Effects of Imidazoline and Nonimidazoline $\alpha_2$-Adrenergic Agents on Intracellular Cyclic AMP and Thromboxane B$_2$ Concentrations in Canine and Leporine Platelets

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ABSTRACT
We have previously demonstrated leporine platelets lack imidazoline (I) receptors and effects of imidazoline $\alpha_2$-adrenergic agents on platelet aggregation differ in dogs and rabbits. In order to compare the effects of various $\alpha$-adrenergic agents on canine and leporine intraplatelet cAMP ([cAMP]$_i$) and thromboxane B$_2$ ([TXB$_2$]), elevations of both forskolin-induced [cAMP], and adrenaline-collagen-induced [TXB$_2$], were determined using immunoassay. The $\alpha_2$-adrenoceptor antagonists were ineffective in canine platelets, but the $\alpha_2$-adrenoceptor agonists adrenaline inhibited forskolin-induced increase in [cAMP]. Adrenaline was ineffective in leporine platelets, but $\alpha_2$-adrenoceptor antagonists were effective on forskolin-induced increase in [cAMP]. Adrenaline-collagen-induced elevation of [TXB$_2$] in canine platelets was not inhibited by the non-imidazoline $\alpha_2$-adrenoceptor agonist xylazine, but was inhibited by $\alpha_2$-adrenoceptor antagonists, an imidazoline $\alpha_2$-adrenoceptor agonist (naphazoline) and an imidazoline compound (antazoline). In contrast, $\alpha_2$-adrenoceptor agonists were ineffective on [TXB$_2$], in leporine platelet-rich plasma and all drugs were ineffective on [TXB$_2$] in leporine washed platelets, demonstrating various imidazoline or nonimidazoline $\alpha_2$-adrenergic agents effectively inhibited forskolin-induced elevation of [cAMP], and adrenaline-collagen-induced [TXB$_2$], and the effective drugs differed between dogs and rabbits. We propose imidazoline $\alpha_2$-adrenergic agents suppress cAMP production via the $\alpha_2$-adrenoceptor while exerting a negative control on TXB$_2$ generation via the arachidonic acid-thromboxane A$_2$ pathway.

Keywords: Adrenoceptors, collagen, forskolin, platelet aggregation, thromboxane A$_2$

INTRODUCTION
Mammalian platelets vary widely in their responses to catecholamines and other $\alpha$-adrenergic agents (Sinakos and Caen, 1967; Kerry et al., 1984; Naselsky et al., 2001). Adrenaline is a weak platelet agonist, the function of which is primarily to sensitize platelets to other activating agents (Ardlie et al., 1985; Hjemdahl et al., 1994). Physiological adrenaline concentrations enhance shear-dependent platelet aggregation and platelet-to-platelet interaction on collagen (Goto et al., 1992; Mustonen and Lassila, 1996). The $\alpha_2$-adrenoceptors on human platelet membranes has been demonstrated by binding studies (Motulsky et al., 1980; Motulsky and Insel, 1982; Lanza and Cazenave, 1985) and adrenaline-induced platelet aggregation is thought to be mediated by $\alpha_2$-adrenoceptor stimulation (Grant and Scrutton, 1979; Lasch and Jakobs, 1979; Pinthong et al., 2004). Although $\alpha_2$-adrenoceptors are coupled to G$_i$, which mediates adenyllyl cyclase (AC) inhibition, the decrease in intraplatelet cAMP concentrations ([cAMP]) cannot account for many observed physiological responses (Savi et al., 1996; Yang et al., 2002; Khan et al., 1999). It is reported that
α2-adrenoceptors also couple to non G, proteins, accelerating sodium-hydrogen ion exchange and stimulating an increase in phospholipase A2 (PLA2) activity that results in increased thromboxane A2 (TXA2) formation (Khan et al., 1999; Mustonen et al., 2001). However, adrenaline-induced intraplatelet events are, to a great extent, unclear. Clonidine, an imidazoline α2-adrenoceptor agonist, has a complex effect on platelets. In humans, clonidine induces platelet aggregation and potentiates adenosine diphosphate (ADP)-stimulated platelet aggregation, but inhibits adrenaline-stimulated platelet aggregation (Hsu et al., 1979; Shattil et al., 1981). Imidazoline α2-adrenergic agents can bind to nonadrenergic imidazoline binding sites (I1 and I2 receptors) that are distinct from α2-adrenoceptors in human platelets (Michel et al., 1990; Zonnenschein et al., 1990; Piletz and Sletten, 1993; Piletz et al., 1996a). A limited number of studies have attempted to clarify the direct effects of imidazoline agents on platelet aggregation; however, some studies have indicated that there may be a dysregulation of platelet α2-adrenoceptors and I1 receptors in depression (Garcia-Sevilla et al., 1981; Piletz et al., 1996b, c).

Imidazoline α2-adrenergic agents also exert complex effects on mammalian platelet aggregation (Hikasa et al., 1999; Yokota et al., 2013a, b). Although, the mechanism is unclear, I receptor-mediated actions of imidazoline compounds may interact with adrenergic signaling via the α2-adrenoceptor. We previously reported that selected imidazoline α2-adrenergic agents blocked adrenaline-potentiated aggregation in canine platelets with a rank order different from α2-adrenoceptor activity (Hikasa et al., 1999). Furthermore, we demonstrated that the effects of imidazoline adrenergic agents on leporine platelet aggregation differed from those observed in dogs (Yokota et al., 2013a). It was also demonstrated that leporine platelets expressed I1 receptors instead of I2 receptors, whereas canine platelets expressed both I1 and I2 receptors (Hikasa et al., 2013). Comparative studies on the effects of imidazolines on canine and leporine intraplatelet signaling may be important for characterizing platelet receptors and may be useful for elucidating the biological function of imidazoline receptors. This study aimed to investigate the effects of imidazoline α2-adrenergic agents on [cAMP], and on intraplatelet thromboxane B, concentrations ([TXB2]), a stable metabolite of TXA2 (Hamberg et al., 1975), in canine and leporine platelets.

### MATERIALS AND METHODS

**Drugs:** The following drugs were used (with their sources indicated in parentheses). The L-adrenaline (Tokyo Kasei, Ind. Co., Japan); antazoline HCl, naphazoline HCl, yohimbine HCl, forskolin, indomethacin, 3-isobutyl-1-methylxanthine (IBMX) and SQ22536 (Sigma Chemical Co., St. Louis, Mo., USA); phentolamine mesylate (Ciba-Geigy Corp., Hyogo, Japan); atipamezole HCl and medetomidine HCl (Farmos Group Ltd, Finland); xylazine HCl (Bayer, Germany); ADP and collagen (Baxter, Japan). Adrenaline was dissolved in 0.04 M L−1 HCl solution and then diluted with physiological saline solution (PSS). Indomethacin was dissolved in 5% NaHCO3 solution and then diluted with PSS. All other drugs were dissolved in PSS.

**Preparation of citrated platelet plasma and washed platelet suspensions:** Six healthy adult beagle dogs (three intact males and three females) and seven adult male Japanese white rabbits (weighing 2.5-3.5 kg) were used for this study. For the [TXB2] assay using citrated platelet plasma, jugular blood samples were collected in plastic syringes containing 3.5% sodium citrate solution at a ratio of 1 part anticoagulant to 9 parts blood from dogs or rabbits. In addition, considering the influence of platelet aggregation, blood specimens were collected from dogs or rabbits without anesthesia. The study protocol was approved by the Animal Research Committee of Tottori University. The blood was centrifuged for 15 min at 110-140 g to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by centrifuging PRP for 15 min at 1500 g. The final platelet count was adjusted to 25-30×10^4 µL−1 by dilution with PPP.

For the assays of [cAMP], or [TXB2], using washed platelets, jugular blood samples were collected from dogs and rabbits in plastic syringes containing citrate dextrose-A (ACD-A) solution (40 mM L−1 citrate, 100 mM L−1 sodium citrate, 135 mM L−1 dextrose, pH 4.5) at a ratio of 1 part ACD-A to 6 parts blood. PRP was prepared by centrifugation at 90-100 g for 15 min. The platelets were isolated by centrifugation at 500 g for 15 min and gently resuspended in a wash solution that consisted of calcium-free modified Tyrode’s buffer (130 mM L−1 NaCl, 5 mM L−1 KCl, 14 mM L−1 sodium citrate, 10 mM L−1 Tris, 2 mM L−1 HEPES, 7 mM L−1 NaHCO3, 1 mM L−1 MgCl2, 6 mM L−1 dextrose, 0.1% gelatin, pH 7.35). The mixture was then centrifuged at 500 g for 15 min and the washing procedure was repeated. The washed platelets were finally resuspended in modified Tyrode’s buffer containing 1.8 mM L−1 CaCl2. The final platelet count was adjusted to 25-30×10^4 µL−1 by dilution with modified Tyrode’s buffer containing 1.8 mM L−1 CaCl2.

**Measurement of [cAMP]:** Washed platelets (180 µL) were preincubated in the presence of 1 mM L−1 IBMX (a phosphodiesterase inhibitor) for 10 min at 37°C and then treated with an aliquot (22 µL) of the drug and 22 µL of forskolin and incubated for 10 min at 4°C. After incubation, the reaction was terminated with 5% trichloroacetic acid and the platelets were separated by centrifugation at 1200 g for 10 min at 4°C. Subsequently, the pellet was removed and supernatants were stored and frozen at -20°C. The quantity of cAMP was determined by a radioimmunoassay kit (Yamasa Shoyu, Tokyo, Japan).

**Measurement of [TXB2]:** Washed platelets or PRP (180 µL) were preincubated for 10 min at 37°C and then treated with an aliquot (24 µL) of the drug, adrenaline (24 µL) and collagen (24 µL) and were incubated for 10 min at 37°C. After incubation, the reaction was terminated with 2.5 mM L−1 EDTA and 25 mM L−1 indomethacin and the platelets were separated by centrifugation at 1200 g for 10 min at 4°C. The
pellet was removed and supernatants were stored and frozen at -20°C. The quantity of TXB₂ was determined by enzyme immunoassay kit (Cayman Chemical Co., MI, USA).

**Data analysis:** All data were expressed as Means±SEM. Data were statistically compared by Student’s t-test or Wilcoxon-Mann-Whitney test, based on the F value. Differences were considered significant when p<0.05. One-way ANOVA followed by the Dunnett’s test was used for intragroup comparisons of concentration-related cAMP levels.

**RESULTS**

**Effects of adrenaline and ADP on [cAMP], in washed canine platelets:** The platelet agonist ADP at concentrations of 2-10 µM L⁻¹, which approximately elicits maximal platelet aggregation (Hikasa et al., 1999), did not potentiate [cAMP] in washed canine platelets (data not shown). Furthermore, a combination of adrenaline (final concentration; 10 µM L⁻¹) and ADP (10 µM L⁻¹), which produces maximal platelet aggregation (Hikasa et al., 1999), was also ineffective on [cAMP], in washed canine platelets (data not shown).

**Effects of forskolin on [cAMP], in washed canine platelets:** The effect of forskolin (1, 10 and 100 µM L⁻¹) was pretested for its ability to increase [cAMP], and the inhibitory effect of SQ22536, an AC inhibitor that was previously identified as a positive control, was validated in washed canine platelets (Fig. 1a). Forskolin increased [cAMP], in a concentration-dependent manner. The forskolin (10 µM L⁻¹)-induced increase in [cAMP], in washed canine platelets was significantly inhibited by SQ22536 (100 µM L⁻¹). For evaluation of inhibitory effects of the imidazoline and nonimidazoline α-adrenoceptor agents, 10 µM L⁻¹ forskolin was selected to induce the elevation of [cAMP], in washed canine platelets.

**Effects of imidazoline and nonimidazoline α-adrenoceptor agents on forskolin-induced elevation of [cAMP], in washed canine platelets:** Adrenaline (100 µM L⁻¹), the imidazoline adrenoceptor agonists naphazoline (100 µM L⁻¹) and medetomidine (100 µM L⁻¹) and the nonimidazoline adrenoceptor agonist xylazine (100 µM L⁻¹) inhibited forskolin-induced elevation of [cAMP], in washed canine platelets (Fig. 1b). In comparison, the imidazoline adrenoceptor antagonists phenolamine (100 µM L⁻¹) and atipamezole (100 µM L⁻¹) and the nonimidazoline adrenoceptor antagonist yohimbine (100 µM L⁻¹) did not significantly inhibit forskolin-induced increase in [cAMP]. The imidazoline compound antazoline (100 µM L⁻¹), which is devoid of α-adrenergic activity, also had no effect on forskolin-induced elevation of [cAMP].

**Effects of forskolin on [cAMP], in washed leporine platelets:** The effect of forskolin (1, 10 and 100 µM L⁻¹) was pretested for its ability to increase [cAMP]. Furthermore, the inhibitory effects of SQ22536 (100 µM L⁻¹) and adrenaline (100 µM L⁻¹) were evaluated in washed leporine platelets (Fig. 2a). Forskolin increased [cAMP], in a concentration-dependent manner and produced a significant increase at a concentration of 100 µM L⁻¹. The forskolin (100 µM L⁻¹)-induced increase in [cAMP], was significantly inhibited by SQ22536 (100 µM L⁻¹), whereas the high adrenaline concentration (100 µM L⁻¹) did not inhibit forskolin-induced elevation of [cAMP], in washed leporine platelets. For the following evaluation of inhibitory effects of the imidazoline and nonimidazoline α-adrenoceptor agents, the concentration of 100 µM L⁻¹ forskolin was chosen to elevate [cAMP], in washed leporine platelets.
Effects of imidazoline and nonimidazoline α-adrenoceptor agents on forskolin-induced elevation of [cAMP] in washed leporine platelets: The imidazoline adrenoceptor agonists naphazoline (100 µM L⁻¹) and medetomidine (100 µM L⁻¹) inhibited forskolin-induced elevation of [cAMP], in washed leporine platelets (Fig. 2b). Moreover, the α₂-adrenoceptor antagonists phentolamine (100 µM L⁻¹) and yohimbine (100 µM L⁻¹) exerted significant inhibitory effects on forskolin-induced elevation of [cAMP], in washed leporine platelets. Atipamezole (100 µM L⁻¹) and antazoline (100 µM L⁻¹) had no effect on forskolin-induced elevation of [cAMP], in washed leporine platelets; in contrast, adrenaline was also ineffective on the forskolin-induced elevation of [cAMP], in washed leporine platelets.

Effects of imidazoline and nonimidazoline α-adrenoceptor agents on adrenaline-collagen-induced elevation of [TXB₂] in canine PRP and washed platelets: A combination of adrenaline (10 µM L⁻¹) and collagen (2 µg mL⁻¹), with which maximum and full aggregation effects were reproducibly observed in dogs (Hikasa et al., 1999), was used for the [TXB₂] induction stimuli. Similar results were obtained for [TXB₂] using canine PRP or washed canine platelets. Collagen (2 µg mL⁻¹) alone did not increase [TXB₂], in canine PRP or washed platelets, but the combination of collagen and adrenaline induced a significant elevation of [TXB₂], in both PRP and washed platelets (Fig. 3). Adrenaline-collagen-induced increase in [TXB₂] was completely inhibited by...
α2-adrenoceptor antagonists (phenotamine and yohimbine; 100 µM L−1), the imidazoline adrenoceptor agonist naphazoline (100 µM L−1) and the imidazoline compound antazoline (100 µM L−1) in both PRP and washed platelets. In contrast, the nonimidazoline adrenoceptor agonist xylazine (100 µM L−1) did not inhibit adrenaline-collagen-induced elevation of [TXB2] in either PRP or washed platelets.

Effects of imidazoline and nonimidazoline α-adrenoceptor agents on adrenaline-collagen-induced elevation of [TXB2] in leporine PRP and washed platelets: A combination of adrenaline (10 µM L−1) and collagen (1 µg mL−1), with which the maximum aggregation effects were reproducibly observed in rabbits (Yokota et al., 2013a), was used for the [TXB2] induction stimuli. Collagen (1 µg mL−1) alone did not increase [TXB2], but the combination of collagen and adrenaline significantly increased [TXB2] in leporine PRP (Fig. 4a). The αα-adrenoceptor antagonists (phenotamine and yohimbine; 100 µM L−1) and antazoline (100 µM L−1) completely inhibited adrenaline-collagen-induced elevation of [TXB2], whereas the αα-adrenoceptor agonists naphazoline (100 µM L−1) and xylazine (100 µM L−1) did not significantly inhibit adrenaline-collagen-induced elevation of [TXB2] in leporine PRP. In contrast, neither collagen (1 µg mL−1) alone nor the combination of collagen and adrenaline produced a significant elevation of [TXB2] in washed leporine platelets (Fig. 4b). Furthermore, none of the test agents exhibited any effect on adrenaline-collagen-induced elevation of [TXB2] in washed rabbit platelets.

DISCUSSION

In the present study using washed canine platelets, adrenaline and αα-adrenoceptor agonists (naphazoline, medetomidine and xylazine) inhibited forskolin-induced [cAMP]. These results support that αα-adrenoceptors are abundant on canine platelets, similar to human platelets (Motulsky et al., 1980; Shattil et al., 1981; Motulsky and Insel, 1982; Lanza and Cazenave, 1985; Hikasa et al., 2013) and that [cAMP] is negatively controlled by the suppression of AC activities via αα-adrenoceptors. Moreover, antazoline, an imidazoline agent lacking αα-adrenoceptor activity and αα-adrenoceptor antagonists, with imidazoline activity (phenotamine and atipamezole), were ineffective on [cAMP]. These results indicate that platelet aggregation resulting from [cAMP] inhibition in dogs were not primarily conducted through I receptors, but through αα-adrenoceptors, similar to that in humans (Grant and Scrutton, 1979; Hsu et al., 1979; Weber et al., 1999). In contrast, adrenaline was ineffective on [cAMP] in washed leporine platelets. Selected αα-adrenoceptor antagonists partially inhibited [cAMP] in washed leporine platelets, although none of the tested αα-adrenoceptor antagonists inhibited [cAMP] in dogs. These results suggest the possibility that agonistic action on αα-adrenoceptors on leporine platelets may not contribute to down regulation of forskolin induced elevation of [cAMP].

We have previously demonstrated that the effects of imidazoline adrenergic agents on leporine platelet aggregation differed from those observed in dogs (Yokota et al., 2013a) and that leporine platelets do not express I receptors (Hikasa et al., 2013). The current results may suggest the possibility that some kind of cooperative action exists between I2 receptors and αα-adrenoceptors in regard to [cAMP] inhibition via αα-adrenoceptors because of lack of I1 receptors in rabbits. In addition, it has also been clarified by saturation binding assays that the composition ratios of αα-adrenoceptor, I1 receptor and I2 receptor densities in canine platelets are about 3:1:3, whereas that in leporine platelets are about 1:0:4 (Hikasa et al., 2013). The differences between leporine and canine [cAMP], in inhibitory effects of certain compounds may be due to the differences in relative contributions of each receptors in leporine and canine platelets.

Fig. 4(a-b): Effects of imidazoline and nonimidazoline αα-adrenoceptor agents on adrenaline-collagen-induced elevation of [TXB2] in leporine (a) PRP and (b) washed platelets. A combination of adrenaline (10 µM L−1) and collagen (1 µg mL−1) was used to stimulate [TXB2]. This combination was determined to elicit reproducible maximum aggregation in leporine platelets. Each test agent was applied at a final concentration of 100 µM L−1. *Significant difference from the adrenaline-collagen stimulus combination shown by the gray nonhatched column (t-test or Wilcoxon-Mann-Whitney test, depending on the F value, p<0.05)
Although the adrenaline-induced signaling pathway remains unclear, it is known that α₂-adrenoceptor agents stimulate PLA₂, activity resulting in increased formation of TXA₂ via nonGi-proteins (Khan et al., 1999; Mustonen et al., 2001). Furthermore, although the signaling pathway through the I₁ and I₂ receptor subtypes has not been fully elucidated, it is suggested that I receptors are linked to G-protein and that activation of PLA₂, leading to the release of arachidonic acid (AA) and the subsequent generation of prostaglandins plays a major role in the physiological actions elicited by stimulation of I receptors (Ernsberger et al., 1995; Khan et al., 1999; Mustonen et al., 2001). In the present study, adrenaline-potentiated collagen increased [TXB₂], in canine platelets was completely blocked by α₂-adrenoceptor antagonists. Furthermore, interestingly, imidazoline agent (antazoline) and the imidazoline α₂-adrenoceptor agonist (naphazoline) also inhibited the elevation of [TXB₂]. These results suggest that the adrenaline-augmented signal pathway of collagen induced elevation of [TXB₂], may be controlled and/or modified by imidazoline activities through I receptors. With regard to the combination of adrenaline and collagen, it is assumed that the Ca²⁺-AA-TXA₂ pathway via Gq-proteins operates in parallel with AC-cAMP signaling via Gi-proteins (Khan et al., 1999; Mustonen et al., 2001; Yokota et al., 2013a). Imidazoline α₂-adrenoceptor agents may nullify the inhibition of the AC-cAMP pathway by α₂-adrenoceptor block and/or suppress the Ca²⁺-AA-TXA₂ pathway and subsequent increase in [TXB₂], through I receptors. In contrast, in leporine platelets, the imidazoline α₂-adrenoceptor agonists naphazoline and xylazine were ineffective on [TXB₂], in platelet-rich plasma and all drugs were ineffective on [TXB₂], in washed platelets. The differences between leporine and canine [TXB₂], in inhibitory effects of certain compounds may be also due to the differences in relative ratios of α₂-adrenoceptor, I₁ receptor and I₂ receptor densities between leporine and canine platelets. In general, various aggregation factors, such as fibrin, vWF and selected glycoproteins are present in PRP and the possibility of interactions with these platelet aggregation factors must be considered (Jung et al., 1981; Beguin et al., 1999). Although it remains unclear that all agents were ineffective on [TXB₂], in leporine washed platelets, it is possible that, particularly in leporine blood platelets, collagen, adrenaline, and/or particular drugs interacted with these aggregation factors. Further examination of this issue will be necessary to reveal the mechanisms underlying the inconsistent effects of imidazoline α₂-adrenoceptor agents on leporine platelet aggregation.

CONCLUSION

This study demonstrated that adrenaline and α₂-adrenoceptor agonists effectively inhibited forskolin-induced elevation of [cAMP], in canine platelets, whereas, adrenaline was ineffective on [cAMP], in washed leporine platelets but α₂-adrenoceptor antagonists partially inhibited [cAMP], in washed leporine platelets. On the other hand, adrenaline-potentiated collagen increased [TXB₂], in canine platelets was completely blocked not only by α₂-adrenoceptor antagonists, but also by imidazoline agent and the imidazoline α₂-adrenoceptor agonist. In contrast, in leporine platelets, the imidazoline α₂-adrenoceptor agonists were also ineffective on [TXB₂], in platelet-rich plasma and all drugs were ineffective on [TXB₂], in washed platelets. It can be presumed that the cause of differences in the effectiveness of the drugs between dogs and rabbits is due to the differences in relative ratios of α₂-adrenoceptor, I₁ receptor and I₂ receptor densities between leporine and canine platelets. We propose imidazoline α₂-adrenergic agents suppress cAMP production via the α₂-adrenoceptor while exerting a negative control on TXB₂ generation via the arachidonic acid–thromboxane A₂ pathway.

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