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Comprehensive characterization of platelet function in dogs with hyperadrenocorticism

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Contributions

SK (Sanggu Kim), DL, and SK (Soochong Kim) conceived the study and wrote the manuscript. SK (Sanggu Kim) and DL performed the experiments and analyzed the data. PKC, HK, and BK contributed to the experimental design and writing of the manuscript. All authors critically revised the manuscript.

Disclosures

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Data-sharing statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Abstract

Hyperadrenocorticism (HAC) leads to a hypercoagulable state and contributes to the risk of thromboembolic disease. Hypercoagulation in HAC occurs in both humans and dogs. Platelets play a major role in thrombosis and hemostasis, but no study has investigated platelet function in dogs with HAC. Thus, we aimed to characterize the platelet function and its molecular mechanism in dogs with HAC by using platelets isolated from normal dogs and dogs with HAC. This prospective cross-sectional study included 7 dogs with HAC and 15 normal dogs. Various platelet functional responses including platelet aggregation and dense-granule secretion were evaluated. 2-MeSADP- and low concentration of thrombin-induced platelet aggregation and secretion were significantly inhibited in dogs with HAC compared to normal dogs. Furthermore, the pre-incubation of platelets with prednisolone inhibited 2-MeSADP- and thrombin-induced platelet aggregation and secretion only in normal dog platelets, whereas no additional inhibitory effect was shown in dogs with HAC confirming a role of excessive cortisol in platelet function. In addition, 2-MeSADP- and thrombin-induced platelet aggregation and post-adrenocorticotropic hormone (ACTH) cortisol levels showed a negative correlation. Moreover, 2-MeSADP- and thrombin-induced thromboxane A_2 (TxA_2) generation was significantly inhibited in dogs with HAC compared to normal dogs, confirming the role of cortisol in TxA_2 generation. Finally, thrombin-induced ERK and AKT phosphorylation were significantly inhibited in dogs with HAC. In conclusion, excessive cortisol in dogs with HAC affects platelet function by suppressing TxA_2 generation through the regulation of ERK and AKT phosphorylation.

Introduction

Hyperadrenocorticism (HAC), also known as Cushing's syndrome, is a common endocrine disorder, caused by pathologically excessive production of cortisol. Clinical manifestations of HAC include polydipsia, polyuria, panting, alopecia, abdominal distention, hepatomegaly, and thromboembolism has been occasionally presented in both humans and dogs with HAC (1). Although there is insufficient evidence to support a direct link between HAC and the development of thrombosis (2), it is classically accepted that HAC leads to a hypercoagulable state and contributes to the development of spontaneous thromboembolism (3). Understanding mechanisms involved in the effects of HAC on coagulability is important because thrombosis and thromboembolism are potentially life-threatening complications with marked mortality (4). To prevent the risk of thromboembolic disorders, administration of antiplatelet agents and/or anticoagulants is considered in patients with HAC that have other risk factors for thrombosis, such as pancreatitis, sepsis, and cancer (2).

Various mechanisms explain a hypercoagulable state in HAC, including elevated procoagulant factors, decreased antithrombin, and inhibited fibrinolysis (5, 6). In addition, thrombocytosis, one of the common clinicopathologic findings consistent with HAC (7), could largely be attributed to hypercoagulability. It is well known that platelets play an important role in thrombosis and hemostasis and induce various thrombotic events triggered by several metabolic diseases upon activation, and thus regulate coagulative activity in the circulation. The majority of prior research that assessed the impact of glucocorticoids on platelet function used whole blood or PRP which contained procoagulant components including fibrinogen, vWF, and factor VIII that undoubtedly impacted the results of the investigations. There hasn't been any investigation on the effect of endogenous cortisol on washed platelet function in HAC-affected patients.

Contradictory to the prevalent knowledge regarding the excessive cortisol causing hypercoagulability in HAC-affected patients, in our recent *in vitro* investigation, we discovered that prednisolone, a glucocorticoid, when pre-incubated with washed platelets, inhibits the $cPLA_2$-mediated TxA_2 production, suggesting that glucocorticoids have an inhibitory effect on platelet function (8). A recent study has also demonstrated that glucocorticoids inhibit platelet aggregation in equine platelet-rich plasma (9). Considering these findings, an assumption is proposed that endogenous glucocorticoids would lead to the inhibition of platelet function, even though the hypercoagulable state has been previously widely known in patients with HAC. Hypercoagulation in HAC occurs in both humans and dogs, and it is 1,000 times more common in dogs than in humans, making it easier to recruit a patient group (10-12). Thus, we aimed to investigate the effect of excessive endogenous cortisol on platelet function and the molecular mechanism involved by using washed platelets and activating with various agonists in dogs affected with HAC.

In this study, we have found that 2-MeSADP- and thrombin-induced platelet aggregation and dense granule secretion are inhibited in dogs with HAC compared to normal dogs. The prednisolone pre-incubated platelets inhibited 2-MeSADP- and thrombin-induced platelet aggregation and secretion only in normal dog platelets, whereas no additional inhibitory effect was shown in dogs with HAC indicating an inhibitory role of excessive endogenous cortisol on platelet function. We demonstrated that 2-MeSADP- and thrombin-induced platelet aggregation had a negative correlation with post-adrenocorticotropic hormone (ACTH) cortisol. We have further shown that 2-MeSADP- and thrombin-induced platelet TxA_2 generation is inhibited in dogs with HAC compared to normal dogs, suggesting that endogenous cortisol exerts its inhibitory effect on platelets by regulating TxA_2 generation. Finally, we have shown that 2-MeSADP- and thrombin-induced ERK and AKT phosphorylation is inhibited in HAC-affected dogs. We conclude that excessive cortisol in dogs with HAC has an inhibitory role in platelet function by inhibiting TxA_2 generation through the regulation of ERK and AKT phosphorylation.

Methods

Animals

This prospective study included 7 dogs that were newly diagnosed with HAC and 15 normal dogs. All dogs were presented and examined at Chungbuk National University Veterinary Teaching Hospital between July 2021 and May 2023. All samples were collected with informed client consent, and this study was approved by the Institutional Animal Care and Use Committee of Chungbuk National University (CBNUA-1569-21-02).

HAC was initially suspected when a dog had a minimum of 1 compatible clinical sign or examination finding as described in the ACVIM Consensus Statement regarding the diagnosis of HAC (1). Diagnosis of HAC was based on the results of the ACTH stimulation test (ACTH ST) or low-dose dexamethasone suppression test (LDDST). Diagnosis of HAC was confirmed when the cortisol concentration was >26 μg/dl 1 hour after IV administration of 250 μg/dog synthetic tetracosactrin or when the cortisol concentration was >1.4 μg/dl 8 hours after IV administration of 0.01 mg/kg IV dexamethasone. Differentiation between pituitary-dependent HAC and adrenal-dependent HAC was based on ultrasonographic findings and/or plasma concentrations of endogenous ACTH. Dogs with systemic infection, cancer, and thrombocytopenia, and dogs that administered glucocorticoids or non-steroidal anti-inflammatory drugs within 14 days of presentation were excluded. Normal dogs were included based on the unremarkable findings on history, physical examinations, CBC, serum biochemistry, and urinalysis.

Sample collection

Whole blood samples were obtained from the jugular vein. To prevent any potential impacts that synthetic tetracosactrin or dexamethasone might have on platelet functions, blood was collected before an ACTH ST or LDDST from dogs with a significant clinical suspicion of having HAC.

Platelet count measurement

The platelet counts were analyzed using a whole blood sample of 250 μl collected into an EDTA tube. Analysis was performed using a blood cell count machine (IDEXX ProCyte Dx, IDEXX Laboratories, Inc., Westbrook, ME, USA) showing <3% coefficient of variation for the CBC. Platelet counts greater than $484 \times 10^3/\mu l$ were defined as thrombocytosis.

Washed platelet preparation

Whole blood from dogs in 3.8% sodium citrate was centrifuged at 350 x *g* for 10 min at room temperature, the supernatant was collected, and 1 μM prostaglandin E_1 was added. Platelet pellets were produced by centrifuging the supernatant at 1000 x g for 10 min. The platelet number was adjusted to 1×10^8 cells/ml in Tyrode's buffer containing 0.05 units/ml of apyrase.

Platelet aggregation and secretion were measured by Lumi-aggregometer, TxA_2 generation was measured by ELISA, and phosphorylation of signaling molecules was detected by Western blot analysis. Please refer to the Online Supplementary Methods.

Statistical analysis

The data were expressed as the median and interquartile range (IQR). Fisher's exact test was used to compare the proportion of females and thrombocytosis between the two groups. The Mann-Whitney U-test and unpaired t-test were used to compare the age, body weight, platelet counts, the extent of

platelet aggregation and dense granule secretion, TxA_2 generation, and protein phosphorylation between normal dogs and dogs with HAC.

Results

Demographic characteristics of the dogs

Seven dogs with HAC and 15 normal dogs were included in this study. The demographic characteristics of the dogs are presented in Table 1. No significant differences were observed in age ($P = 0.07$), body weight ($P = 0.62$), and sex ($P = 0.65$) between normal dogs and dogs with HAC.

Of the 7 dogs with HAC, 2 dogs each were confirmed as HAC by ACTH ST or LDDST. The other 3 dogs were not confirmed by ACTH ST and were confirmed by additional LDDST. Of the 7 dogs with HAC, 5 had pituitary-dependent HAC and 2 had adrenal-dependent HAC. In 7 dogs with HAC, 6 dogs showed polyuria/polydipsia, 4 dogs showed hepatomegaly, 3 dogs showed panting, and 2 dogs each showed polyphagia, abdominal distention, and endocrine alopecia.

Comparison of platelet counts between normal dogs and dogs with HAC

Four of 15 normal dogs (4/15, 26.7%) and three of 7 dogs with HAC (3/7, 42.9%) showed thrombocytosis. The proportion of thrombocytosis was not significantly different between normal dogs and dogs with HAC ($P = 0.63$). No significant difference was observed in platelet counts between normal dogs (median, 350 [IQR, 225–526] × 10^3/μl) and dogs with HAC (479 [360–579] × 10^3/μl, $P = 0.22$, Figure 1).

2-MeSADP- and thrombin-induced platelet aggregation and secretion are inhibited in dogs with HAC

In order to check the functional difference between normal dogs and dogs with HAC in platelets, Platelets were activated with 2-MeSADP and thrombin, and platelet aggregation and dense granule secretion were measured. As illustrated in Figure 2A, platelet aggregation induced by 2-MeSADP was significantly decreased in dogs with HAC. Furthermore, low concentrations of thrombin-induced platelet aggregation and thrombin-induced dense granule secretion were significantly decreased in dogs with HAC in Figure 2B.

Prednisolone affects 2-MeSADP- and thrombin-induced platelet aggregation and secretion only in normal dogs

In order to determine the effect of cortisol difference between normal dogs and dogs with HAC in platelets, we pre-incubated prednisolone and compared agonist-induced platelet aggregation and dense granule secretion. As shown in Figure 3, prednisolone inhibited 2-MeSADP-induced platelet aggregation only in normal dogs and there was no difference in 2-MeSADP-induced platelet aggregation between normal dogs and dogs with HAC. In addition, prednisolone inhibited low concentrations of thrombin-induced platelet aggregation and thrombin-induced platelet dense granule secretion only in normal dogs and there was no difference in thrombin-induced platelet aggregation and secretion between normal dogs and dogs with HAC.

2-MeSADP- and collagen-induced platelet aggregation are inhibited in the presence of prednisolone in *in vitro* models of HAC

The preceding data indicated that prednisolone pre-incubation in normal dog platelets was highly comparable to HAC dog platelets. We used this *in vitro* modeling for further validation of the impact of glucocorticoids on platelets. As shown in Figure 4A, 2-MeSADP-induced platelet aggregation and dense granule secretion were inhibited in the presence of prednisolone. In order to reflect the condition of HAC patients, we measured 2-MeSADP-induced platelet aggregation and secretion following fibrinogen pre-incubation, which is known to be elevated in HAC patients. Fibrinogen slightly enhanced 2-MeSADP-induced platelet aggregation and dense granule secretion in the presence or absence of prednisolone, but fibrinogen did not have any additional effect on 2-MeSADP-induced platelet aggregation and secretion in the presence of prednisolone. (Figure 4C). To further check the effect of glucocorticoids on non-GPCR-mediated pathways, we activated platelets with collagen. As shown in Figure 4E, platelet aggregation and dense granule secretion were inhibited by prednisolone only upon stimulation with a low concentration of collagen.

2-MeSADP- and thrombin-induced TxA_2 generation are inhibited in dogs with HAC

To confirm the effect of cortisol on platelets in dogs with HAC, we stimulated the platelets with 2-MeSADP and thrombin and compared the amount of generated TxA_2. As shown in Figure 5, 2-MeSADP and thrombin-induced TxA_2 generation were significantly inhibited in dogs with HAC compared to normal dogs, confirming the inhibitory effect of cortisol on TxA_2 generation.

Thrombin-induced ERK and AKT phosphorylation are inhibited in dogs with HAC

To determine the molecular mechanism involved in the regulation of TxA_2 generation by cortisol, we evaluated thrombin-induced ERK and AKT phosphorylation in dogs with HAC. Both ERK and AKT phosphorylation induced by thrombin were significantly decreased in dogs with HAC compared to normal dogs (Figure 6). This result confirms that excessive cortisol negatively regulates ERK and AKT phosphorylation.

2-MeSADP- and thrombin-induced Platelet aggregation and post-ACTH cortisol have a negative correlation

To check whether platelet function is affected in dogs with HAC, the relationship between platelet aggregation and post-ACTH cortisol was examined. As shown in Figure 7, the extent of 100 nM 2-MeSADP- and 0.2 U/ml thrombin-induced platelet aggregation and post-ACTH cortisol levels showed a negative correlation.

Discussion

HAC is a potentially life-threatening disease that causes obesity, diabetes, and hypertension, with approximately 5% of human patients dying within 10 years and 50% of patients dying within 510 days in dogs (13, 14). All of these symptoms are linked to thromboembolic illness, and around 10% of patients with HAC died as a result of serious thromboembolism (15). Therefore, numerous researches have been done to determine whether coagulopathy caused by HAC is associated with coagulation factors (16, 17). However, there are few studies that explore the impact of endogenous cortisol on

coagulopathy in dogs with HAC, specifically focusing on platelets, which play a crucial role in thrombosis. Furthermore, the importance is greater in dogs, which have a 1000 times higher incidence of HAC than humans (11, 12). Thus, in this study, we evaluated the functions of platelets in dogs with naturally occurring HAC and the molecular mechanism involved and minimized the effects of external factors such as coagulation factors and blood cells by using the washed platelets with adjusted numbers.

Activated platelets cause hemostasis and eventually contribute to coagulation through interaction with coagulation factors (18). Platelet GPCRs are clinically important GPCRs that regulate platelet functional responses through various G-protein-mediated signaling. Therefore, to understand the effect of endogenous cortisol on platelet function in HAC-affected dogs, we stimulated the washed platelet with major GPCR agonists 2-MeSADP and thrombin that promote the development of coagulation. Interestingly, we observed that ADP- and low concentrations of thrombin-induced platelet aggregation were significantly inhibited in the dogs with HAC, contradicting the widely accepted notion that HAC could lead to hypercoagulation and contribute to the formation of thromboembolism. Furthermore, both low and high concentrations of thrombin-induced dense granule secretion were significantly decreased in dogs with HAC. Some earlier studies using a platelet function analyzer reported that ADP- and collagen-induced platelet aggregation were not affected in patients with endogenous hypercortisolism compared to the healthy controls (19). On the contrary, dogs with HAC have been demonstrated to have decreased platelet function (20). However, these studies were conducted using whole blood samples where there is the presence of many coagulative and procoagulative factors including various blood cells which can modulate platelets' response to various stimuli. Although the mechanisms involved are unclear, it has been reported that glucocorticoids regulate GPCR signaling in platelets (21). ADP causes platelet aggregation by activation of G_q-coupled $P2Y_1$ and G_i-coupled $P2Y_{12}$ receptor-mediated signaling pathways (22). Likewise, thrombin activates platelets through protease-activated receptors (PARs) by coupling to G_q and $G_{12/13}$ (23). Our data suggest that endogenous cortisol has a negative role in regulating ADP and thrombin receptor-mediated platelet function. Unlike ADP which requires granule secretion for complete platelet aggregation, thrombin is a more potent agonist and requires secretion only at lower concentrations of thrombin stimulation to cause platelet aggregation. This might be the reason why although both low and high concentrations of thrombin-induced dense granule secretion were inhibited, only low concentrations of thrombin-induced platelet aggregation in dogs with HAC.

Excessive glucocorticoids are metabolized in the body, causing various pathophysiological conditions, including anti-inflammatory and immunosuppressive states, and these factors may be sufficiently potent to influence platelet function by increasing glucocorticoid activity (24). To ensure that endogenous cortisol directly affects platelet functional response, we pre-incubated the platelets of both normal dogs and dogs with HAC with prednisolone, which has approximately four times the glucocorticoid activity of cortisol. This treatment mimics the condition of HAC, which is known as iatrogenic Cushing's syndrome in humans, and provides a model to investigate the direct effects of glucocorticoids on platelet function. We have recently demonstrated that prednisolone has an inhibitory effect on platelet functional responses *in vitro* using murine platelets (8). Consistent with our recent finding, prednisolone pre-incubation inhibited 2-MeSADP-induced aggregation, and thrombin-induced aggregation and secretion only in normal dogs' platelets whereas no additional inhibitory effect was shown in dogs with HAC platelets (Figure 2 vs Figure 3). In addition, there were no significant differences in these agonists-induced platelet aggregation and secretion between normal dogs and dogs with HAC in prednisolone pre-incubated platelets, confirming that glucocorticoid has a direct effect on platelet function. Additionally, we checked the effect of prednisolone on non-GPCR mediated signaling by stimulating the platelets with collagen. Consistently, prednisolone only affected low concentration of collagen-induced platelet aggregation and secretion which is dependent on TxA_2

generation, further indicating that prednisolone regulates TxA_2 generation-mediated platelet function (25).

Importantly, activated platelet generates TxA_2 that acts as a positive-feedback mediator by recruiting circulating platelets, activating them via thromboxane prostanoid receptor by coupling to G_q and $G_{12/13}$ to form a stable hemostatic plug (26, 27). It is well known that ADP causes the platelet TxA_2 generation that acts as a positive feedback mediator in activating platelet secretion and the resultant secondary wave of aggregation (26, 28). Likewise, thrombin also induces TxA_2 generation in platelets, which contributes to the potentiation of platelet aggregation and secretion induced by thrombin (29, 30). PARs also indirectly cause secreted ADP-induced G_i coupled-$P2Y_{12}$ receptor-mediated platelet aggregation (31). We checked whether cortisol regulates platelet function by regulating TxA_2 generation in HAC dogs by measuring the TxA_2 release. Our data showed that both ADP and thrombin-induced TxA_2 generation were significantly inhibited in dogs with HAC compared to normal dogs, confirming that cortisol regulates the positive feedback effect of TxA_2 generation in dogs with HAC. It is well established that ADP-induced secretion and the resultant secondary wave of aggregation are dependent on the positive feedback effect of generated TxA_2 (32). However, unlike ADP, it has been shown that platelet aggregation only requires a positive feedback effect of generated TxA_2 when stimulated with a low concentration of thrombin (29, 30). TxA_2 generation and subsequent granule secretion play a role only at the low concentration of thrombin-induced platelet activation This may be the reason why endogenous cortisol affected only low concentrations of thrombin-induced platelet aggregation while inhibiting both low and high concentrations of thrombin-induced TxA_2 generation (Figure 5B) as well as secretion (Figure 2B) in dogs with HAC. Taken together, our data confirms that endogenous cortisol regulates GPCR-mediated platelet function by inhibiting TxA_2 generation in HAC-affected dogs.

In order to find the molecular mechanism involved in the regulation of platelet function by endogenous cortisol, we checked ERK and AKT phosphorylation. ERK phosphorylation is one of the most important upstream signaling molecules that regulate TxA_2 generation downstream of $P2Y_{1,}$ $P2Y_{12,}$ and PARs in platelets (33). It has been demonstrated that thrombin-induced TxA_2 generation is mediated by the $P2Y_{12}$ receptor through the regulation of PI3K-AKT-ERK phosphorylation (34). Additionally, glucocorticoids also have been shown to activate ERK1/2 in the PC12 cells (a pheochromocytoma cell) and vascular smooth muscle cells (35, 36). Consistently, we found that thrombin-induced ERK and AKT phosphorylation were significantly inhibited in dogs with HAC, demonstrating that excessive cortisol negatively regulates ERK and AKT phosphorylation. However, thrombin-induced ERK and AKT phosphorylation were partially inhibited in dogs with HAC in the present study. Furthermore, we recently demonstrated that prednisolone significantly inhibits ADP-induced cPLA2 phosphorylation, resulting in TxA_2 suppression and inhibited ERK phosphorylation in platelets (8). When platelets are activated by various agonists, TxA_2 generation and secreted ADP further enhance ERK and AKT phosphorylation through positive feedback loops. Therefore, inhibition of TxA_2 generation and subsequent ADP secretion by excess cortisol in HAC leads to inhibition of ERK and AKT phosphorylation, leaving only ERK and AKT phosphorylation by inside-out signaling.

Platelets play a direct role in primary hemostasis and contribute to coagulation by interacting with coagulation factors (18). However, hypercoagulation appears in patients with HAC. This seems to be related to an increase in the coagulation factor and fibrinogen, overwhelming the contribution of platelets, although platelet function decreases in patients with HAC. However, bleeding complications are most common when platelet function is low. For example, bleeding complications are commonly present in disorders with impaired platelet function, such as Glanzmann thrombasthenia, gray platelet syndrome, and Chediak-Higashi syndrome (37-39). Consistently, prolonged bleeding times have been observed in human Cushing's syndrome patients, which were reduced after adrenalectomy (40). This

phenomenon may be explained by the compensatory increase in platelet count observed in HAC patients (Figure 1), which attempts to offset decreased platelet function. However, the contribution of elevated fibrinogen levels to platelet function appears insufficient (Figure 4C), potentially resulting in bleeding complications despite the hypercoagulable state.

For effective treatment and alleviation of the symptoms in disorders with thromboembolism, such as HAC, platelet functions must be precisely defined. However, platelet aggregation by light transmission aggregometry, which represents platelet functions, requires blood collection, which may be hazardous to HAC patients with thromboembolic symptoms, and it is challenging to implement in the hospital. Therefore, an easily obtained criterion is required to characterize platelet function in HAC patients. So, we investigated the relationship between platelet count, basal cortisol, post-ACTH cortisol, and the extent of platelet aggregation. Our data demonstrated that platelet count, basal-cortisol, and post-ACTH cortisol showed negative relationship with the extent of platelet aggregation. However, basal-cortisol can appear variable even in the normal physiological state such as circadian patterns, physical activity, and stress. Also, additional factors that may influence platelet counts include environmental exposure, nutritional intake, and genetic variations between breeds. In contrast, the post-ACTH cortisol level which is an important criterion for HAC diagnosis, has minimal variability other than basal-cortisol and platelet count. Consequently, this would be an ideal way to overcome the realistic limitation of measuring platelet function in HAC patients and could provide an accurate direction for treatment.

The present study showed that platelet functions, including agonists-induced platelet aggregation and thrombin-induced dense granule secretion, were decreased in dogs with HAC. These findings are interesting in that they contradict the widely accepted notion that HAC could lead to hypercoagulation and contribute to the formation of thromboembolism (3, 41). Previous studies using thromboelastography showed a hypercoagulable state in dogs with HAC and the causes of it are explained by the increase of coagulation factors and thrombocytosis (5, 20). Furthermore, other factors than hypercoagulation could contribute to the formation of thromboembolism in dogs with HAC. Systemic hypertension usually occurs secondary to HAC (42) and it can lead to endothelial dysfunction and an increase of plasma levels of tissue-type plasminogen activator, increasing the risk of thromboembolism (43, 44). In addition, adrenal gland tumors, which account for about 15% of the causes of HAC in dogs, can further contribute to hypercoagulability because carcinoma can increase levels of fibrinogen (45). In other words, dogs with HAC have multifactorial coagulation disorders that are combined with the decrease of platelet functions and hypercoagulable states; therefore, delicate therapeutic strategies would be needed to prevent the formation of thrombosis in dogs with HAC.

If there are no other risk factors for thrombosis in dogs with HAC, administration of antithrombotic drugs is not recommended because HAC alone is not associated with a high risk of thromboembolism (2, 46). However, if there are other concomitant risk factors for thrombosis, the administration of antithrombotic drugs should be considered to reduce the risk of thrombosis (2, 46). The antithrombotic drug consists of antiplatelet agents and anticoagulant agents. Considering that dogs with HAC have reduced platelet function, the use of antiplatelet agents may seem theoretically invalid. However, antiplatelet agents would be considered the mainstay of prevention of thrombosis in dogs with HAC because platelets play a major role in clot formation (47) and dogs with HAC usually had increased platelet number. Because antiplatelet agents are associated with an increased risk of bleeding (48), the negative effects of cortisol on platelet functions should be considered and caution should be taken when administrating antiplatelet agents in dogs with HAC. In addition, our results, which showed a decreased platelet function in dogs with HAC contrary to conventional belief, emphasize the importance of other systemic effects such as systemic hypertension and adrenal tumors in the formation of thrombosis. Therefore, management of other systemic factors that contribute to

thrombosis formation by controlling systemic hypertension or surgical resection of adrenal tumors would be significantly beneficial to prevent thromboembolic disorders in dogs with HAC. Furthermore, HAC shows similar symptoms in both humans and dogs (10). To overcome the limitation that HACs in humans are relatively rare and difficult to investigate using large populations, experiments utilizing dogs are predicted to aid humanity in terms of translational medicine.

In conclusion, we demonstrate that the dogs with HAC have impaired platelet function due to excessive cortisol by inhibition of TxA_2 generation, thereby regulating ERK and AKT phosphorylation.

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Table 1. Demographic features for dogs included in this study

Variable	Normal dogs (N = 15)	Dogs with HAC (N = 7)	*P* value
Age (years)	8.8 (5.8–11.1)	10.2 (8.8–16.0)	0.07
Body weight (kg)	6.9 (5.3–10.9)	7.1 (5.3–7.5)	0.62
Sex (number)			
Females/males	6/9	4/3	0.65
Basal-cortisol (μg/dl)	3.8 (1.6–4.9)	5.3 (3.3–12.7)	0.14
Post-cortisol (μg/dl)	7.1 (5.9–11.7)	27.9 (24.9–39.8)	0.0002

Data are expressed as median (IQR) or number. N: number; HAC: hyperadrenocorticism.

Figure Legends

Figure 1. Scatterplot of blood platelet counts in normal dogs and dogs with hyperadrenocorticism. Data are shown as medians and IQRs. The Mann-Whitney *U* test.

Figure 2. Comparison of platelet aggregation and dense granule secretion between normal dogs (n = 15) and dogs with hyperadrenocorticism (n = 7). Washed canine platelets were stimulated with (A) 30, 50, 100, and 200 nM 2-MeSADP and (B) 0.2, 0.3, 0.5, and 1 U/ml thrombin for 3.5 min and platelet aggregation and dense granule secretion were measured by Lumi-aggregometer under stirring conditions. (C) Aggregation and dense granule secretion were quantified in normal dogs and dogs with hyperadrenocorticism from panels A and B. Data are illustrated as mean ± SE. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

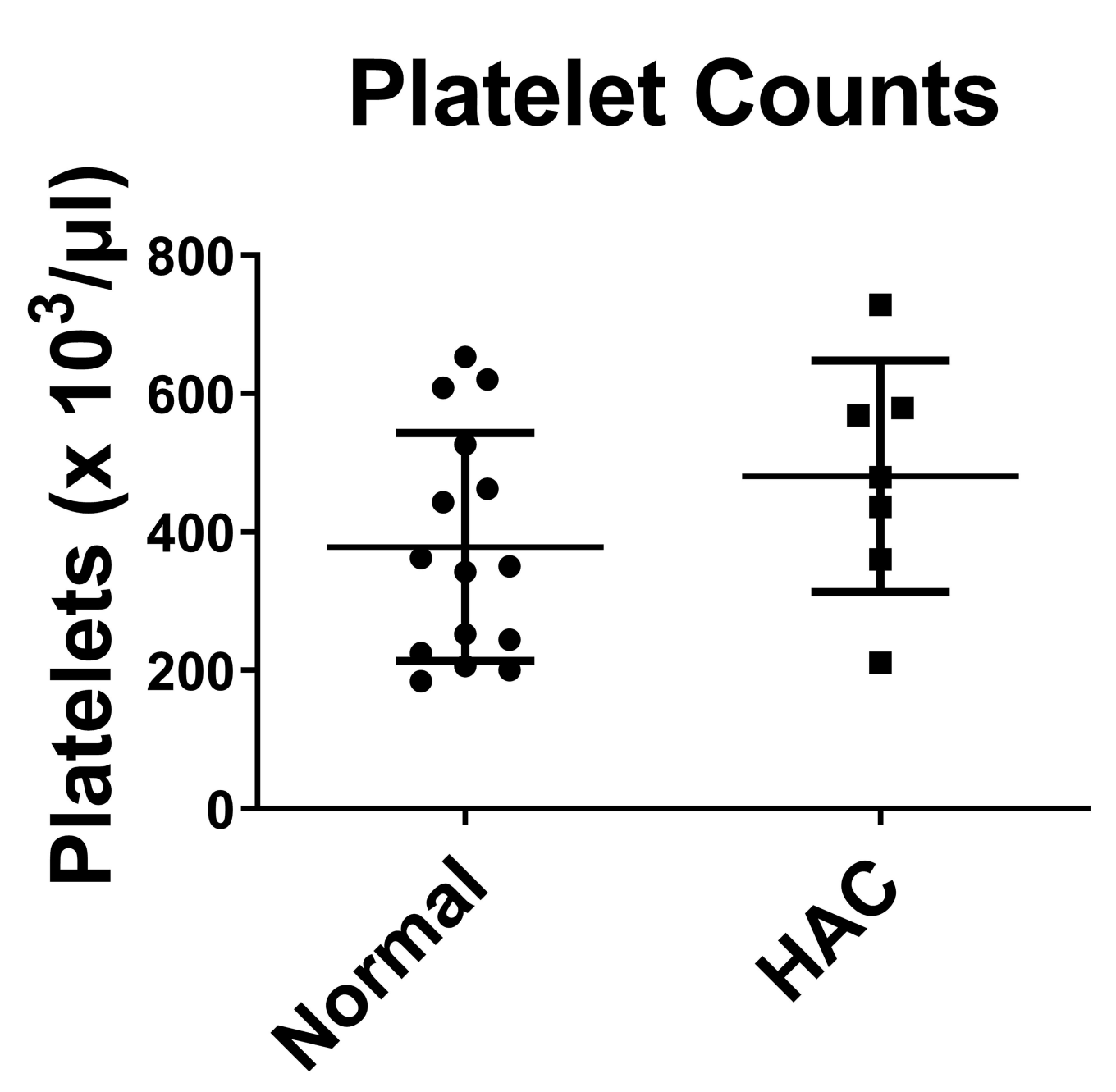
Figure 3. Comparison of prednisolone pre-incubated platelet aggregation and dense granule secretion between normal dogs (n = 15) and dogs with hyperadrenocorticism (n = 7). Washed canine platelets were preincubated with 1000 nM prednisolone and stimulated with (A) 30, 50, 100, and 200 nM 2-MeSADP and (B) 0.2, 0.3, 0.5, and 1 U/ml thrombin for 3.5 min and platelet aggregation and dense granule secretion were measured by Lumi-aggregometer under stirring conditions. (C) Aggregation and dense granule secretion were quantified in normal dogs and dogs with hyperadrenocorticism from panels A and B. Data are illustrated as mean ± SE.

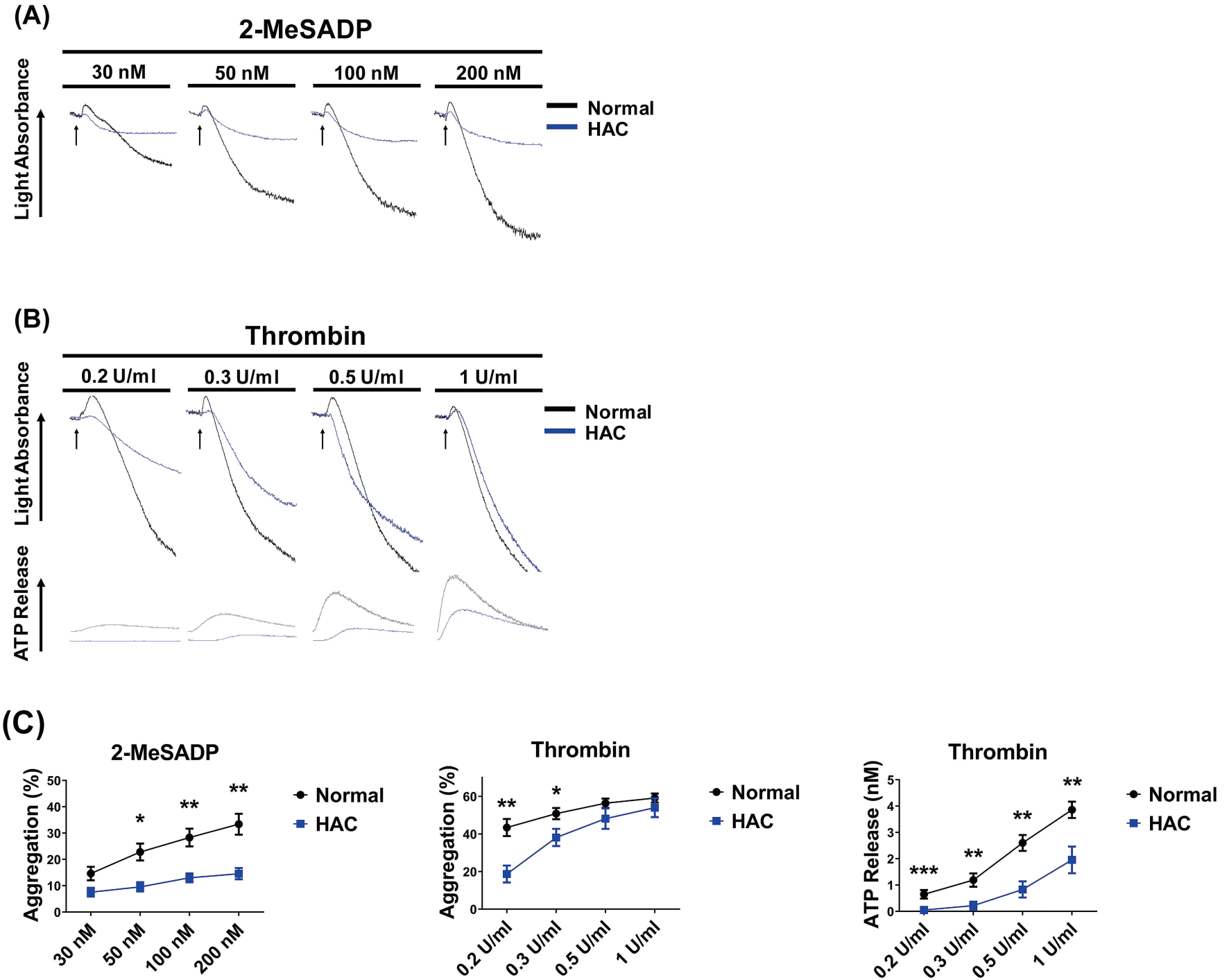
Figure 4. Comparison of platelet aggregation and dense granule secretion in normal dogs (n = 5) with and without prednisolone pre-incubation. Washed canine platelets were stimulated with (A) 30, 50, and 100 nM 2-MeSADP, (C) 30 and 100 nM 2-MeSADP with 100 μg/ml fibrinogen, and (E) 2 and 5 μg/ml collagen for 3.5 min and platelet aggregation and dense granule secretion were measured by Lumi-aggregometer under stirring conditions. (B, D, F) Aggregation and dense granule secretion were quantified from panels A, C, and E. Data are illustrated as mean ± SE. **, $p < 0.01$.

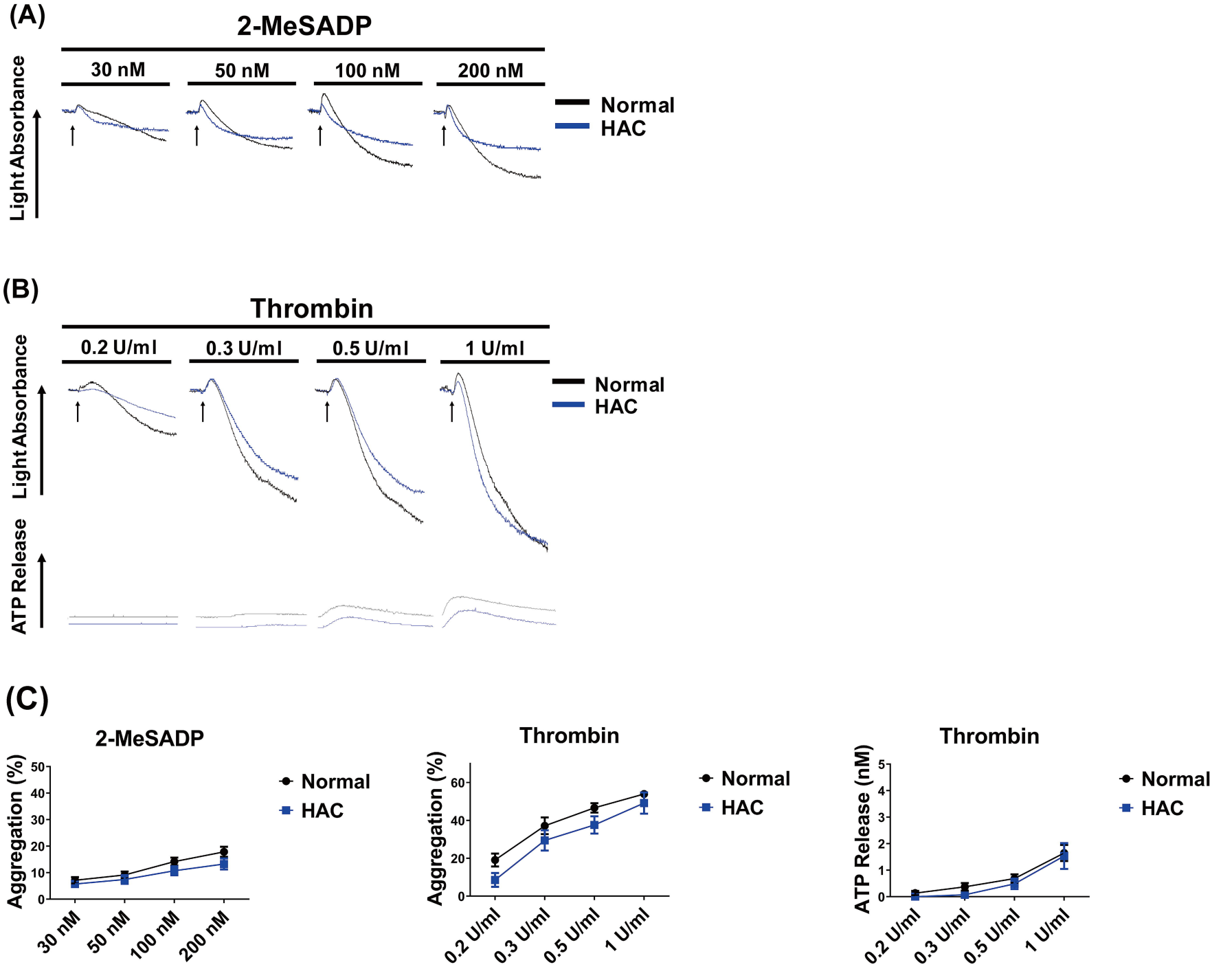
Figure 5. Comparison of 2-MeSADP- and thrombin-induced TxA_2 generation between normal dogs and dogs with hyperadrenocorticism. Washed canine platelets were stimulated with (A) 30, 50, and 100 nM 2-MeSADP and (B) 0.2 and 1 U/ml thrombin for 3.5 min under stirring conditions and thromboxane B_2 (TxB_2) generation was measured. Data are illustrated as mean ± SE. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

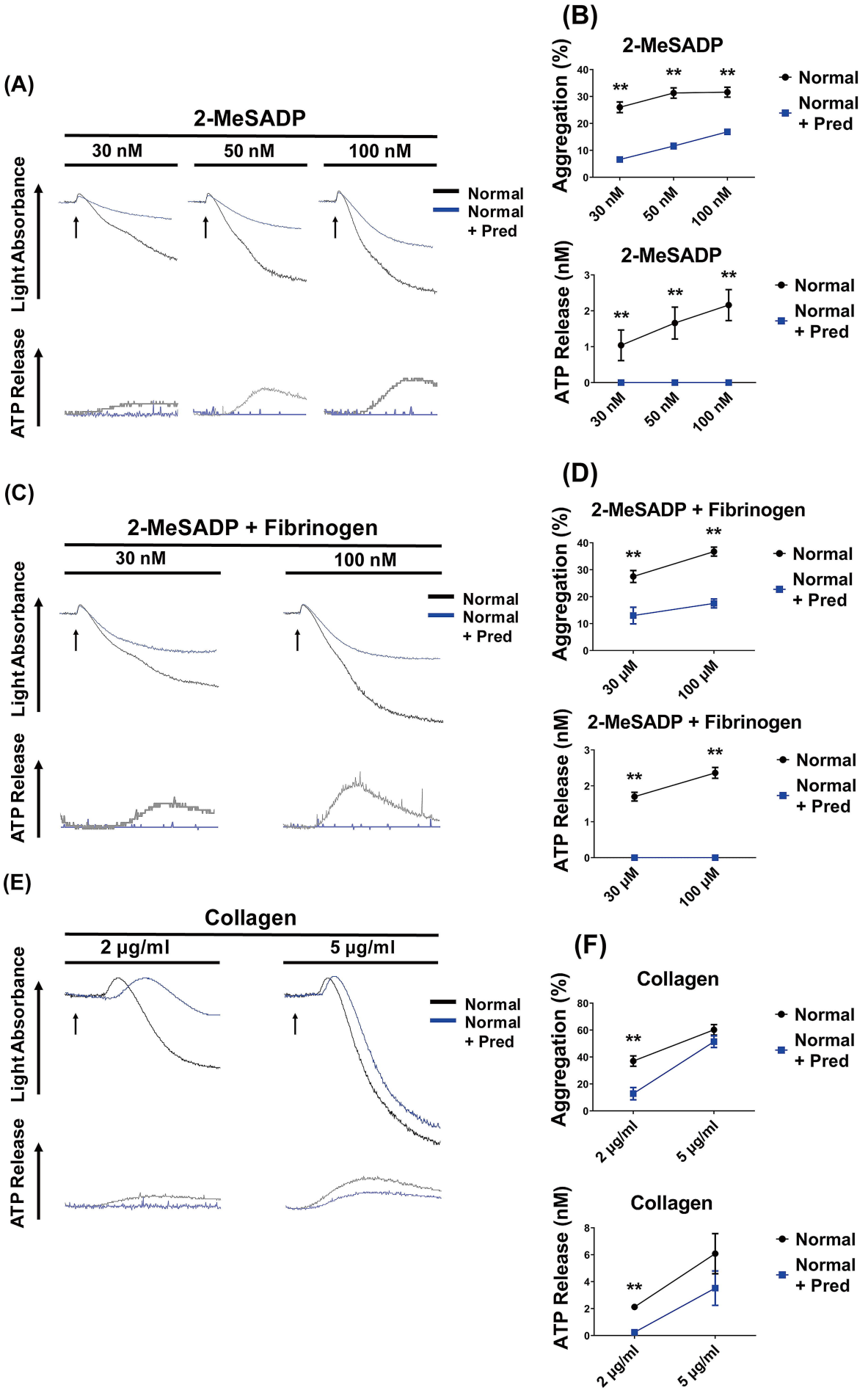
Figure 6. Comparison of thrombin-induced ERK and AKT phosphorylation between normal dogs and dogs with hyperadrenocorticism. Washed canine platelets were stimulated with 0.2 U/ml thrombin for 2 min under stirring conditions. The membrane was probed with anti-phospho-ERK, anti-ERK, anti-phospho-AKT, or anti-AKT antibodies. Data are representative of three independent experiments. (B) Quantification of ERK phosphorylation and (C) AKT phosphorylation are shown as mean ± SE. ***, $p < 0.001$; ****, $p < 0.0001$.

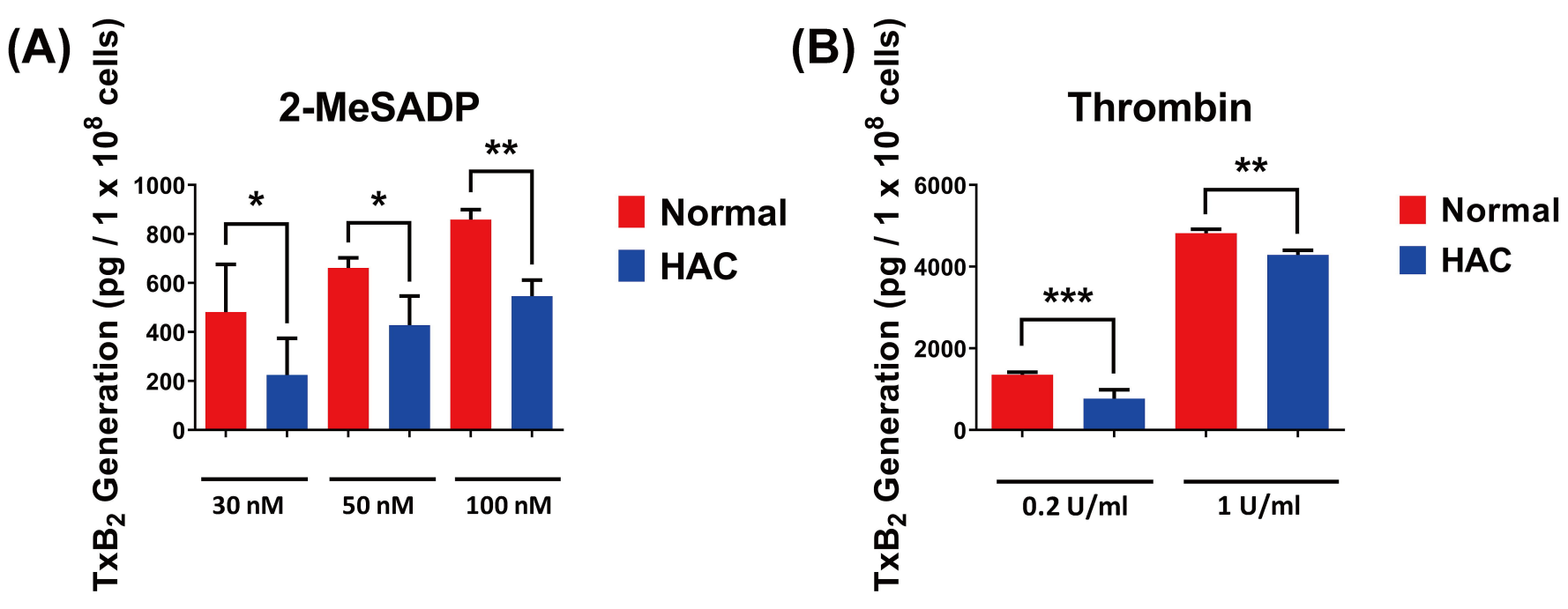
Figure 7. Relationship between platelet aggregation and post-ACTH cortisol in normal dogs and dogs with hyperadrenocorticism. Correlations between platelet aggregation induced by (A) 100 nM 2-MeSADP and (B) 0.2 U/ml thrombin and post-ACTH cortisol were depicted as plots.

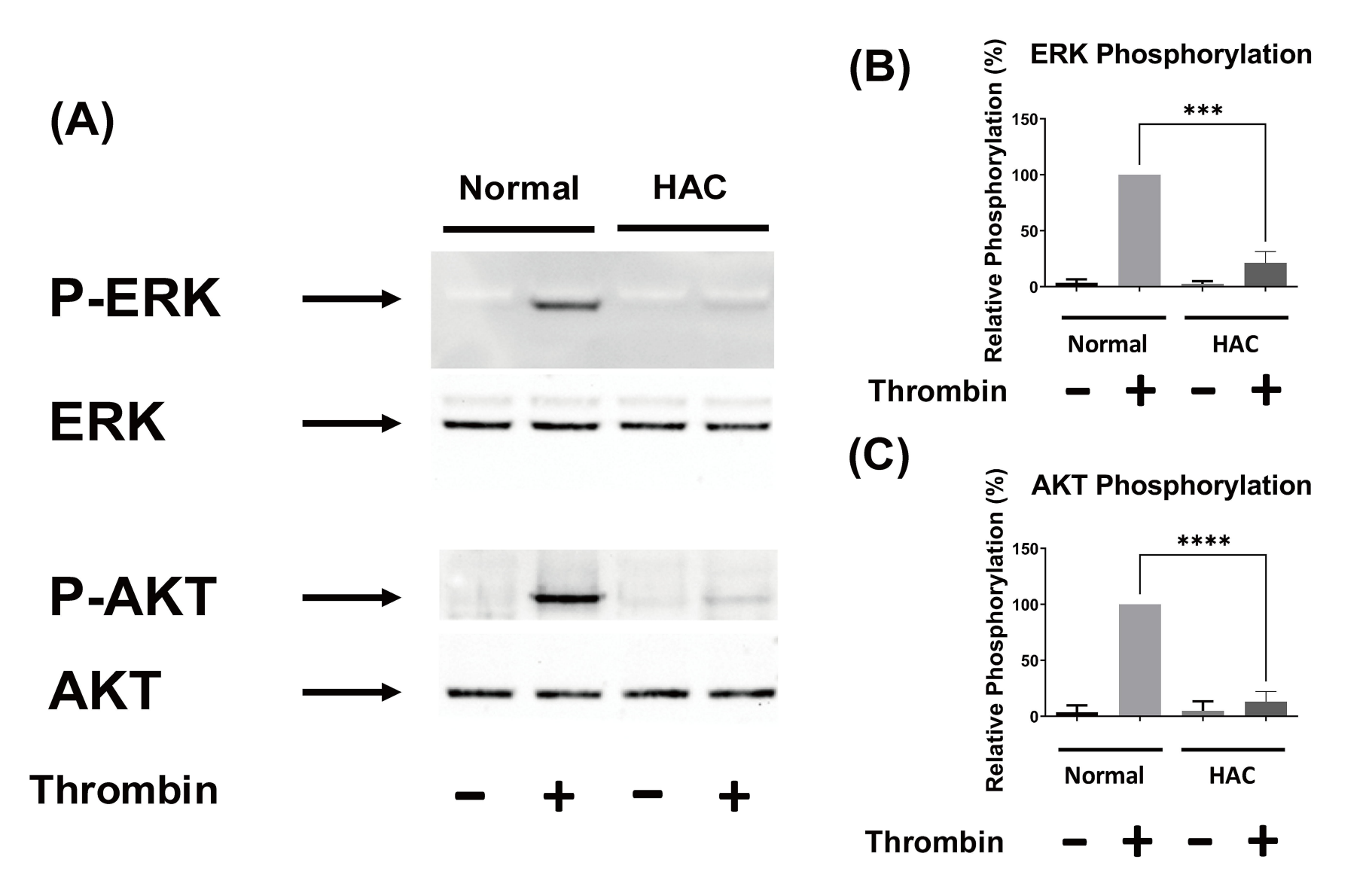

Platelet Counts
Platelets (x 10^3/µl)
800
600
400
200
0
Normal
HAC

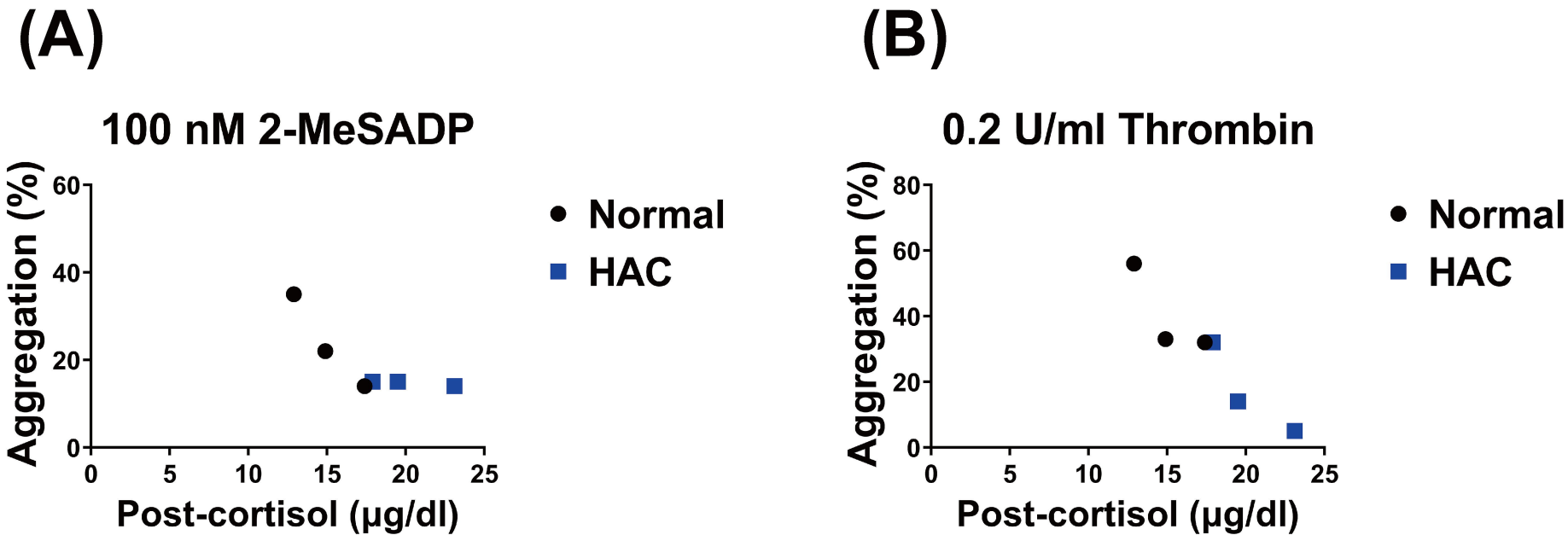

(A)
2-MeSADP
30 nM
50 nM
100 nM
200 nM
Light Absorbance
Normal
HAC
(B)
Thrombin
0.2 U/ml
0.3 U/ml
0.5 U/ml
1 U/ml
Light Absorbance
ATP Release
Normal
HAC
(C)
2-MeSADP
Aggregation (%)
Normal
HAC
30 nM
50 nM
100 nM
200 nM
Thrombin
Aggregation (%)
Normal
HAC
0.2 U/ml
0.3 U/ml
0.5 U/ml
1 U/ml
Thrombin
ATP Release (nM)
Normal
HAC
0.2 U/ml
0.3 U/ml
0.5 U/ml
1 U/ml


(A)
2-MeSADP
30 nM
50 nM
100 nM
200 nM
Light Absorbance
Normal
HAC
(B)
Thrombin
0.2 U/ml
0.3 U/ml
0.5 U/ml
1 U/ml
Light Absorbance
ATP Release
Normal
HAC
(C)
2-MeSADP
Aggregation (%)
0
10
20
30
40
50
30 nM
50 nM
100 nM
200 nM
Normal
HAC
Thrombin
Aggregation (%)
0
20
40
60
0.2 U/ml
0.3 U/ml
0.5 U/ml
1 U/ml
Normal
HAC
Thrombin
ATP Release (nM)
0
1
2
3
4
5
0.2 U/ml
0.3 U/ml
0.5 U/ml
1 U/ml
Normal
HAC


(A)
2-MeSADP
30 nM
50 nM
100 nM
Light Absorbance
ATP Release
Normal
Normal + Pred
(B)
2-MeSADP
Aggregation (%)
30 nM
50 nM
100 nM
Normal
Normal + Pred
2-MeSADP
ATP Release (nM)
30 nM
50 nM
100 nM
Normal
Normal + Pred
(C)
2-MeSADP + Fibrinogen
30 nM
100 nM
Light Absorbance
ATP Release
Normal
Normal + Pred
(D)
2-MeSADP + Fibrinogen
Aggregation (%)
30 µM
100 µM
Normal
Normal + Pred
2-MeSADP + Fibrinogen
ATP Release (nM)
30 µM
100 µM
Normal
Normal + Pred
(E)
Collagen
2 µg/ml
5 µg/ml
Light Absorbance
ATP Release
Normal
Normal + Pred
(F)
Collagen
Aggregation (%)
2 µg/ml
5 µg/ml
Normal
Normal + Pred
Collagen
ATP Release (nM)
2 µg/ml
5 µg/ml
Normal
Normal + Pred


(A)
2-MeSADP
TxB2 Generation (pg / 1 x 10^8 cells)
1000
800
600
400
200
0
*
*
**
30 nM
50 nM
100 nM
Normal
HAC
(B)
Thrombin
TxB2 Generation (pg / 1 x 10^8 cells)
6000
4000
2000
0
***
**
0.2 U/ml
1 U/ml
Normal
HAC


(A)
Normal
HAC
P-ERK
ERK
P-AKT
AKT
Thrombin
- + - +
(B)
ERK Phosphorylation
Relative Phosphorylation (%)
***
150
100
50
0
Normal
HAC
Thrombin
- + - +
(C)
AKT Phosphorylation
Relative Phosphorylation (%)
****
150
100
50
0
Normal
HAC
Thrombin
- + - +


(A)
100 nM 2-MeSADP
Aggregation (%)
0
20
40
60
0
5
10
15
20
25
Post-cortisol (μg/dl)
Normal
HAC
(B)
0.2 U/ml Thrombin
Aggregation (%)
0
20
40
60
80
0
5
10
15
20
25
Post-cortisol (μg/dl)
Normal
HAC

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.