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Characterization of platelet aggregation responses in microminipigs: Comparison with miniature pigs and the influence of dual antiplatelet administration of aspirin plus prasugrel

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ABSTRACT

We aimed to characterize platelet aggregation responses and the impact of dual antiplatelet therapy in microminipigs. In this in vitro study, both adenosine-5′-diphosphate (ADP, 5–50 μM) and collagen (2–20 μg/ml) induced concentration-related platelet aggregation in the microminipigs; 20 μM ADP and 5 and 12.5 μg/ml collagen were selected for further ex vivo studies. Aspirin plus prasugrel were administered orally for 7 days (n = 4/each group). Ex vivo platelet aggregation was analyzed on Day 1 (1 and 4 h after administration), Day 4 (4 h), and Day 7 (4 h) under three different prasugrel dosing regimens: LD0/MD1 (1 mg/kg/day), LD0/MD3 (3 mg/kg/day), and LD10/MD1 (10 mg/kg loading dose and 1 mg/kg/day maintenance dose). Aspirin (10 mg/kg/day) was administered to all groups. In the presence of aspirin, prasugrel at 3 and 10 mg/kg significantly inhibited ADP-induced platelet aggregation on Day 1. On Days 4 and 7, significant inhibition of platelet aggregation (IPA) was also observed in each group. With 5 μg/ml collagen-induced platelet aggregation, all three groups showed significant IPA at 4 h on Day 1 or later. In 12.5 μg/ml collagen-induced platelet aggregation, all groups showed significant effects on Days 4 and 7; however, the 30%–35% IPA was considerably lower than that (50%–60%) found with 5 μg/ml collagen. In Clawn miniature pigs, similar inhibitory patterns were observed for both ADP- and collagen-induced ex vivo platelet aggregation. In conclusion, these results indicated that microminipigs as well as miniature pigs may represent useful experimental animals for thrombosis research.

Introduction

Acute coronary syndrome (ACS) is one of the leading causes of death worldwide [1]. Coronary plaque rupture often leads to platelet activation/aggregation and thrombus formation, with consecutive vessel ischemia leading to myocardial infarction (MI) [2]. Thus, activated platelets play a central role in the pathogenesis of MI. Patients with ACS, particularly those subject to percutaneous coronary intervention (PCI), need to be treated with dual antiplatelet therapy (DAPT) consisting of aspirin combined with a $P2Y_{12}$ receptor antagonist [3–5]. Prasugrel is a third generation thienopyridyl prodrug [6,7] that effects potent and specific inhibition of the platelet $P2Y_{12}$ adenosine-5′-diphosphate (ADP) receptor [8,9]. Prasugrel can provide more consistent and greater $P2Y_{12}$ inhibition than clopidogrel [10]. The more optimal pharmacokinetics and pharmacodynamics of prasugrel provide more effective platelet inhibition as well as greater clinical benefits in patients with ACS undergoing PCI [11,12].

Compared with other experimental animals, such as mice, rats, and rabbits, pigs provide more useful animal models of cardiovascular disease since their anatomy, physiology, feeding, and sleep cycles are similar to those of humans [13,14]. Consequently, domestic pigs are often used in nonclinical cardiovascular research [15–17]. However, because of their large body size, experimentation with domestic pigs presents several difficulties, such as handling, maintenance, and necessity of large quantities of the drug. Commercially available experimental miniature pigs, such as Clawn, Göttingen, and Yucatan minipigs, are smaller than domestic pigs, and a large number of nonclinical studies have been reported using these miniature pigs [18–22]. However, these miniature pigs are still too large to be widely used in pharmacological research. The microminipig (MMPig, Fuji Micra Inc., Shizuoka, Japan) has recently been established as an experimental animal [23–25]. Several studies have demonstrated its utility in a variety of experimental cardiovascular applications [14,26–28]. However, to our knowledge, there have been no reports regarding platelet aggregation in microminipigs, an important shortcoming given the central role of platelet aggregation in ACS/atherothrombotic disease.

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The present study aimed to characterize ADP and collagen mediated platelet aggregation responses in microminipigs. In addition, the effects of DAPT with prasugrel and aspirin were compared using microminipigs and Clawn miniature pigs to further clarify the suitability of microminipigs for the study of cardiovascular disease.

Materials and Methods

Materials

Sodium citrate and methylcellulose (MC) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ADP (MCM ADP) and collagen (MCM collagen H) were purchased from LMS Co., Ltd. (Tokyo, Japan). Prasugrel hydrochloride (prasugrel) was provided by Ube Industries, Ltd. (Yamaguchi, Japan). Aspirin was purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA) or Sigma-Aldrich Corp. (St. Louis, Missouri, USA).

Animals

Male Clawn miniature pigs, purchased from NARC Corporation (Chiba, Japan), were used for the present study. Male microminipigs were purchased from Fuji Micra Inc. and used for the present study. These animals' weights are shown in Table 1. Each animal's general condition was checked at receipt, and the animals were housed in individual cages. The pigs were then subjected to 2 weeks of quarantine and acclimatization. During this period, each animal's general condition was observed daily. All animal studies were approved by the Institutional Animal Care and Use Committee (NISSEI BILIS Co., Ltd., LSI Medience Corporation and Daiichi Sankyo Co., Ltd.).

Drug Administration

Prasugrel and aspirin were suspended in 0.5% (w/v) MC solution. The doses for each agent are shown in Table 1. The suspension of prasugrel or aspirin was orally administered once a day for 7 days to each pig with a volume of 0.5 ml/kg (miniature pigs) or 1 ml/kg (microminipigs). Prasugrel doses used for this series of platelet function studies were based on the results from other platelet aggregation studies using rats, dogs, and monkeys [8,29]. Based on a previous report [30], an antithrombotic dose of 10 mg/kg/day aspirin was administered to each pig.

Platelet Aggregation

Platelet aggregation was measured before the first study drug administration (prevalues) and on Day 1 (1 and 4 h after the first administration), Day 4 (4 h after administration), and Day 7 (4 h after administration). A 10 ml sample of citrated blood was collected via catheters placed in the femoral artery (miniature pigs) or jugular vein (microminipigs), and then centrifuged to obtain platelet-rich plasma (PRP) (120 × *g* for miniature pigs or 220 × *g* for microminipigs, 10 min, room temperature) and platelet-poor plasma (PPP) (1300 × *g* for miniature pigs or 2200 × *g* for microminipigs, 15 min, room temperature), respectively. The number of platelets was counted, and PRP was diluted with PPP to produce 300×10^3/μl platelets. After count adjustment, 200 or 240 μl of PRP was stirred for 1–1.5 min at 37 °C prior to the addition of ADP (final concentration: 20 μM) or collagen (final concentration: 5 μg/ml for miniature pigs or 5 and 12.5 μg/ml for microminipigs) to induce platelet aggregation. These concentrations of ADP and collagen (submaximal and maximal concentrations) were selected based on preliminary in vitro experiments (Fig. 1 for microminipigs and unpublished data for miniature pigs). Platelet aggregation was measured using 12-channel automated platelet aggregometers (MCM HEMA TRACER 313 M, MC Medical, Inc., Tokyo, Japan) for 10–15 min with maximum platelet aggregation (MPA) automatically recorded.

Table 1
Doses and body weights in each experimental group.

Group	N	Body Weight (kg)	Aspirin (mg/kg/day)	Prasugrel LD (mg/kg)	Prasugrel MD (mg/kg/day)
Microminipigs					
LD0/MD1	4	7.80 ± 0.21	10	-	1
LD0/MD3	4	7.76 ± 0.18	10	-	3
LD10/MD1	4	7.76 ± 0.22	10	10	1
Clawn miniature pigs					
LD0/MD1	4	24.28 ± 1.08	10	-	1
LD0/MD3	4	23.63 ± 1.28	10	-	3
LD10/MD1	4	27.00 ± 1.43	10	10	1

Abbreviations: LD, loading dose; MD, maintenance dose.

Statistical Analysis

Data are presented as mean ± standard error (SE). The extent of inhibition of platelet aggregation (IPA) at each time point in each animal was calculated as the percent of MPA at pre (before administration) according to the following equation: [(MPA at pre − MPA at each time point)/MPA at pre] × 100. The MPA value in each group was analyzed by Dunnett's multiple comparison testing to compare the MPA at each time point against the MPA at pre. A probability level of <0.05 was accepted as significant. Statistical analysis of differences was performed using the SAS System for Windows ver. 8.2 or ver. 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the interlocking biological experiment data analysis system EXSAS (ver. 7.16, Armsystex Co., Ltd., Osaka, Japan) or EXSUS (ver. 7.7.1, CAC EXICARE Corporation, Tokyo, Japan).

Results

ADP- and Collagen-induced in Vitro Platelet Aggregation

To characterize platelet aggregation in microminipigs and select the appropriate concentration(s) of agonists in the further ex vivo study, we measured in vitro platelet aggregation induced by ADP (5–50 μM) and collagen (2–20 μg/ml). As shown in Fig. 1, both ADP and collagen induced platelet aggregation in a concentration-related manner. Based on these results, 20 μM of ADP and 5 and 12.5 μg/ml of collagen were selected for the microminipig ex vivo study. Fig. 2 shows typical platelet aggregation tracings induced by ADP and collagen in microminipigs; tracings were qualitatively similar to those observed in other experimental animals and humans.

Effect of DAPT on ADP-induced Ex Vivo Platelet Aggregation

The aspirin and prasugrel doses administered to each group are shown in Table 1. In microminipigs, platelet aggregation induced by 20 μM ADP at prevalue (pre) was 65 ± 4% (n = 4) for LD0/MD1, 63 ± 2% (n = 4) for LD0/MD3, and 58 ± 3% (n = 4) for LD10/MD1 (Table 2). Prasugrel plus aspirin resulted in dose-related IPA at 1 and 4 h after the first dosing (Fig. 3); the effects of 3 and 10 mg/kg of prasugrel were statistically significant (Table 2). On Days 4 and 7, all groups showed significant and similar IPA. On Day 4, IPA in LD0/MD1, LD0/MD3, and LD10/MD1 was 40 ± 10% (n = 4), 42 ± 6% (n = 4), and 42 ± 6% (n = 4), respectively. On Day 7, IPA in groups LD0/MD1, LD0/MD3, and LD10/MD1 was 47 ± 3% (n = 4), 48 ± 7% (n = 4), and 58 ± 3% (n = 4), respectively (Fig. 3).

In Clawn miniature pigs, platelet aggregation induced by 20 μM ADP at pre was 56 ± 1% (n = 4) for LD0/MD1, 57 ± 1% (n = 4) for LD0/MD3, and 52 ± 2% (n = 4) for LD10/MD1 (Table 2). All doses of prasugrel plus aspirin resulted in significant IPA at 1 and 4 h after the first dosing, with little difference between 3 and 10 mg/kg of prasugrel (Table 2 and Fig. 4). Similar to the microminipigs, on Days 4 and 7, all miniature pig