Mitochondrial ATP generation in stimulated platelets is essential for granule secretion but dispensable for aggregation and procoagulant activity

Paresh P. Kulkarni,[1] Mohammad Ekhlak,[1] Vijay K. Sonkar[2] and Debabrata Dash[1]

[1]Center for Advanced Research on Platelet Signaling and Thrombosis Biology, Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh and [2]Department of Molecular and Human Genetics, Institute of Science, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Correspondence: DEBABRATA DASH: ddash.biochem@gmail.com

doi:10.3324/haematol.2021.279847

Mitochondrial ATP generation in stimulated platelets is essential for granule secretion but dispensable for aggregation and procoagulant activity

Short Title: Mitochondrial ATP fuels platelet granule secretion

Paresh P. Kulkarni[1], Mohammad Ekhlak[1], Vijay K. Sonkar[2], Debabrata Dash[1]*

1 Center for Advanced Research on Platelet Signaling and Thrombosis Biology, Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, India

2 Department of Molecular and Human Genetics, Institute of Science, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, India

***Correspondence**: Prof. Debabrata Dash, Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, India, Tel: +91-9336910665, Fax: +91-542-2367568, E-mail: ddash.biochem@gmail.com

Supplementary figures: Fig. S1 to S3

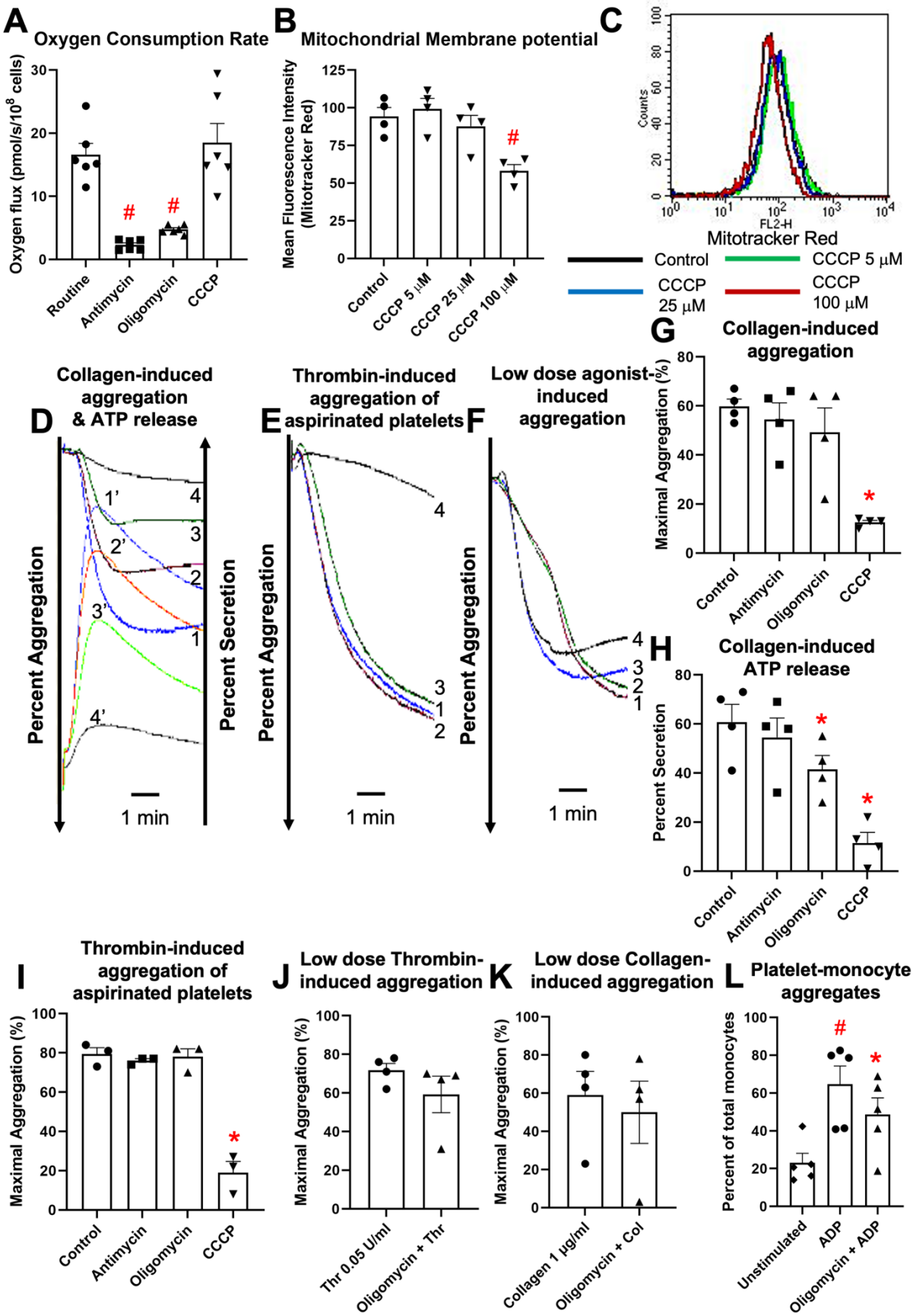


Figure S1. Washed human platelets were pre-treated with vehicle (control), antimycin (2 µg/ml), oligomycin (10 µg/ml) or CCCP (100 µM) for 15 min at room temperature.

A and B, bar diagrams indicating oxygen consumption rates and mitochondrial membrane potential, respectively. **C,** corresponding histogram overlay plot representing Mitrotracker red (Invitrogen #M7512) fluorescence. **D,** representative tracings showing collagen (2 μg/ml)-induced aggregation (1-4) or ATP release (1'-4') of platelets pre-treated with vehicle (1,1'), antimycin (2,2'), oligomycin (3,3') or CCCP (4,4'). **G and H,** corresponding bar diagram quantifying mean platelet aggregation and platelet dense granule secretion in different samples, respectively. **E,** representative tracings showing human thrombin (0.2 U/ml)-induced aggregation of aspirinated platelets pre-treated with vehicle (1), antimycin (2), oligomycin (3) or CCCP (4). **I,** corresponding bar diagram quantifying mean thrombin (0.2 U/ml)-induced aggregation of aspirinated platelets. **F,** representative tracings showing thrombin (0.05 U/ml)(1,2) or collagen (1 μg/ml)(3,4) induced aggregation of platelets pre-treated with vehicle (1,3) or oligomycin (2,4). **J, and K,** corresponding bar diagram quantifying mean platelet aggregation induced by thrombin (0.05 U/ml) and collagen (1 μg/ml), respectively. Whole blood samples labelled with platelet- (APC-anti-CD41a)(BD Biosciences 559777) or leukocyte- (FITC-anti-CD14)(BD Biosciences 555397) specific antibodies were pre-treated with vehicle (control) or oligomycin (10 μg/ml) for 15 min at room temperature in the dark followed by addition of ADP (20 μM). **L,** bar diagrams quantifying mean percent platelet-monocyte aggregates in different samples. Each dot represents an independent observation. Data are presented as mean ± SEM. * represents $p<0.05$ with respect to vehicle-treated agonist-stimulated platelets. # represents $p<0.05$ with respect to vehicle-treated unstimulated platelets. Significance in difference of means was tested by repeated measures ANOVA and Dunnett's multiple comparison test. Thr, Thrombin.

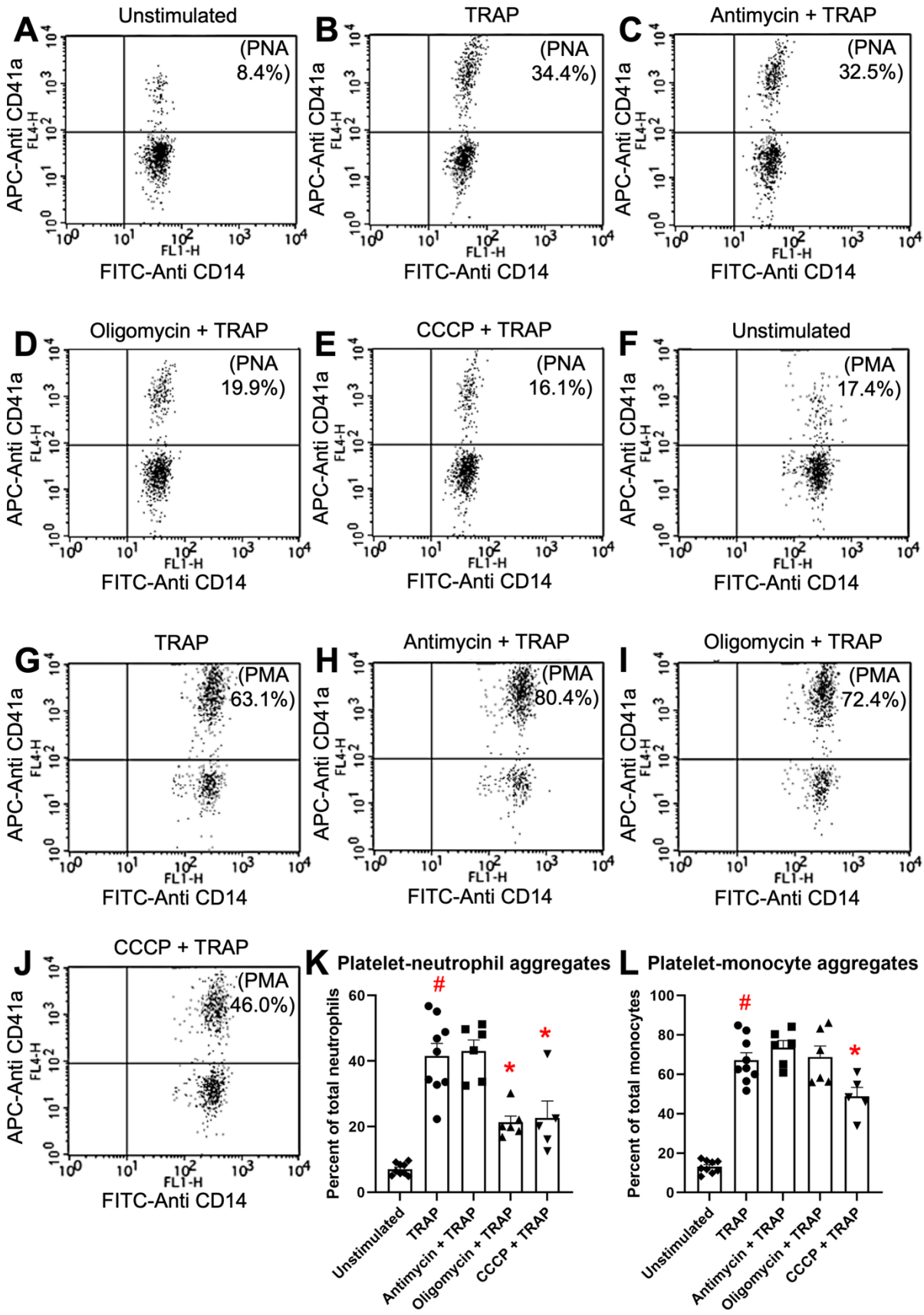
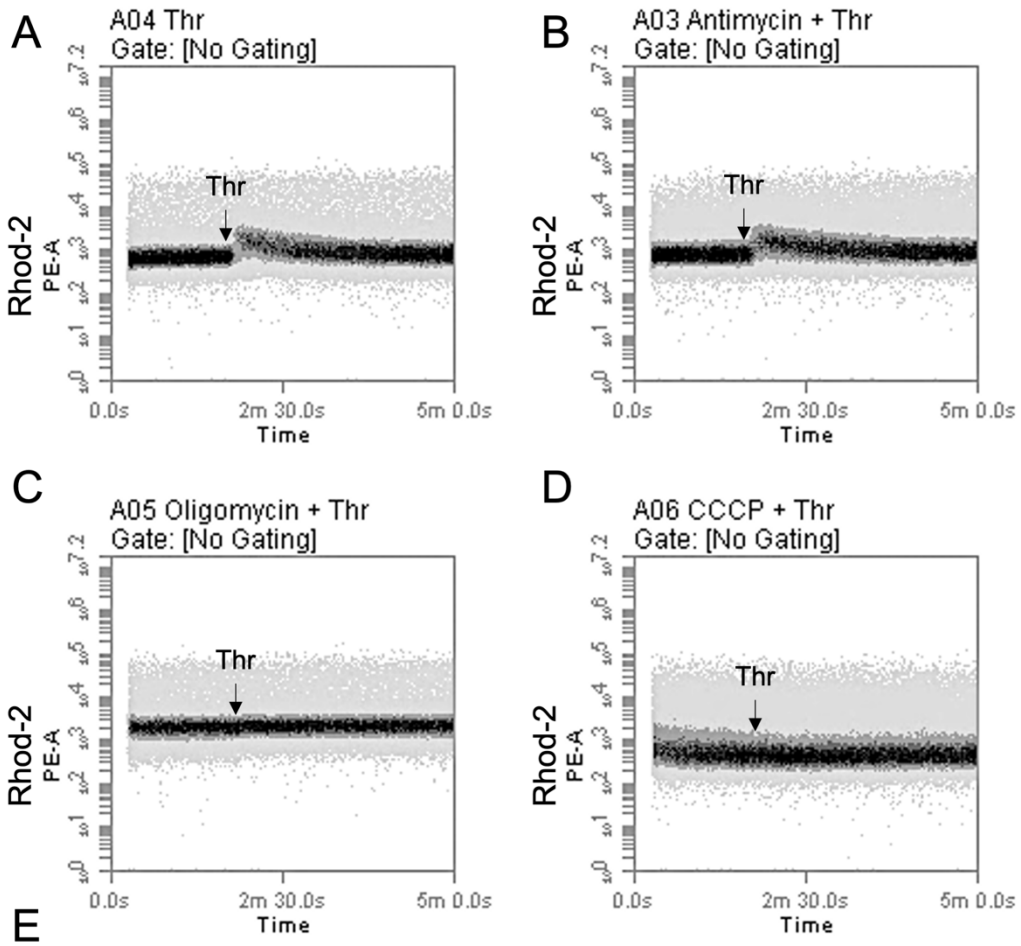


Figure S2. Whole blood samples labelled with platelet- (APC-anti-CD41a) (BD Biosciences #559777) or leukocyte- (FITC-anti-CD14) (BD Biosciences #555397)

specific antibodies were pre-treated with vehicle (control), antimycin (2 μg/ml), oligomycin (10 μg/ml) or CCCP (100 μM) for 15 min at room temperature in the dark followed by addition of TRAP (10 μM). **A-E,** dot plots representing platelet-neutrophil aggregates (right upper quadrant) in unstimulated platelets, or TRAP-stimulated platelets pre-treated with vehicle, antimycin, oligomycin or CCCP, respectively. **F-J,** dot plots representing platelet-monocyte aggregates (right upper quadrant) in unstimulated platelets, or TRAP-stimulated platelets pre-treated with vehicle, antimycin, oligomycin or CCCP, respectively. The numbers in parentheses indicate platelet-leukocyte aggregates as a percentage of total events in the leukocyte gate. **K and L,** corresponding bar diagrams quantifying mean percent platelet-neutrophil and platelet-monocyte aggregates in different samples, respectively. Each dot represents an independent observation. Data are presented as mean ± SEM. * represents $p<0.05$ with respect to vehicle-treated TRAP-stimulated platelets. # represents $p<0.05$ with respect to vehicle-treated unstimulated platelets. Significance in difference of means was tested by repeated measures ANOVA and Dunnett's multiple comparison test. PNA, Platelet-neutrophil aggregates; PMA, Platelet-monocyte aggregates.



E

Control (Vehicle-pre-treated agonist-stimulated platelets)	OXPHOS / Glycolysis	mPTP formation ROS generation ATP synthesis	PS exposure Integrin activation Granule release	Procoagulant activity Platelet aggregation Thrombus Reinforcement
Antimycin (Complex III inhibitor) No mitochondrial ATP synthesis No mitochondrial oxidation of energy substrates	OXPHOS / Glycolysis	mPTP formation ROS generation ATP synthesis	PS exposure Integrin activation Granule release	Procoagulant activity Platelet aggregation Thrombus Reinforcement
Oligomycin (F_oF_1 ATP synthase inhibitor) No mitochondrial ATP synthesis Limited mitochondrial oxidation of energy substrates	OXPHOS / Glycolysis	mPTP formation ROS generation ATP synthesis	PS exposure Integrin activation Granule release	Procoagulant activity Platelet aggregation Thrombus Reinforcement
CCCP (Protonophore & Uncoupler) No mitochondrial ATP synthesis Maximal mitochondrial oxidation of energy substrates	OXPHOS / Glycolysis	mPTP formation ROS generation ATP synthesis	PS exposure Integrin activation Granule release	Procoagulant activity Platelet aggregation Thrombus Reinforcement

Figure S3. Washed human platelets labelled with Rhod-2-AM (Invitrogen #R1244) were pre-treated with either vehicle (control), antimycin (2 μg/ml), oligomycin (10

μg/ml) or CCCP (100 μM) for 15 min at room temperature in the dark, followed by addition of thrombin (0.5 U/ml). **A-D,** representative time-series dot plots exhibiting Rhod-2 fluorescence in vehicle, antimycin, oligomycin and CCCP pre-treated platelets, respectively. **E,** scheme depicting the effects of mitochondrial uncoupler and inhibitors on platelet bioenergetics and agonist-induced functional responses. Thr, Thrombin.