SUPPLEMENTAL MATERIALS

Anti-platelet effect of carbon monoxide is mediated by NAD^+ and ATP depletion

Short title: **Effects of CO on platelet function & bioenergetics**

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Supplemental methods

1.1. Preparation of inactive CORM-A1 (iCORM-A1)

Inactive CORM-A1 (iCORM-A1) was prepared by acidification as described previously[1] at a concentration of 25 mM and stored in aliquots at -20°C.

1.2. Analysis of Cellular Bioenergetics using Extracellular Flux Technology – technical details and calculations

To measure mitochondrial function and glycolysis in isolated human platelets, a Seahorse Bioscience XFe96 Analyzer was used. One day before the experiment, a sensor cartridge was hydrated in XF calibrant and maintained at 37°C in air without CO_2. On the day of the experiment, freshly isolated platelets were suspended in assay medium at a density of 2×10^5 per ml and introduced into the Seahorse XFe96-well plates (50 µl per well, giving a number of 10×10^6 of platelets per well) followed by centrifugation (700g, 5 min). Platelets adhered to the surface and remained adhered throughout the assay. After centrifugation, 130 µl of assay medium was added to each well for a total volume of 180 µl per well and platelets were incubated with bicarbonate-free low buffered assay medium for one hour at 37°C in air without CO_2 prior to the beginning of the assay. Changes in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed over time by sequential injections of reagents in ports A, B, C and D. In the mitochondrial stress test, PBS or CORM-A1 or iCORM-A1 were injected into port A, 1 µg/ml oligomycin into port B, 0.3 µM/1 mM FCCP/pyruvate into port C and 0.5 µM/0.5 µM rotenone/antimycin A into port D. Concentrations of oligomycin, FCCP, rotenone and antimycin A were optimized (data not shown). Mitochondrial stress response enabled determination of the following key parameters of mitochondrial function: acute response, proton leak, maximal respiration, spare respiratory capacity, ATP production and non-mitochondrial oxygen consumption. The bioenergetics parameters (presented in Fig. 1C) were calculated as follows: acute response (mitochondrial respiration; last OCR value before oligomycin minus last OCR value before PBS or CORM-A1), ATP-linked respiration (last OCR value before oligomycin minus minimum OCR value after oligomycin), proton leak (minimum OCR value after oligomycin minus non-mitochondrial OCR), maximal respiration (maximum OCR value after FCCP minus – non-mitochondrial OCR), spare capacity (maximum OCR value after FCCP minus last OCR measurement before PBS or CORM-A1), non-mitochondrial oxygen consumption (minimum OCR measurement after rotenone/antimycin A), acute response (glycolysis; last ECAR value before oligomycin minus last ECAR value before PBS or CORM-A1), oligomycin-induced glycolysis (maximum ECAR value after oligomycin minus last ECAR value before oligomycin). Additionally, we calculated acute response and oligomycin-induced changes in ECAR.

In experiments with pre-treatment of platelets with CORM-A1 prior to thrombin treatment, CORM-A1 was injected into port A, 0.1 U/ml thrombin into port B, 1 µg/ml oligomycin or 0.3 µM/1 mM FCCP/pyruvate into port C and 0.5 µM/0.5 µM rotenone/antimycin A into port D. We analyzed the effects of CORM-A1 on thrombin-induced changes in OCR ($\Delta(\text{Thr-A1})_{\text{mt. respiration}}$) and in ECAR ($\Delta(\text{Thr-A1})_{\text{glycolysis}}$), calculated based on the differences in OCR and ECAR before and after thrombin injection. Based on the experiments with oligomycin, we analyzed the ability of platelets pre-treated with CORM-A1 and thrombin to further maximize their glycolysis in response to oligomycin, reflecting a spare glycolytic capacity (Δ(O-Thr)), while based on the experiments with FCCP/pyruvate, we analyzed the ability of platelets pre-treated with CORM-A1 and thrombin to further maximize their mitochondrial respiration in response to FCCP, reflecting a spare respiratory capacity (Δ(FCCP-Thr)). Bioenergetic parameters of mitochondrial respiration and glycolysis (presented in Fig. 2E) were calculated as follows: $\Delta(\text{Thr-A1})_{\text{mt. respiration}}$ as a difference between third OCR value after thrombin and last OCR value before thrombin, Δ(FCCP-Thr) as a difference

between maximum OCR value after FCCP and last OCR value before FCCP; $\Delta(\text{Thr-A1}\Delta)_{glycolysis}$ as a difference between third ECAR value after thrombin and last ECAR value before thrombin; Δ(O-Thr) as a difference between maximum ECAR value after oligomycin and last ECAR value before oligomycin.

1.3. Lactate dehydrogenase activity measurements

Measurements lactate dehydrogenase activity (LDH) were performed by the enzymatic photometric methods using an automatic Pentra 400 (Horiba, Japan) biochemical analyzer according to the manufacturer's instructions. Samples were suspended in assay buffer at a density of $2x10^5$ platelets/µl; treated with PBS or CORM-A1 (10–1000 µM) or lysis buffer (hypotonic phosphate buffer, 20 mM; positive control) for 8 min at 37°C, followed by centrifugation (1000g, 10 min) and measurement of LDH activity in supernatant.

1.4. Analysis of GAPDH activity

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity was analyzed in samples of washed platelets (WP; suspended in PBS containing 1 g/l glucose and 2 mM glutamine, in a number of 300×10^6 per 0.5 ml per sample) untreated or incubated for 8 min with CORM-A1. Samples were centrifuged (700g, 5 min, RT), washed one time with ice-cold DPBS and lysed in 200 µl of 20 mM phosphate buffer (pH 7.0) for 10 min on ice followed by centrifugation (12 000g, 1 min, RT) as described by Schmidt and Dringen[2]. 20 µl of supernatant was transferred to 96-well plate and GAPDH activity was determined using the method described by Bisswanger[3], briefly, 180 µl of assay mixture (triethanolamine 94 mM, glyceraldehyde 3-phosphate 0.9 mM, NAD^+ 1 mM and potassium dihydrogen arsenate 3 mM) was added and recording of absorbance at 340 nm was immediately started (every 1 min for 10 min, RT). Phosphate buffer was used as a blank. Concentration of NADH was calculated based on absorption coefficient for NADH: $\varepsilon_{340} = 6.3 \times 10^3$ l mol^{-1} cm^{-1}. Exponential curve was fitted to the obtained results and activity of GAPDH was calculated as a coefficient of slope tangent to the curve at the time-point 0, followed by normalization for protein content (Fig. VA). Protein content was measured using BCA Protein Assay Kit (Sigma-Aldrich). To confirm the validity of the method we performed measurement of GAPDH activity in MBA-MD-231 breast cancer cells treated with iodoacetic acid (IAA), which is an inhibitor of GAPDH (Fig. VB).

1.5. Analysis of PFK-1 activity

Phosphofructikinase 1 (PFK-1) activity was analysed in samples of washed platelets (WP; suspended in PBS containing 1 g/l glucose and 2 mM glutamine, in a number of 500×10^6 per 0.5 ml per sample) untreated or incubated for 30 min with CORM-A1, which were processed for the analysis according to the protocol described by Almeida[4] with minor changes. WP were sonicated for 10 min in 150 µl of 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 1 mM EGTA, 10 mM dithiothreitol and protease inhibitor cocktail (Sigma-Aldrich, P2714) and centrifuged (16 600g, 30 min, 4℃). Supernatants were transferred into fresh tubes and used for the assay. PFK-1 activity was determined according to the procedure described by Ishikawa[5] by measuring of NADH oxidation, which correlates with formation of fructose-1,6-bisphoshate. The assay mixture contained 50 mM Tris-HCl buffer (pH 8.0), 1 mM fructose-6 phosphate, 1 mM ATP, 2 mM $MgCl_2$, 0.16 mM NaDH, 2.6 mM dithiothreitol, 1 mM EDTA, 5 mM $(NH_4)_2SO_4$, 0.4 U/ml aldolase, 2.5 U/ml triose-phosphate isomerase (TPI) and 0.4 U/ml glyceraldehyde 3-phosphate dehydrogenase (GAPDH); fructose-6 phosphate and ATP were added just before start of each measurement. 450 µl of the assay mixture was mixed with 50 µl of sample and the absorbance at 340 nm was measured continuously for 5 min (Perkin Elmer). Protein concentration was measured with Bradford Reagent (Quick Start Bradford 1×Dye Reagent; Bio-Rad) in 10× diluted samples, according to the manufacture’s protocol.

1.6. Myoglobin assay for quantitation of CO release from CORM-A1

The assay was performed as described by Fayad-Kobeissi[6] with minor changes. Heart myoglobin solution was dissolved in phosphate buffered saline (PBS, pH 7.4) at a concentration of 66 µM, converted to deoxymyoglobin (dMb) with sodium dithionite (0.2%) and maintained at 37℃. CORM-A1 was added to dMb and incubated at 37℃. After 1, 2, 5 or 8 min of incubation (accordingly to the timing of platelet aggregation) dMb (without CORM-A1) and carboxymyoglobin (MbCO) spectrum (500 – 600 nm) were recorded using UV-VIS spectrophotometer (Perkin Elmer) at room temperature.

Supplemental Figures and Figure Legends

Figure I

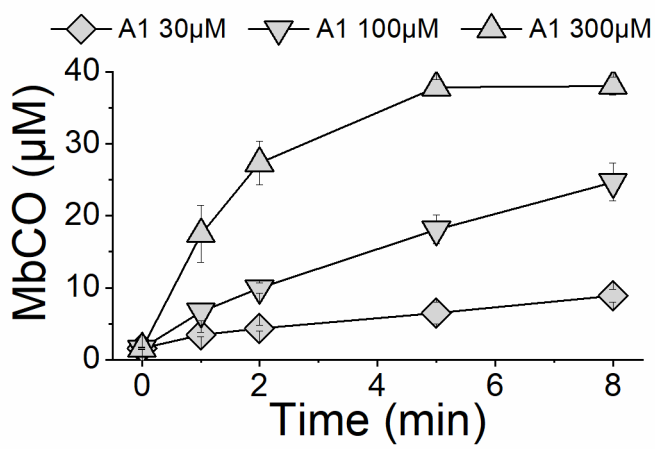

Fig. I. Quantification of CO released from CORM-A1 using the myoglobin assay. Time and concentration-dependent changes in myoglobin (MbCO) concentrations after addition of 30, 100 or 300 μM CORM-A1 to the solution of deoxymyoglobin (dMB) in the time corresponding to platelet aggregation assay. Data represent means ± SD of three independent experiments.

Figure II

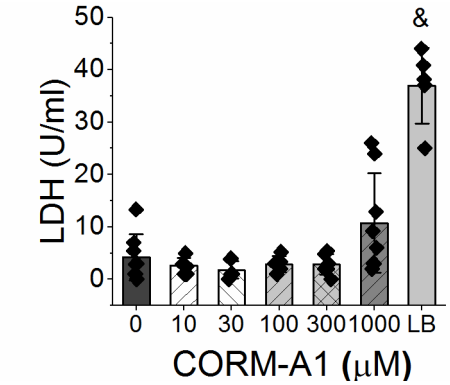

Fig. II. Cytotoxicity test in platelets treated with CORM-A1. Activity of lactate dehydrogenase (LDH) released from platelets treated with PBS, CORM-A1 or lysis buffer (hypotonic phosphate buffer, 20 mM). Data represent the means ± SD of 3–5 independent experiments; $^{\&}$ $P < 0.0001$ as compared to PBS group.

Figure III

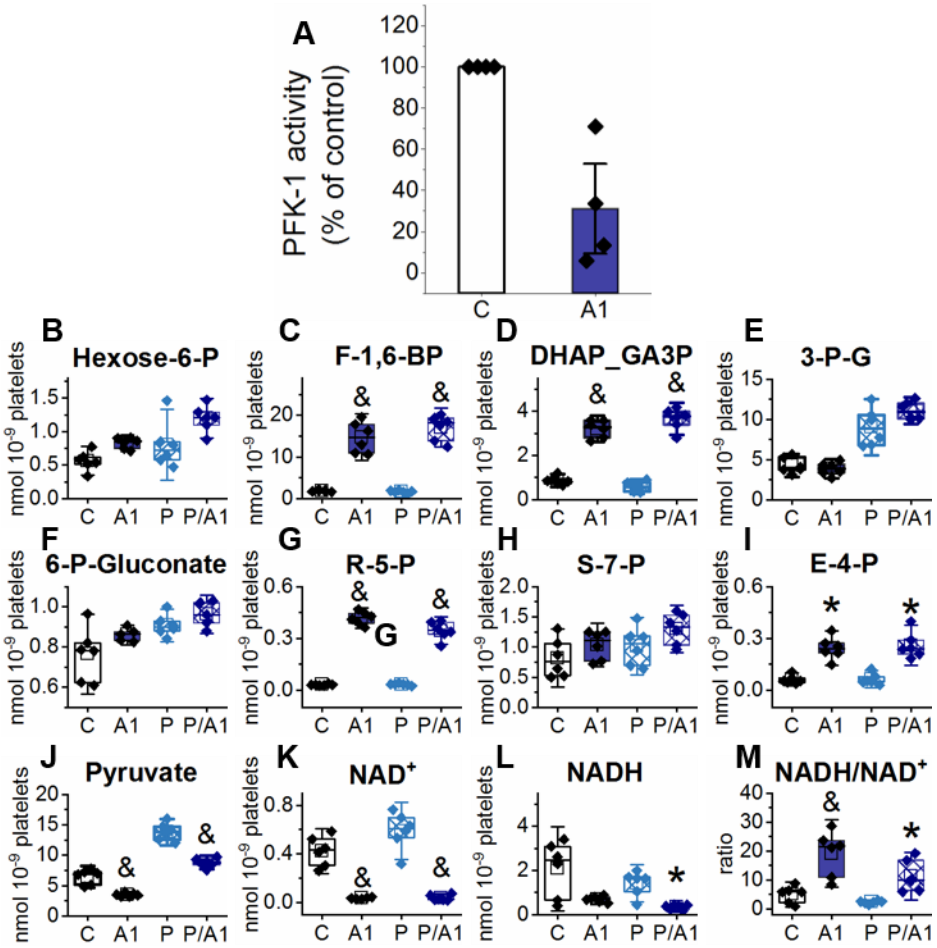

Fig. III. Effect of prolonged incubation of CORM-A1 on phosphofructokinase-1 (PFK-1) activity and metabolomics in human platelets. (A) Human washed platelets (WP) were suspended in PBS containing glucose (1 g/l) and glutamine (2 mM) and incubated for 30 min with PBS (control; C) or CORM-A1 (300 µM). CORM-A1 induced inhibition of PFK-1 to 31+/-14.6 % of control (mean +/-SEM; four independent experiments, 1–4 replicates in each experiment). For statistical analysis Student t-test was performed demonstrating no significant difference between the tested groups. (B–M) Human washed platelets (WP) were suspended in PBS containing glucose (1 g/l) and glutamine (2 mM) without (C, A1) or with pyruvate (100 µM; P, A1/P) and incubated for 30 min with PBS (control; C) or CORM-A1 (300 µM). Data demonstrate concentrations of selected metabolic by-products of glycolysis or mitochondrial respiration, presented as means ± SD (nmol/10^9 platelets) of three independent experiments, n = 2 replicates in each experiment. For statistical analysis, one-way ANOVA was performed (* $P <$ 0.05, $^{\&}$ $P <$ 0.0001).

Figure IV

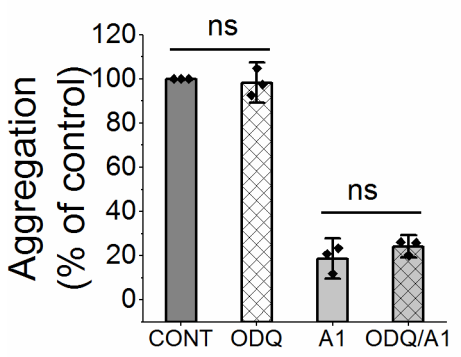

Fig. IV. The lack of ODQ effect on CORM-A1–induced inhibition of platelet aggregation. Aggregation of WP pre-incubated for 1 min with DMSO or ODQ (inhibitor of soluble guanyl cyclase; 10 μM), then treated with water or 300 μM CORM-A1, followed by activation with thrombin (0.1 U/ml). Data represent the means ± SD of free independent experiments.

Figure V

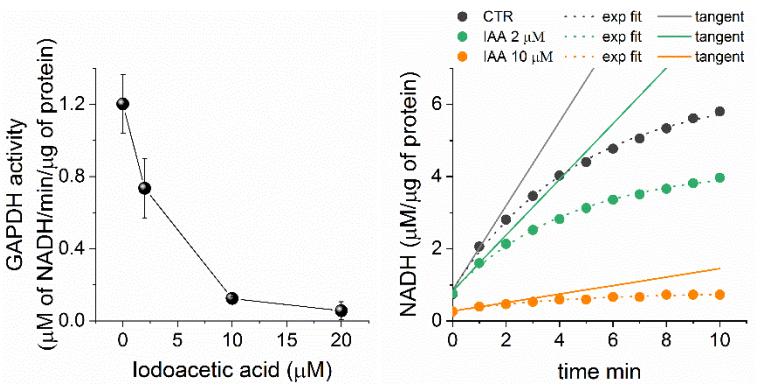

Fig. V. Effect of iodoacteic acid (IAA) on activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in breast cancer cells. (A) GAPDH activity in MDA-MB-231 breast cancer cells treated for 30 minutes with IAA (0, 2, 10, 20 μM). (B) GAPDH activity was calculated based on the rate of NADH concentration, produced in cell homogenates by GAPDH enzyme, and normalized to protein level. The measurement of absorbance (λ = 340 nm) was started immediately after addition of reaction mixture (containing NAD^+ and glyceraldehyde 3-phosphate as substrates and potassium dihydrogen arsenate to inhibit a back reaction) and proceed for 10 min. Exponential curve (y = y0 + A*exp(R0*x); Origin Pro 2019) was fitted to the obtained data points and an initial velocity was calculated as a slope of tangent at 0 point (A*R0).

Major Resources Table

Reagents	Source	Persistent ID
Adenosine 5’-triphosphate disodium salt hydrate (ATP)	Sigma-Aldrich	Cat# A2383, CAS: 34369-07-8
Albumin bovine Fraction V	SERVA	Cat# 1193.04, CAS: 9048-46-8
Aldolase from rabbit muscle	Sigma-Aldrich	Cat# A2714, CAS: 9024-52-6
Ammonium sulfate (NH4)2SO4	Sigma-Aldrich	Cat# A4418, CAS: 7783-20-2
Antimycin A	Sigma-Aldrich	Cat# A8674; CAS: 1397-94-0
BCA Kit for Protein Determination	Sigma-Aldrich	Cat# BCA1
Bradford Reagent (Quick Start Bradford 1 × Dye Reagent	Bio-Rad	Cat# 500-0205
Collagen	CHRONO-LOG	Cat# 385
CORM-A1	Sigma-Aldrich	Cat# SML0315, CAS: 17363-08-5
78c	Merc/Calbiochem	Cat# 5.38763, CAS: 1700637-55-3
2-Deoxy-D-Glucose	Sigma-Aldrich	Cat# D8375, CAS: 154-17-6
Dimethyl malonate (DMM)	Sigma-Aldrich	Cat# 136441, CAS: 108-59-8
Dimethylsulfoxide (DMSO)	Life Technologies	Cat# D12345, CAS: 67-68-5
Dithiothreitol (DTT)	BioShop	Cat# DTT 001.5, CAS: 3483-12-3
DPBS	Lonza	
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)	Sigma-Aldrich	Cat#C2920; CAS: 370-86-5
D-Fructose 6 phosphate dipotassium salt	Sigma-Aldrich	Cat# F1502, CAS: 103213-47-4
Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA)	Sigma-Aldrich	Cat# E1644, CAS: 6381-92-6
Ethyl glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA)	Sigma-Aldrich	Cat# E4378, CAS: 67-42-5
Glucose	Merck	Cat# 1.08337.1000; CAS: 50-99-7
Glutamine	Sigma-Aldrich	Cat#G3126; CAS: 56-85-9
DL-Glyceraldehyde 3-phosphate	Sigma-Aldrich	Cat# G5251, CAS: 591-59-3
Glyceraldehyde 3-phosphate Dehydrogenase from rabbit muscle (GAPDH)	Sigma-Aldrich	Cat# G2267, CAS: 9001-50-7
GSK2837808A	Cayman	Cat# 20626, CAS: 1445879-21-9
Hydrochloric acid	Sigma-Aldrich	Cat# 258148, CAS: 7647-01-0
Iodoacetic acid (IAA)	Sigma-Aldrich	Cat# I4386, CAS: 64-69-7
Magnesium chloride	Sigma-Aldrich	Cat# M8266, CAS: 7786-30-3
Myoglobin from equine heart	Sigma-Aldrich	Cat# M1882, CAS: 100684-32-0
β-Nicotinamide adenine dinucleotide hydrate (NAD)	Sigma-Aldrich	Cat# N6522, CAS: 53-84-9
β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH)	Sigma-Aldrich	Cat# N8129, CAS: 606-68-8
Oligomycin	Sigma-Aldrich/Calbiochem	Cat#495455; CAS: 1404-19-9
3PO	Merc/Calbiochem	Cat# 525330; CAS: 18550-98-6

Potassium arsenate monobasic (KH2AsO4)	Sigma-Aldrich	Cat# A6631, CAS: 7784-41-0
Potassium dihydrogen phosphate (KH2PO4)	Merck	Cat# 1.04873.1000, CAS: 778-77-0
Potassium phosphate dibasic (K2HPO4)	Sigma-Aldrich	Cat# 60356, CAS: 7758-11-4
Protease inhibitor cocktail	Sigma-Aldrich	Cat# P2714
Rotenone	Sigma-Aldrich	Cat#R8875, CAS: 83-79-4
Seahorse XF Base Minimal DMEM	Agilent Technologies	Cat# 102353-100
Sodium dithionite	Sigma-Aldrich	Cat# 157953, CAS: 7775-14-6
Sodium pyruvate	Sigma-Aldrich	Cat# P5280, CAS:113-24-6
Thrombin	BIOMED-LUBLIN	BioTrombina400
Triosephosphate Isomerase from rabbit muscle	Sigma-Aldrich	Cat# T6258, CAS: 9023-78-3
Tris Base	Calbiochem	Cat# 648311, CAS: 77-86-1
UK-5099	Tocris Bioscience	Cat#4186; CAS: 56396-35-1

Supplemental References

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4. Almeida A, Moncada S, Bolaños JP. Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. *Nat Cell Biol* 2004;6(1):45-51.

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