On-line Supplemental Methods

Mice

All animal experiments were conducted under a protocol approved by the Harvard Medical Area standing committee on animals at Brigham and Women’s Hospital in accordance with the U.S. National Institutes of Health or in agreement with the German guidelines for use of live animals approved by the Institutional Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen. C57BL/6 mice (aged 8-12 weeks) were treated intraperitoneally (i.p.) with 500 U/kg 5’-NT with or without 40 mg/kg 8-(p-sulfophenyl)theophylline hydrate (8-SPT, Sigma, St. Louis, MO), a non-specific adenosine receptor (AR) antagonist 1 or an equivalent volume of saline. After 30 min, mice were anesthetized with sodium pentobarbital (70 mg/kg) and blood was drawn via cardiac puncture into a 0.1 volume of sodium citrate (3.8%) or tail bleeding time was assessed as described below. In additional experiments, A2a\(^{+/+}\) or A2a\(^{-/-}\) mouse blood was used to test aggregation ex vivo.

Whole Blood Aggregation

Whole blood aggregation studies were completed on the 560VS aggregometer (Chronolog, Havertown, PA) within 3 h of collection. The protocol for blood donations from healthy volunteers was approved by the institutional review board and written informed consent was obtained from each individual donor prior to blood donation. Blood from healthy human volunteers or mice was drawn into a
0.1 volume of sodium citrate (3.8%), subsequently diluted 1:2 with saline and
preincubated at 37°C for 5 min in an aggregometer cuvette (Chronolog,
Havertown, PA). Platelet activation was started by the addition of one of the
following platelet agonists: 5 µg/mL collagen, 10 µM ADP, 50 µM epinephrine,
0.5 µM arachidonic acid, or 0.1 U/mL thrombin (Chronolog, Havertown, PA) and
percent aggregation was measured for 6 min. In some experiments, whole blood
was treated with 5 U/mL 5'-NT purified from Crotalus atrox venom (Sigma) with
and without 500 µM 5'-[αβ-methylene] diphosphate (APCP, Sigma), a specific
inhibitor of ecto-5'-NT 3; 200 µM 8-SPT (Sigma), a non-specific AR antagonist 1;
100 µM 5'-N-ethylcarboxamidoadenosine (NECA, Sigma), a non-specific AR
agonist 4; 200 µM PSB1115 (Tocris, Ellisville, MO), a specific A2bAR antagonist
5; or saline prior to the addition of a platelet agonist.

Tail Bleeding and Time to Occlusion

Bleeding times were assessed using an adaptation of the method we previously
described 6. Briefly, mice were anesthetized with sodium pentobarbital (70 mg/kg
body weight, i.p.) and placed on a temperature-controlled heating table to
maintain body temperature at 37°C. The mice were then secured with their tails
facing downward and perpendicular to their bodies. After being pulled through a
1.5-mm-diameter template, the tails were transected with a scalpel blade and
bled onto a Whatman filter paper. The filter paper was dabbed to the wound
every 30 seconds without disrupting the forming clot. Any blood dripping during
the 30-sec intervals was allowed to drop freely onto the filter. The experiment was continued until bleeding stopped completely. If bleeding continued after 20 min, bleeding was stopped by cauterization to prevent hypovolemic shock.

**Gel Permeation Chromatography**

5'-NT (30 U x 2) fractions were separated by gel permeation chromatography on a Nucleogel GFC 1000-8 column (Machery-Nagel, Düren, Germany). The mobile phase consisted of 1 L of water containing 19 mM potassium dihydrogen phosphate and 45 mM sodium chloride (pH 7.0). With a flow rate of 0.6 mL/min and a wavelength of 215 nm, the fractions for each peak were collected, evaporated to dryness by a lyophilizator, and reconstituted in 300 μl of water (pH 7.4). The units for each peak were not determined. Each fraction (50 μl) was added to whole human blood (diluted 1:2 with saline) prior to the addition of ADP and percent aggregation was measured.

**Adenosine Measurements**

2 mL of heparinized blood in the presence or absence of 0.2 mM dipyridamole (GensiaSicor, Irvine, CA, a non-specific inhibitor of equilibrative nucleoside transporters) was treated with 5 U/mL 5'-NT or saline and immediately added to 6 mL of 0.6 N perchloric acid at 0°C. The tubes were vortexed for 1 minute, placed on ice, and centrifuged within 10 min at 3,350 g for 10 min at 4°C. Adenosine concentrations were measured in perchloric acid extracts from whole blood as described previously. Briefly, all samples were supplemented with a
known amount of N-6-methyladenosine as the internal standard. The supernatant was adjusted to a pH of 5.5-6.5 by adding 2 M potassium carbonate. The precipitated potassium perchlorate was discarded after centrifugation at 20,000 g and the supernatant was applied onto a solid-phase extraction column (BondElut, ICT, Bad Homburg, Germany). Elution of the compounds was performed with 0.1 M HCl, and the eluate was analyzed by HPLC with UV detection using a Nucleosil 100 C18 (3 µm, 125x4 mm i.d.) column. Eluent consisted of solvent A (10 mM ammonium dihydrogenphosphate and 0.6 M heptanesulfonic acid sodium salt in 3% methanol) as the ion-pair forming agent and solvent B (solvent A containing 10% acetonitrile). Remote control, data acquisition, and quantification of peak areas were performed with Peak Simple Software 3.12 by SRI.

**Statistical analysis.**

All values are presented as the mean ± standard deviation (SD) of n independent experiments. All data were subjected to Student’s unpaired t test or one-way ANOVA, followed by the Student-Newman-Keuls post-hoc test using SigmaStat software (SPSS). Differences were considered significant at p < 0.05.

**References**


