Comprehensive characterization of platelet function in dogs with hyperadrenocorticism

Sanggu Kim,[1*] Dohee Lee,[2*] Preeti Kumari Chaudhary,[1] Hakhyun Kim,[2] Byeong-Teck Kang[2] and Soochong Kim[1]

[1]Laboratory of Veterinary Pathology and Platelet Signaling, College of Veterinary Medicine, Chungbuk National University and [2]Laboratory of Veterinary Internal Medicine, College of Veterinary Medicine, Chungbuk National University, Cheongju, Republic of Korea

**SK and DL contributed equally as first authors.*

Correspondence: S. Kim
skim0026@cbu.ac.kr

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Supplemental Methods

Materials

The following reagents were purchased from Sigma Aldrich (St. Louis, MO, USA): 2-MeSADP, thrombin, apyrase, prostaglandin E1 (PGE1), sodium citrate, and prednisolone. Anti-phospho-ERK (Thr202/Tyr204), anti-total-ERK, anti-phospho-AKT (Ser473), and anti-total-AKT antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). HRP-linked secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Thromboxane B_2 (TxB_2) ELISA kit was purchased from Enzo Life Sciences (Exeter, UK). Synthetic tetracosactrin was purchased from Novartis (Basel, Switzerland) and dexamethasone was purchased from Jeil Pharmacology (Daegu, Republic of Korea). All other consumable reagents were of analytic grade.

Platelet aggregation and secretion

Washed platelets were placed in a Lumi-aggregometer (Chrono-Log, Havertown, PA, USA) at 37 °C and stirred at 900 rpm to measure the light transmission. Before stimulating with agonists, washed platelets were pre-incubated for 5 min with 1000 nM prednisolone. The prednisolone concentration was established based on the 2 mg/kg (830 nM) dose, which has been demonstrated to induce iatrogenic Cushing's syndrome in several previous studies. Platelet ATP release was measured using luciferin luciferase reagent to evaluate platelet dense granule secretion.

TxA_2 generation measurement

Washed platelets were adjusted at a concentration of 2×10^8 platelets/ml. Platelets were stimulated with an agonist for 3.5 min in a Lumi-aggregometer and the response was terminated by snap freezing. Samples were stored at -80 °C till TxB_2 levels were measured. TxB_2 levels were assessed using an ELISA kit in accordance with the manufacturer's manuals.

Western blot analysis

2-MeSADP and thrombin were used to stimulate washed platelets for 2 min and the reaction was stopped with 6.6 N perchloric acid. Samples were centrifuged (10,000 rpm, 3 min) and washed with distilled water. Washed samples were centrifuged (11,000 rpm, 3 min), re-suspended with Laemmli sample buffer, and heated for 10 min. Protein samples were loaded on a 10% SDS PAGE and transferred onto PVDF membranes. Membranes were blocked by incubation with SuperBlock® blocking buffer (Thermo Fisher Scientific, Waltham, MA, USA). The membrane was incubated with anti-phospho-ERK (Thr202/Tyr204), anti-total-ERK, anti-phospho-AKT (Ser473), or anti-AKT antibodies overnight with gentle agitation. The membranes were incubated in appropriate secondary antibodies and chemiluminescence substrate (Pierce, Rockford, IL, USA) for detecting immune reactivity.