Arachidonic acid depletion extends survival of cold-stored platelets by interfering with the [glycoprotein Ibα – 14-3-3ζ] association

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Online Supplementary Design and Methods

Materials

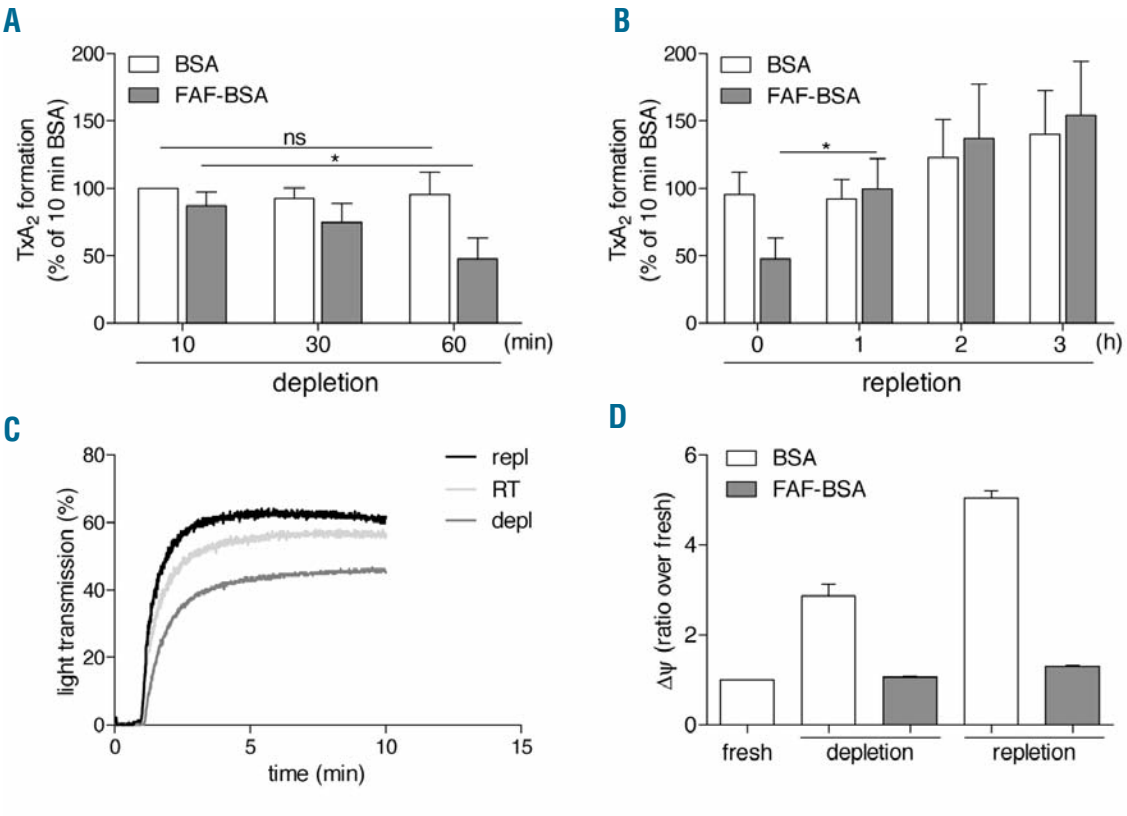
We used the following products (with sources): hematin (Alfa Aesar, Ward Hill, MA, USA), arachidonic acid (AA; Bio/Data Corporation, Horsham, PA, USA), biotin-AA, cyclo-oxygenase (COX) activity assay buffer (Cayman Chemical, Ann Arbor, MI, USA), BSA fraction V (BSA) and fatty acid free-BSA (FAF-BSA), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1), indomethacin, luminol (Sigma-Aldrich, St Louis, MO, USA), BSA for blotting (MP Biomedicals, Solon, OH, USA), cell tracker green, (Molecular Probes, Invitrogen, Carlsbad, CA, USA), 5-chloromethyl fluorescein diacetate (CMFDA), lipo-oxygenase-inhibitor 5, 8, 11-eicosatriynoic acid (ETI, Cayman Chemical, Ann Arbor, MI, USA), Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA), prostacyclin (PGI_2, Cayman Chemical, Ann Arbor, MI, USA), P38MAPK substrate ATF-2 fusion protein, kinase buffer (Cell Signaling Technology, Danvers, MA, USA), P38MAPK-inhibitor SB203580 and cytochrome P450 mono-oxygenase inhibitor SK&F96365 (Alexis Biochemicals/Enzo Lifesciences BVBA, Zandhoven, Belgium), *o*-sialoglycoprotein endopeptidase (OSGE, Cederlane Laboratories, Hornby, Ontario, Canada), and TxA_2 Enzyme Immuno Assay (EIA) kit (Assay Designs, Ann Arbor, MI, USA). Thromboxane receptor (TPα) antagonist SQ30741 was a kind gift from Bristol-Meyers-Squibb (Maarssen, The Netherlands).

Antibodies used for western blotting were directed against GPIbα (clone SZ2, Beckman Coulter, Marseille, France), COX-1 (Abcam, Cambridge, UK), phospho ATF-2 (Thr 171), total Bad (Zymed, Invitrogen, Carlsbad, CA, USA), Bad Ser 136, Phospho-P38MAPK (Thr180/Tyr182), total P38MAPK, secondary horseradish peroxidase-labeled anti-rabbit antibodies (Cell Signaling Technology, Danvers, MA, USA), Bad (Enzo life science, Farmingdale, NY, USA), 14-3-3ζ (C-16, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were: Alexa-680 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and IRDYe 800CW (LI-COR Biosciences, Lincoln, NE). Antibodies for immunoprecipitation were against GPIbα (AK2, Santa Cruz Biotechnology), total Bad (Cell signaling Technology, Danvers, MA, USA) and 14-3-3ζ (V-16, Santa Cruz Biotechnology).

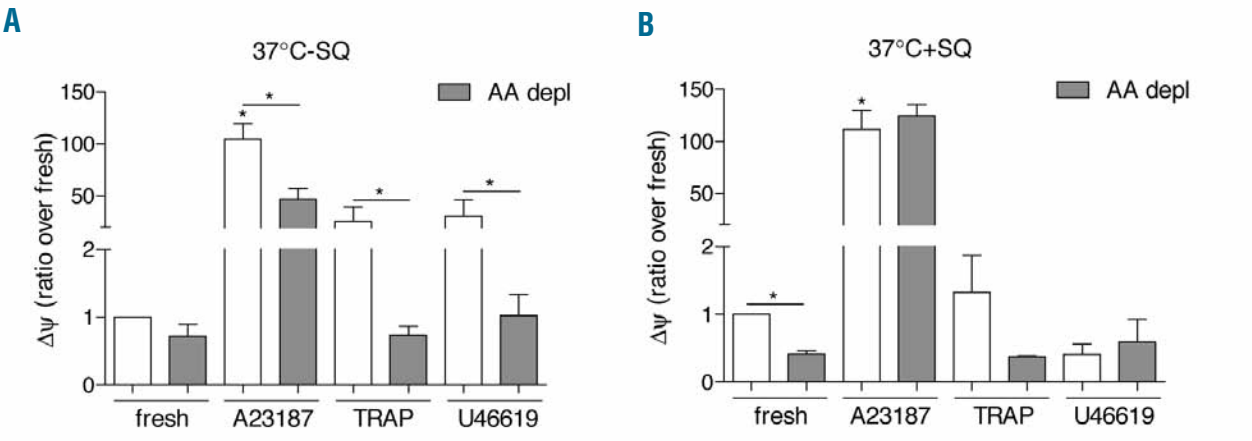
For studies shown in the *Supplementary Appendix*, we used the following products (with sources): calcium ionophore A23187 (Calbiochem, Darmstadt, Germany), fibrinogen (Enzyme Research Laboratories, South Bend, IN, USA), thrombin receptor (PAR1)-activating peptide (TRAP, SFLLRN, Bachem, Switzerland), and TxA_2-mimetic U46619 (Cayman Chemical, Ann Arbor, MI, USA). The P_2Y_{12} blocker AR-C69931MX was a kind gift from Astra Zeneca, Loughborough, UK.

Hemostatic properties after arachidonic acid depletion/repletion

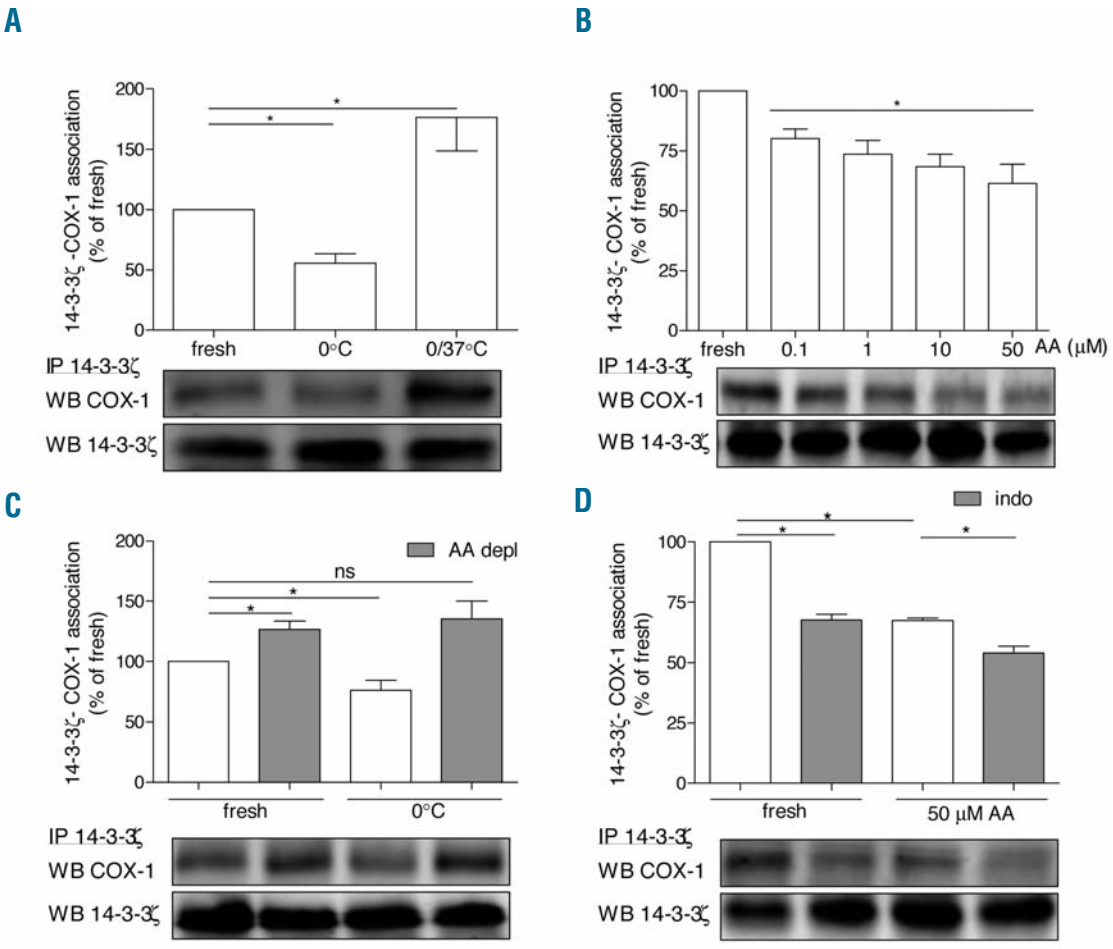
AA-depleted platelets were prepared by incubation with FAF-BSA using BSA as a control. AA-repleted platelets were prepared by subsequent incubation with AA. In non-stirred suspensions, TxA_2 was measured upon stimulation with 5 μM TRAP (10 min, 37°C). Aggregation induced by 20 μM TRAP was measured in an optical aggregometer (Chronolog Corporation, Haverford, PA, USA) at 37°C with stirring at 900 rpm in the presence of 100 nM AR-C69931MX and 100 μg/mL fibrinogen.



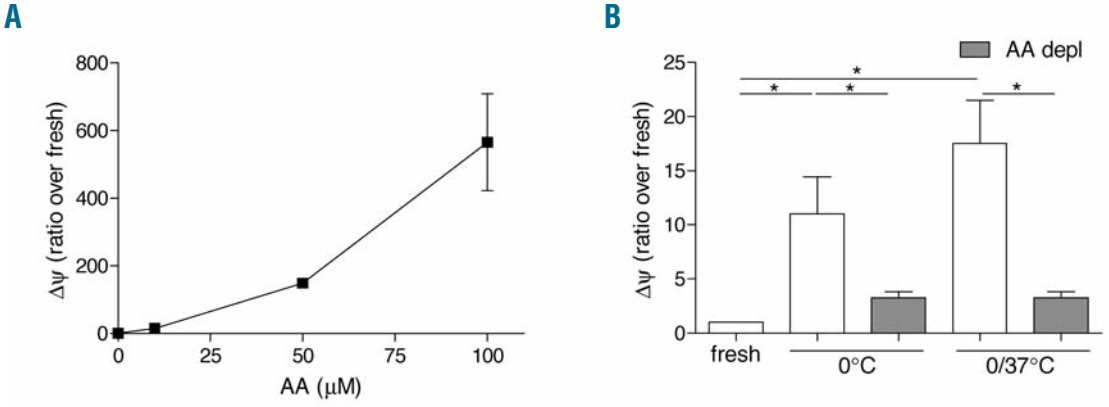
Online Supplementary Figure S1. Reversible modulation of TxA_2 formation by AA de-/repletion. (A) Depletion of platelet-AA. Platelets were incubated in buffer with BSA (open bars) and FAF-BSA (gray bars) for the indicated times at 0°C. Samples were washed in the presence of PGI_2, placed at 37°C for 30 min to restore responsiveness and incubated with 5 μM TRAP (10 min, 37°C) to induce TxA_2 formation. (B) Repletion of platelet-AA. Platelets incubated with FAF-BSA for 1 h at 0°C were washed and incubated in buffer containing 1 mM AA for the indicated times. Following a second washing step, TRAP induced TxA_2 formation was measured. (C) Reversible modulation of aggregation. Room temperature-stored platelets (RT), AA-depleted platelets (60 min with FAF-BSA, 0°C) and AA-repleted platelets (AA-depleted platelets incubated for 1 h with 1 μM AA, 37°C) were stimulated with 20 μM TRAP in the presence of the P2Y12 blocker AR-C69931MX and aggregation was measured at 37°C. Mean aggregations were 57.3 ± 2.1, 42.3±3.1 and 60.0±2.8 % respectively. (D) AA-repletion does not initiate apoptosis. The change in mitochondrial membrane potential ($\Delta\Psi$) was measured in platelets incubated under the conditions described for (C). Data are means ± SEM (n=3) with statistically significant differences indicated by an asterisk ($P<0.05$).



Online Supplementary Figure S2. Free arachidonic acid contributes to agonist-induced platelet apoptosis. Effect of AA-depletion on apoptosis induction at 37°C in the absence (A) and presence (B) of the TPα-blocker SQ30741. Platelets were incubated at 0°C for 4 h with normal BSA (open bars) and FAF-BSA (gray bars) and then washed: apoptosis induction was analyzed by measuring the change in mitochondrial membrane potential, $\Delta\Psi$. Platelets were stimulated without stirring with Ca^{2+}-ionophore A23187 (3 μM, with 2 mM extracellular Ca^{2+}), TRAP (20 μM) and the TxA_2-analog U46619 (10 μM).



Online Supplementary Figure S3. Arachidonic acid dissociates the [14-3-3ζ-COX-1] complex. Measurement of the [14-3-3ζ - COX-1] association. (A) Fresh, 0°C- and 0/37°C-treated platelets. (B) Fresh platelets and platelets incubated with 100 nM, and 1, 10 and 50 μM AA for 10 min at 0°C. (C) Fresh and 0°C-treated platelets incubated with normal BSA (open bars) and FAF-BSA (AA depl, gray bars). (D) Effect of indomethacin. Fresh platelets and platelets incubated with 50 μM AA (10 min, 0°C) in the absence and the presence of indomethacin.



Online Supplementary Figure S4. Arachidonic acid triggers apoptosis in murine platelets. (A) Addition of AA induces ΔΨ-change in murine platelets. The ΔΨ-change in fresh platelets and platelets incubated with 10, 50 and 100 μM AA for 10 min at 0°C. (B) AA depletion lowers the induction of cold-induced apoptosis. The ΔΨ-change in fresh 0/37°C-treated platelets incubated with FAF-BSA to lower platelet-AA (AA depl, gray bars) and normal BSA (open bars). Data are means ± SEM (n=3) with significant differences ($P<0.05$) compared with fresh platelets and between treatments indicated by (*).