide 6 (TRAP-6) and collagen. The plate was shaken at 1,200 rpm for 5 min and absorbance was read at 405 nm using a Flexstation 3 plate reader (Molecular Devices, Wokingham, UK).

2.4. Platelet oxidative state and adhesion

Platelet oxidative state was detected in live cells adhering to collagen [20]. PRP was incubated with 2',7'-dichlorofluorescein (DCFDA) for 15 min in a 96-well plate, followed by an incubation with 30 μM CxxCpep or vehicle control for 10 min. PRP was added onto a coverslip coated with 100 μg/mL Collagen (Nycomed, Munich, Germany) and live images acquired for 10 minutes using a Nikon A1-R confocal microscope (10x objective, Nikon Instruments Europe BV, Amsterdam, Netherlands). Single platelet fluorescence values (excitation at 495 nm/emission 525 nm) of at least 7 platelets and the number of adhered cells based on the fluorescence of each platelet were determined using ImageJ software (NIH,

125 USA).

126

127 **2.5. Detection of platelet outer surface free thiols**

128 Surface-free thiols were detected using an adapted method from [21]. PRP (diluted
129 1:10 v/v in modified Tyrode's HEPES buffer) was incubated with 30 μM CxxCpep or vehicle
130 control for 10 min, followed by the addition of 1 μg/mL collagen-related peptide (CRP) for
131 10 min at room temperature. After platelet activation, 500 μM FITC-tagged Maleimide was
132 added in the dark for 30 min. This solution was diluted 1:25 using modified Tyrode's-HEPES
133 buffer and events were acquired using a BD Accuri flow cytometer (BD Biosciences,
134 Wokingham, UK). Data are expressed as median fluorescence intensity (MFI). CRP was used
135 instead of collagen to avoid clogging the flow cytometer.

136

137 **2.6. Oxygen consumption rate and extracellular acidification rate assays**

138 Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were
139 measured using a Seahorse XFe24 Extracellular Flux Analyser (Agilent Technologies,
140 Stockport, UK). Washed platelets (2.5 x 10^7 cells/well) were resuspended in DMEM assay
141 medium. Platelets were kept at 37 ^{o}C for 30 minutes with 30 μM CxxCpep, 30 μM Scrambled
142 peptide (VGCPAKWCYHEF, synthesized by PeptideSynthetics, Fareham, UK) or vehicle
143 and loaded into the Seahorse XFe24 extracellular flux analyser. Baseline measurements of
144 OCR and ECAR were performed at the beginning of the assay, followed by the addition of
145 thrombin or collagen, oligomycin to inhibit ATP synthase, cyanide p-trifluoro-methoxy
146 phenyl-hydrazone (FCCP) to uncouple oxidative phosphorylation and inhibitors of complex I
147 and III rotenone and antimycin A, respectively. Respiratory parameters were calculated
148 according to [22-24].

149

2.7. Statistical analysis

Statistical analyses were performed on GraphPad Prism 8.0 software (GraphPad Software, San Diego, USA). Bar graphs express mean ± SEM and sample size varied from 3-6 independent repeats. Outliers were determined and excluded by the ROUT test. Statistical analysis was performed through paired one-way or two-way ANOVA and Tukey or Sidak as post-tests, as appropriate.

3. RESULTS

3.1. CxxCpep does not penetrate the outer membrane of platelets

CxxCpep binds to PDI and inhibits platelet aggregation [17], however, it was unclear whether CxxCpep could target different pools of PDI, i.e. intracellular and pecPDI. Therefore, we first assessed whether CxxCpep would be able to penetrate the platelet membrane at either 4°C or 37°C. As shown in Figure 1, the peptide was found on the extracellular milieu (Figure 1B) but not in the cytosolic fraction (Figure 1C). This suggests that CxxCpep does not penetrate the outer membrane of platelets and may target pecPDI, not intracellular PDI.

3.2. CxxCpep reduces collagen-induced platelet aggregation

To further explore the antiplatelet effects of CxxCpep, we measured platelet aggregation (Figure 2). CxxCpep inhibited collagen-induced platelet aggregation, as shown by a 3-fold increase in EC_{50} for collagen (vehicle 0.29 μg/mL *vs* CxxCpep 0.98 μg/mL, $p<0.05$, Figure 2A) based on the maximum aggregation obtained. In parallel, there was no inhibition when platelets were stimulated with TRAP-6. Given that GPVI is the main collagen receptor responsible for platelet aggregation [25], these data led us to investigate the

underlying mode of action of CxxCpep.

3.3. CxxCpep decreases oxidative burst and surface free thiols in GPVI-stimulated platelets

GPVI activation results in an intracellular oxidative burst, mainly regulated by Nox-1 [13], while activated platelets present an increase in outer surface free thiols [26], placing redox processes at the centre of platelet regulation by this receptor. We explored if CxxCpep could affect collagen-induced platelet oxidative burst and surface free-thiol formation upon activation of GPVI with CRP (Figure 3). An intracellular oxidative burst measured by DCF fluorescence levels was detected within 60 seconds of adhesion to collagen (Figure 3A) while CxxCpep decreased DCF fluorescence by ~30% when compared to vehicle (Figure 3A). Importantly, CxxCpep did not affect platelet adhesion (Figure 3B). Maleimide-labeled free thiols increased 3-fold on CRP-activated platelets, while pre-incubation with CxxCpep reduced surface free thiols by ~30% (Figure 3C). Therefore, CxxCpep can control redox modifications triggered by collagen and GPVI activation, presumably through pecPDI inhibition.

3.4. Decreased collagen-induced respiratory burst in platelets treated with CxxCpep

Given that CxxCpep was able to modulate intracellular oxidative burst, we studied if specific sources of intracellular oxidants, such as mitochondrial respiration, were also affected. Indeed, both collagen and thrombin led to increased OCR (Figure 4), however only thrombin led to higher ECAR (Supplementary Figure 1). Collagen increased basal OCR by 38% either after pre-incubation with vehicle or a scrambled peptide (Supplementary Figure 2B); an effect that was abrogated when CxxCpep was pre-incubated (Figures 4B). These effects were not observed in thrombin-stimulated platelets (Figure 4D and E). Addition of a

scrambled peptide decreased maximal and non-mitochondrial respiration in platelets stimulated with collagen (Supplementary Figure 2), however it did not affect collagen-induced respiration, suggesting that the inhibition observed for CxxCpep was not due to assay interference. CxxCpep did not influence the ECAR of platelets stimulated with neither collagen nor thrombin (Supplementary Figure 3). Therefore, CxxCpep modulates mitochondrial respiratory burst in collagen-stimulated platelets.

4. DISCUSSION

In this study, we explored several mechanisms that are regulated by the pecPDI-targeted inhibitor CxxCpep. CxxCpep inhibited platelet aggregation induced by collagen, but not by TRAP-6. This was associated with decreased oxidative burst and levels of outer membrane free thiols in CxxCpep-treated platelets stimulated with collagen or CRP, respectively. Mitochondrial respiration was also decreased in collagen-stimulated platelets pre-treated with CxxCpep, suggesting that pecPDI may somehow regulate mitochondrial function. Altogether, we suggest that CxxCpep modulates collagen-induced activation of platelets through the inhibition of pecPDI by disrupting oxidative burst and mitochondrial respiration.

Our finding that CxxCpep is unable to concentrate in the cytosolic fraction of platelets confirms previous speculations that this peptide was only able to target extracellular proteins [17]. Indeed, there are currently only two small molecule PDI inhibitors unable to cross the outer membrane, namely CxxCpep and isoquercetin [8]. However, quercetin and structurally related flavonoids have been reported to inhibit other molecules in platelets [27], while CxxCpep was unable to potentiate platelet inhibition exerted by anti-PDI antibodies, suggesting this peptide is selective to pecPDI [17].

CxxCpep decreased by 30% the 3-fold increase in outer surface free thiols observed in

CRP-activated platelets. The magnitude of this reduction suggests that CxxCpep does not target unspecific free thiols on the platelet outer surface, in line with previous observations [17]. Since the same concentration of CxxCpep was able to potently decrease platelet aggregation, this suggests that only a fraction of platelet surface free thiols modulate platelet function. However, it is not known if there are GPVI-selective mechanisms that regulate the redox shift of proteins on the platelet membrane upon activation. The observed decrease of membrane-free thiols with CxxCpep can be due to mixed disulphide bonds formed between CxxCpep and pecPDI that prevented maleimide binding, therefore, reducing the levels of surface free thiols, similar to what described for resting platelets [17]. Nevertheless, we have previously shown that 30 μM CxxCpep was able to inhibit platelet activation, while control peptides confirmed that the effects observed were due to vicinal thiols in CxxCpep [11].

Resting platelets rely on aerobic glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) for energy needs [24]. Upon activation, both collagen and thrombin led to a higher platelet oxygen consumption. It is yet unclear if/how collagen affects mitochondrial respiration in platelets despite reports showing similar findings for thrombin-stimulated platelets [24, 28]. Our data suggest that collagen-induced platelet activation relies on OXPHOS. Interestingly, CxxCpep decreased collagen-induced OXPHOS but had no effect when platelets were stimulated with thrombin. We observed lower ATP-linked respiration in CxxCpep-treated platelets stimulated with collagen. It has been shown that lower ATP-linked respiration can lead to reduced levels of secreted ADP [29], which limits platelet activation. Indeed, platelet activation induced by collagen is highly dependent on secondary agonists, such as ADP [30]. Therefore, treatment with CxxCpep may reduce the levels of secreted ADP due to lower mitochondrial respiration.

Despite lower OCR in CxxCpep-treated platelets, there were no changes in the ECAR of platelets stimulated with collagen. In agreement with these observations, PDI knockdown

in human vascular endothelial cells resulted in lower mitochondrial respiration while glycolysis was unaffected [15]. This suggests that pecPDI may influence mitochondrial respiration but not ECAR (regulated by glycolytic and non-glycolytic pathways) in platelets stimulated with collagen. In parallel, CxxCpep treatment decreased maximal ECAR in thrombin-activated platelets following previous findings on inhibition of thrombin-activated platelet aggregation by CxxCpep [17]. Overall, these data suggest an intriguing link between pecPDI and mitochondrial respiration that needs to be further addressed.

In conclusion, we show that the pecPDI inhibitor CxxCpep decreases collagen-induced responses in platelets. Future studies should identify ROS sources affected by CxxCpep in collagen-activated platelets. Mitochondrial respiration may be a key phenomenon regulated by this peptide inhibitor, suggesting the use of CxxCpep as a template for drug discovery programmes that aim to identify novel antiplatelet compounds.

AUTHOR CONTRIBUTIONS

R.S. Gaspar and S. Mansilla designed the study, performed experiments, analyzed data, and drafted the manuscript. V.A. Vieira and L.B. da Silva performed experiments and analyzed data. A. Trostchansky, J.M. Gibbins, L. Castro, and A.M.A. Paes designed the study, supervised protocols, and reviewed the manuscript. All authors agreed on the final version submitted.

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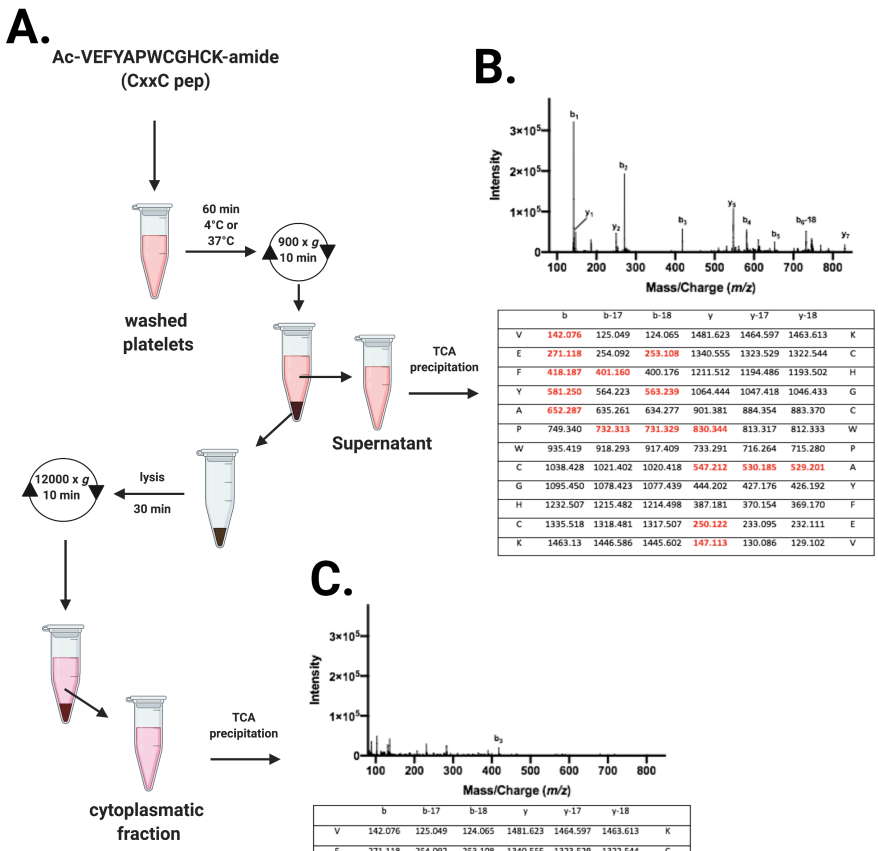
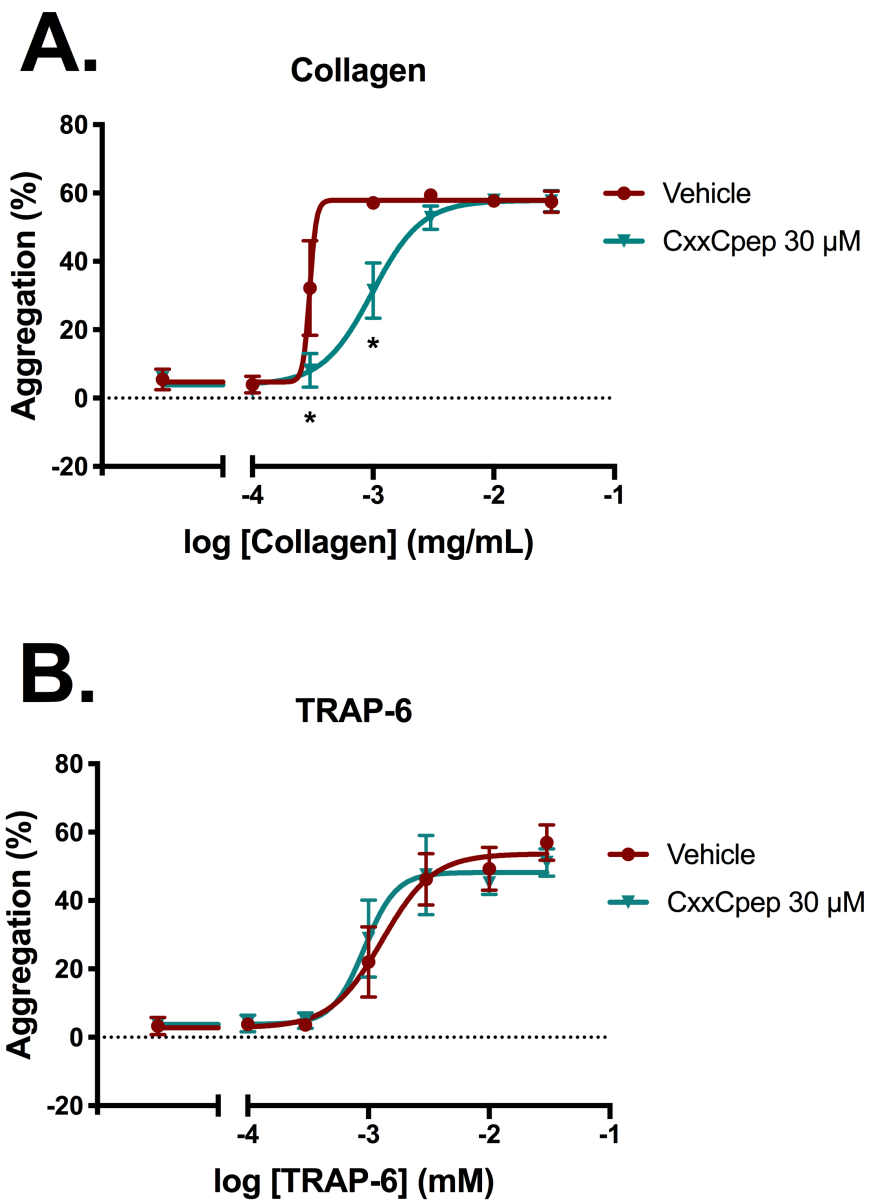
DISPLAY ITEMS



	b	b-17	b-18	y	y-17	y-18	
V	142.076	125.049	124.065	1481.623	1464.597	1463.613	K
E	271.118	254.092	253.108	1340.555	1323.529	1322.544	C
F	418.187	401.160	400.176	1211.512	1194.486	1193.502	H
Y	581.250	564.223	563.239	1064.444	1047.418	1046.433	G
A	652.287	635.261	634.277	901.381	884.354	883.370	C
P	749.340	732.313	731.329	830.344	813.317	812.333	W
W	935.419	918.293	917.409	733.291	716.264	715.280	P
C	1038.428	1021.402	1020.418	547.212	530.185	529.201	A
G	1095.450	1078.423	1077.439	444.202	427.176	426.192	Y
H	1232.507	1215.482	1214.498	387.181	370.154	369.170	F
C	1335.518	1318.481	1317.507	250.122	233.095	232.111	E
K	1463.13	1446.586	1445.602	147.113	130.086	129.102	V

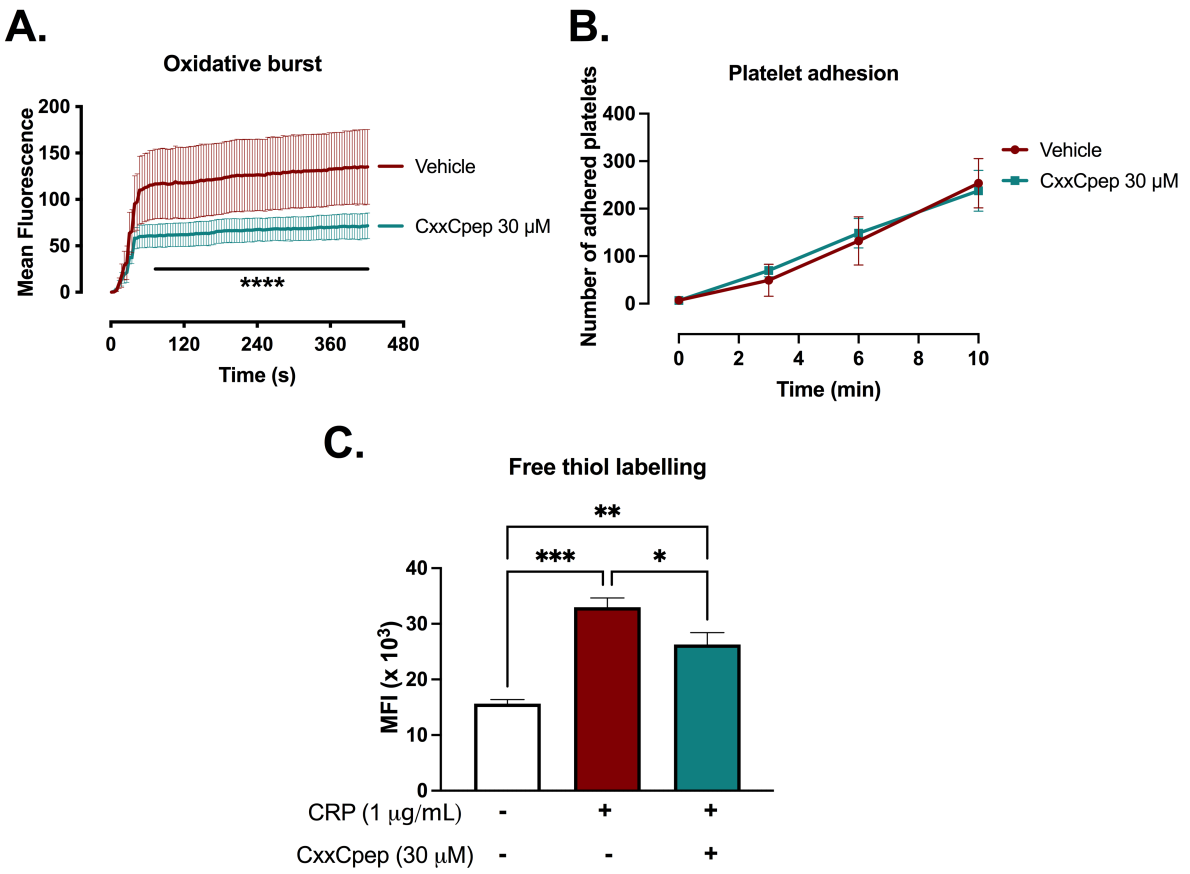
Figure 1. CxxCpep does not reach the platelet cytosol. CxxCpep was incubated with platelets at either 4°C or 37°C for 1h, as explained in Materials and Methods. (A) Schematic representation of supernatant and cytosolic fractions. The y and b series for the CxxCpep were determined by ESI-MS/MS. ESI-MS/MS spectra and corresponding table of detected ions of the supernatant (B) and cytoplasmatic fraction (C) are shown for conditions kept at 37°C. Similar results were obtained at 4°C. CxxCpep ions were marked in red at the tables.



380

381 **Figure 2. CxxCpep inhibits collagen-induced platelet aggregation.** Platelet-rich plasma
382 was incubated with 30 µM CxxCpep for 10 min and stimulated with different concentrations
383 of (A) collagen or (B) thrombin receptor activator 6 (TRAP-6). Platelet aggregation measured
384 as described in Materials and Methods. n = 4 independent donors. Graphs present mean ±
385 SEM and data analyzed using two-way ANOVA and Tukey's post-test. * $p<0.05$ *vs* vehicle.

386



387

388 **Figure 3. CxxCpep decreases collagen-induced oxidative burst and surface-free thiols**
389 **upon GPVI activation.** (A) PRP was incubated with 2',7'-dichlorofluorescein (DCFDA) for
390 15 min, followed by 30 µM CxxCpep or vehicle control for 10 min, and fluorescence
391 measured using a confocal microscope. (B) Number of adhered platelets. (C) PRP was
392 incubated with 30 µM CxxCpep for 10 min and activated with 1 µg/mL CRP. FITC-
393 Maleimide was used to detect free thiols. n = 4 independent donors. Graphs present mean ±
394 SEM and data analyzed using two-way ANOVA (A) and (B) or one-way ANOVA (C) and
395 Tukey's post-test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

396

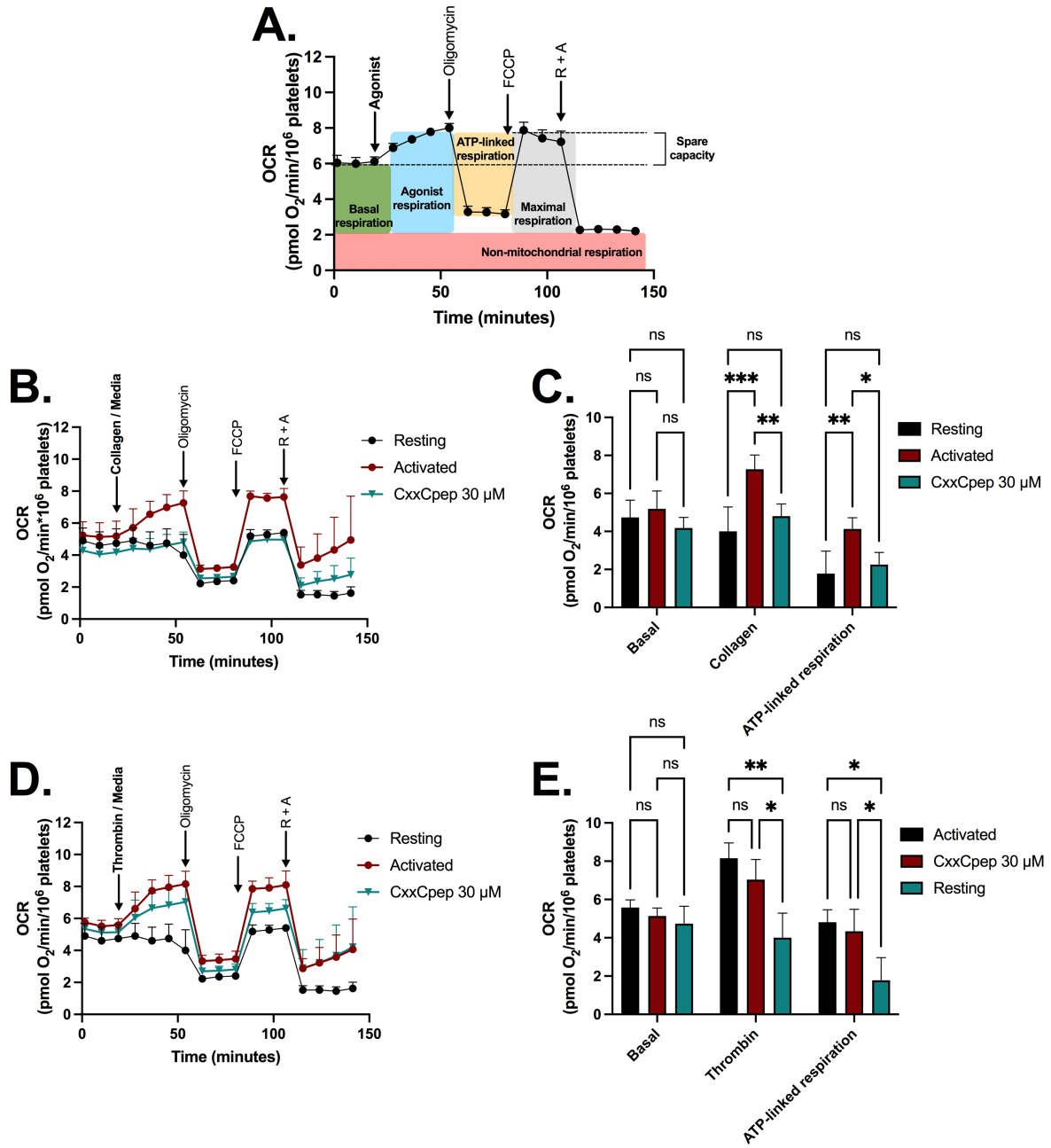


Figure 4. CxxCpep decreases collagen-induced respiratory burst in platelets. The oxygen consumption rate (OCR) of washed platelets was measured with sequential injection of agonist (3 µg/mL collagen or 0.05 U/mL thrombin), 2.5 µM oligomycin, 1.4 µM Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and 2 µM rotenone and antimycin (R+A). (A) Representative OCR curve and definition of spare capacity and basal, agonist-induced, ATP-linked, maximal, and non-mitochondrial respiration. Maximal and non-mitochondrial respiration were not quantified due to interference of a scrambled peptide

(Supplementary Figure 2) (B) Mean curve of resting platelets pre-treated with media or 30 μM CxxCpep for 1 hour before activation with collagen. (C) OCR quantifications of (B). (D) Mean curve of thrombin-activated platelets. (E) OCR quantifications of (D). Graphs show ± SEM (n = 3). Data analysed using paired two-way-ANOVA and Sidak's post-test. * $p<0.05$; ** $p<0.01$; ns non-significant.