

Prasugrel, a Platelet P2Y₁₂ Receptor Antagonist, Improves Abnormal Gait in a Novel Murine Model of Thrombotic Hindlimb Ischemia

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Background—The efficacy of P2Y₁₂ inhibition for the prevention of cardiovascular events in patients with peripheral arterial disease (PAD) has been established. However, the therapeutic effects on ischemic limb complications are less clear. Accordingly, we aimed to develop a novel murine model of thrombotic hindlimb ischemia to reflect that found in patients with PAD exhibiting ischemic limb symptoms. We further investigated the effects of P2Y₁₂ deficiency and P2Y₁₂ inhibition by prasugrel in this model.

Methods and Results—Thrombus formation induced by application of ferric chloride to the femoral artery resulted in a significant reduction in blood flow in the injured limb. In gait analysis using the CatWalk system, moderate difficulties in grounding and weight bearing of the ischemic limb, including reduction of maximum contact area and stance phase duration and increasing in swing phase duration in the ischemic limb, were observed in this model. Blood flow reduction and gait abnormalities gradually recovered over 21 days to levels present before arterial injury. Compared to wild-type (WT) mice, significant increases in blood flow and improvement in gait were observed in P2Y₁₂-deficient mice. In addition, daily oral administration of prasugrel (3 mg/kg per day) to WT mice resulted in significant inhibition of blood flow reduction and gait abnormalities to levels found in P2Y₁₂ deficient mice.

Conclusions—Acute femoral artery thrombosis resulted in hindlimb ischemia and moderate gait abnormalities in mice. In addition, the present study suggests a possible role of P2Y₁₂ in the complications with thrombotic limb ischemia. (*J Am Heart Assoc.* 2016;5:e002889 doi: 10.1161/JAHA.115.002889)

Key Words: femoral artery thrombosis • gait abnormalities • limb ischemia • P2Y₁₂ • prasugrel

Patients with peripheral arterial disease (PAD) have an increased risk of cardiovascular events, such as myocardial infarction, ischemic stroke, and vascular death.^{1,2} Platelets have been considered to play a central role in the development of arterial thrombosis in patients with systemic atherosclerotic vascular disease, including PAD.³ The benefits of antiplatelet therapy for the secondary prevention of cardiovascular events in patients with PAD have been evaluated, and antiplatelet therapy, notably P2Y₁₂ inhibition, has resulted in reduced risk of major adverse cardiovascular events in these patients.^{1,4} On the basis of this evidence, treatments with antiplatelet therapy for the prevention of cardiovascular events in PAD patients have been recom-

mended.^{5,6} Treatment for limb complications of PAD, including intermittent claudication, ischemic pain, or ischemic ulceration, is also a clinical goal; however, the efficacy of P2Y₁₂ inhibition on the progression of ischemic symptoms as the primary end point in PAD patients has not been consistent.^{7–12} Further studies are therefore required to better understand the role for P2Y₁₂ inhibition in controlling ischemic limb complications in patients with PAD. Prasugrel, a third-generation thienopyridine antiplatelet agent used clinically to treat patients with acute coronary syndrome who are undergoing percutaneous coronary intervention,¹³ has been shown to provide effective inhibition of platelet activation and aggregation via the selective inhibition of platelet P2Y₁₂.^{14,15} Furthermore, it has also been shown to be more potent and consistent than clopidogrel for P2Y₁₂ inhibition.¹⁶ While prasugrel's profile would suggest that it would provide benefit in ischemic limb complications, as well as reducing the risk of cardiovascular events through potent and consistent P2Y₁₂ inhibition, the efficacy of prasugrel in PAD remains to be explored in both nonclinical and clinical studies.

To date, critical limb ischemia (CLI) models have been widely used for nonclinical studies of PAD^{17–19}; but in the majority of patients with PAD, the disease is at the mild PAD stage, which is asymptomatic or results in intermittent

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claudication, and only 1% to 3% of patients have CLI.⁵ It is therefore desirable to have an experimental model of mild PAD reflecting this level of disease in PAD patients and thus allowing for more relevant pharmacological interventions. For the present study, we hypothesized that thrombus formation in the mouse femoral artery might cause acute limb ischemia and moderate walking abnormalities similar to those in patients with mild PAD. Additional limitations of current models relate to the evaluation of gait abnormalities in the hindlimb ischemia models. Evaluation of the walking profile in CLI models has mostly involved the use of a treadmill^{20,21}; however, this only allows measurement of walking time and walking distance, and it can only be used to evaluate animals able to walk. A more sensitive and reproducible system to evaluate walking function was therefore required. The CatWalk system (Noldus Information Technology, Wageningen, The Netherlands), a video-based system for automatic gait analysis, allows the determination of a variety of gait parameters from the animal's footprints captured optically.^{22,23} This system has been utilized in animal models of neuropathic pain,²⁴ inflammatory pain,²⁵ spinal cord injury,²³ Parkinson's disease,²⁶ and cerebral infarction.²⁷ However, to our knowledge, there have been no reports of the CatWalk system being used for evaluating PAD models.

In this study, we aimed to develop a novel murine model of thrombotic hindlimb ischemia and to investigate the effects of P2Y₁₂ deficiency and P2Y₁₂ antagonism on blood flow and walking function in the ischemic limb.

Materials and Methods

Materials

Prasugrel hydrochloride (prasugrel) was provided by Ube Industries Ltd. (Ube, Japan). Ferric chloride (FeCl₃) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Gum arabic was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Animals

C57BL/6J mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Mice lacking P2Y₁₂ receptors (P2Y₁₂-deficient mice) were generated as previously described²⁸ with a mixed C57BL/6-129 background and backcrossed for 7 generations with C57BL/6J mice. General observation was conducted once daily during the quarantine, acclimatization, and experimental periods with no abnormalities being observed. All animal studies were approved by the Institutional Animal Care and Use Committee (Nissei Bilis Co., Ltd. and Daiichi Sankyo Co., Ltd.).

Drug Administration

Prasugrel was suspended in a 5% solution of gum arabic. Starting 2 days before the arterial injury (Day -2), prasugrel or the vehicle were administered once a day for 23 days to each mouse by oral gavage with a volume of 10 mL/kg. The prasugrel doses used for the repeated administration studies were based on a previous platelet aggregation study.²⁸

FeCl₃-Induced Arterial Thrombosis

On the day of arterial injury (Day 1), nonfasted mice were restrained on a heating pad under 1.5% isoflurane anesthesia. An incision was made above the femoral artery from the inguinal crease, and the left femoral artery was exposed and gently excised from the femoral nerve and vein. Filter paper (1×2 mm, Qualitative filter paper No. 2; Advantec Toyo Kaisha, Ltd., Tokyo, Japan) saturated with 10% FeCl₃ was placed on the femoral artery at the midpoint between the lateral circumflex femoral artery branch and popliteal artery branch for 3 minutes at 0.9 to 1.1 hours after administering prasugrel or the vehicle. In the sham group, the femoral artery was treated with distilled water in place of the FeCl₃.

Measurement of Blood Flow

Blood flow images were captured of the mice under isoflurane inhalation anesthesia before and 1 hour after the arterial injury or the sham operation on Day 1. On Days 3, 7, and 21, anesthetized mice were laid in a supine position and blood flow images of the hindlimbs were captured with a laser speckle blood flow imager (OZ-1, Omegawave, Inc., Tokyo, Japan). Using the blood flow images for each animal, the relative blood flow was calculated as the ratio of the injured hindlimb value to the normal hindlimb value.

Morphometric Analysis of the Vessel Lesions

After capturing the blood flow images on Day 1, the anesthetized animals were exsanguinated with perfusion of saline via the left ventricle to an incision in the right atrium. Following euthanasia, perfusion fixation was performed with neutral buffered 10% formalin for 5 to 9 minutes, and then the FeCl₃-injured area of the left femoral artery was excised and fixed by immersion in neutral buffered 10% formalin. Paraffin sections were prepared at 3 positions on the femoral arteries: the midsection of the FeCl₃-injured segment, 100 μm distal from the midsection, and 200 μm distal from the midsection. Elastica van Gieson staining was performed at each position. For each of these Elastica van Gieson-stained sections, image analysis software (WinROOF 2013; Mitani Corporation, Tokyo, Japan) was used to determine the area of the lumen (A), the

area surrounded by internal elastic lamina (B), and the area of the thrombus (T). By using each value, lumen stenosis [$100 \times \{B - (A - T)\} / B$] was calculated. Values for the thrombus area and lumen stenosis in the 3 positions were averaged.

Gait Analysis

Gait analysis was performed using the CatWalk system in accordance with the manufacturer's instructions and previously published procedures.^{22,23} Briefly, this system consists of an enclosed glass runway with customized lighting. When the animal's paws make contact with the glass runway, light is reflected downwards and the illuminated contact area is recorded with a high-speed color video camera positioned under the runway. From the position, intensity, duration, and contact area of each paw, the analysis system can calculate multiple gait parameters for the animal.

The gait patterns were investigated on Days -3 (pre), 3, 7, and 21. We analyzed the following 4 parameters: (1) maximum (max) contact area: the surface area contacted by the paw during a stance phase; (2) swing phase duration: the time in seconds in a step cycle during which a paw was not in contact with the glass plate; (3) stance phase duration: the time in seconds in a step cycle during which a paw was in contact with the glass plate; and (4) stride length: the distance between successive placements of the same paw. For each parameter, we calculated as a percentage the ratio of the injured hindlimb value to the contralateral normal hindlimb value.

Statistical Analysis

Data are presented as mean \pm SE. Student *t* tests were used for the comparisons between the wild-type (WT) and P2Y₁₂-deficient mice and between the control and sham groups. A paired *t* test was used for the comparison of the relative blood flow before and 1 hour after arterial injury. Two-way ANOVA was used for the comparison among the genotype (WT/P2Y₁₂ deficiency) and the injury (pre/post). Dunnett's test was used for the comparison between the control and all prasugrel groups. In all the analyses, statistical significance was defined as $P < 0.05$. SAS 9.2 for Windows (SAS Institute Inc., Cary, NC) and EXSUS Ver. 7.7.1 (Arm Systex Co., Ltd., Osaka, Japan) were used for the analyses.

Results

Effects of P2Y₁₂ Deficiency on Thrombus Formation and the Blood Flow of the FeCl₃-Injured Hindlimb

Representative blood flow images of the hindlimbs of WT and P2Y₁₂-deficient mice are shown in Figure 1A. The relative

blood flow of the injured hindlimb before/after arterial injury is shown in Figure 1B. There were no differences in the relative blood flow between WT mice and P2Y₁₂-deficient mice before arterial injury. In the WT mice, a statistically significant reduction in relative blood flow was observed 1 hour after the arterial injury ($P = 0.0008$). In the P2Y₁₂-deficient mice, the relative blood flow after the arterial injury was not significantly reduced, and it was significantly higher than that of the WT mice ($P = 0.0018$). Typical Elastica van Gieson-stained cross-sections of the FeCl₃-injured femoral arteries on Day 1 are shown in Figure 1C. In the morphometric analysis, significantly less thrombus area was observed in P2Y₁₂-deficient mice compared with the WT mice ($P = 0.0021$, Figure 1D). Lumen stenosis was also significantly lower in P2Y₁₂-deficient mice compared to WT mice ($P = 0.0041$, Figure 1E).

Effects of Prasugrel on the Blood Flow of the FeCl₃-Injured Hindlimb

Representative hindlimb blood flow images after arterial injury on Day 1 in the sham, control, and prasugrel groups are shown in Figure 2A. The time course of relative blood flow following arterial injury is shown in Figure 2B. Relative blood flow in the sham group ranged from $97.2 \pm 3.4\%$ to $105.4 \pm 3.1\%$ over the study period. In the control (vehicle) group, relative blood flow of the injured hindlimb was reduced 1 hour after arterial injury on Day 1 and then gradually recovered to pre-injury levels through Day 21. The reduction of relative blood flow in the injured hindlimb was statistically significant compared to the sham group from Day 1 to Day 21; the values for relative blood flow on Days 1, 3, 7, and 21 were $47.7 \pm 1.5\%$ ($P < 0.0001$), $69.9 \pm 2.1\%$ ($P < 0.0001$), $90.3 \pm 3.8\%$ ($P = 0.0265$), and $90.3 \pm 3.4\%$ ($P = 0.0237$), respectively. The lower-dose prasugrel groups showed similar time courses for the relative blood flow; however, similar to what was seen in the P2Y₁₂-deficient mice, prasugrel at 3 mg/kg per day significantly ameliorated the reduction of the relative blood flow on Days 1 and 3 compared to the control group. The values of relative blood flow on Days 1, 3, 7, and 21 were $65.0 \pm 6.0\%$ ($P = 0.0051$), $89.5 \pm 4.5\%$ ($P = 0.0001$), $95.8 \pm 3.7\%$ ($P = 0.6242$), and $102.0 \pm 3.5\%$ ($P = 0.0732$), respectively.

Effects of P2Y₁₂ Deficiency and Prasugrel on Gait Parameters of the FeCl₃-Injured Hindlimb

The effects of P2Y₁₂ deficiency on gait abnormalities in this thrombotic hindlimb ischemia model were evaluated 3 days after arterial injury (Day 3). The maximum contact area of the P2Y₁₂-deficient mice was significantly increased compared to that of the WT mice ($P = 0.0021$); the ischemic-to-normal ratios for the maximum contact area were $94.10 \pm 15.59\%$ and

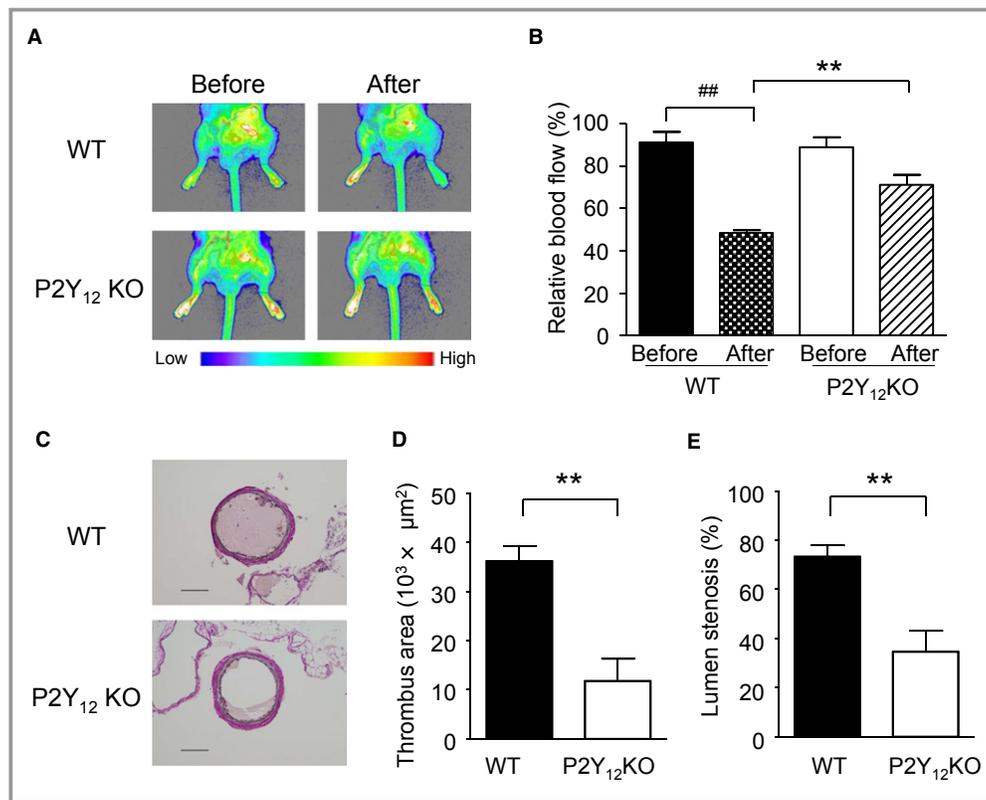


Figure 1. Hindlimb blood flow and pathology in wild-type (WT) and P2Y₁₂-deficient mice. Blood flow images of hindlimbs were captured by laser speckle imaging in WT and P2Y₁₂-deficient (KO) mice. Representative blood flow images and relative blood flow for before and 1 hour after arterial injury are shown in (A) and (B), respectively. The results of 2-way ANOVA were as follows: Genotype: $F(1, 16) = 5.88, P = 0.0275$; Injury: $F(1, 16) = 52.02, P < 0.0001$; and Genotype \times Injury interaction: $F(1, 16) = 8.82, P = 0.0090$. Typical Elastica van Gieson–stained images of the injured arteries are shown in (C). Scale bar, 100 μm . Thrombus area (D) and lumen stenosis (E) were measured morphometrically. Data are presented as mean \pm SE ($n = 5$). ** $P < 0.01$ vs WT mice (Student t test). ## $P < 0.01$ vs before the arterial injury (paired t test).

31.92 \pm 7.64% for the P2Y₁₂-deficient and WT mice, respectively. The swing phase duration in the P2Y₁₂-deficient mice was significantly reduced compared to that for the WT mice ($P = 0.0110$); the ischemic-to-normal ratios for swing phase duration were 109.96 \pm 6.91% and 160.83 \pm 16.56% for the P2Y₁₂-deficient mice and WT mice, respectively. The stance phase duration in P2Y₁₂-deficient mice was significantly increased compared to that for the WT mice ($P = 0.0021$); the ischemic-to-normal ratios for stance phase duration were 97.28 \pm 7.20% and 54.99 \pm 7.63% for the P2Y₁₂-deficient and WT mice, respectively. The stride length of the injured limb did not alter in either the P2Y₁₂-deficient mice or the WT mice.

Next, the effects of prasugrel on gait parameters in this thrombotic hindlimb ischemia model were evaluated on Days –3 (pre), 3, 7, and 21. The time courses in the ratios of gait parameters of the injured hindlimb to normal hindlimb are shown in Figure 3. The gait parameters of the 3 mg/kg per day prasugrel-treatment group were similar to those of the P2Y₁₂-deficient mice. Prior to the arterial injury, there were no

significant differences for all the gait parameters between the sham group and the control group, and between all prasugrel groups and the control group. The maximum contact area in the control group was significantly reduced on Days 3 and 7 compared to the sham group; the ratios of ischemic hindlimb maximum contact area to normal hindlimb maximum contact area on Days 3 and 7 were 47.65 \pm 7.24% ($P = 0.0002$) and 61.24 \pm 7.78% ($P = 0.0002$), respectively (Figure 3A). Prasugrel at 3 mg/kg per day significantly reduced the decrease in the maximum contact area on Days 3 and 7 compared to the control group; the ischemic-to-normal ratios for the maximum contact area on Days 3 and 7 were 107.23 \pm 11.61% ($P = 0.0004$) and 96.61 \pm 9.48% ($P = 0.0083$), respectively (Figure 3A). The swing phase duration in the control group was significantly increased on Days 3 and 7 compared to the sham group; the ischemic-to-normal ratios for swing phase duration on Days 3 and 7 were 178.28 \pm 18.90% ($P = 0.0024$) and 142.51 \pm 12.38% ($P = 0.0035$), respectively (Figure 3B). Prasugrel at 3 mg/kg per day significantly prevented the

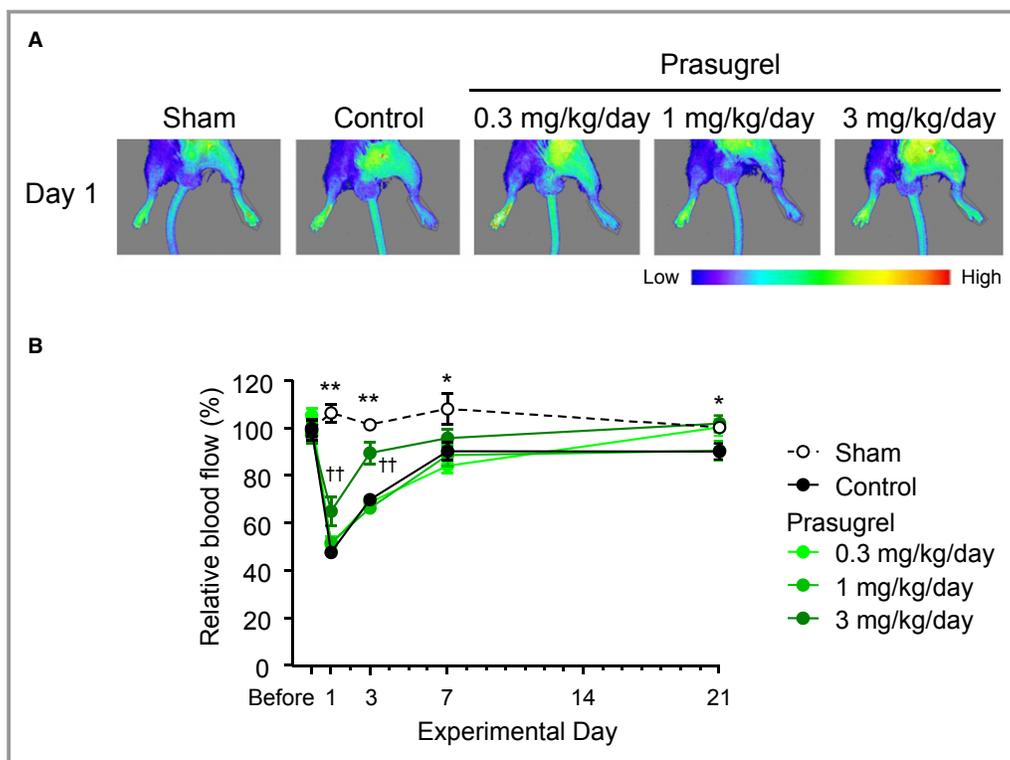


Figure 2. Effects of prasugrel on injured hindlimb blood flow. Representative blood flow images at 1 hour after the arterial injury on Day 1 are shown in (A). Relative blood flow was calculated for each animal at each time point (B). Data are presented as the mean \pm SE (n=10). Before: before the arterial injury. Day 1: 1 hour after the arterial injury. * P <0.05, ** P <0.01 vs control group (Student t test). †† P <0.01 vs control group (Dunnett's test).

increase in swing phase duration on Days 3 and 7 compared to the control group; the ischemic-to-normal ratios for swing phase duration on Days 3 and 7 were $126.10\pm 12.09\%$ ($P=0.0231$) and $106.06\pm 9.50\%$ ($P=0.0400$), respectively (Figure 3B). The stance phase duration in the control group was significantly decreased on Days 3 and 7 compared to the sham group; the ischemic-to-normal ratios for stance phase duration on Days 3 and 7 were $58.09\pm 7.79\%$ ($P=0.0032$) and $72.29\pm 6.60\%$ ($P=0.0025$), respectively (Figure 3C). Prasugrel at 3 mg/kg per day significantly reduced the decrease in stance phase duration on Days 3 and 7 compared to the control group; the ischemic-to-normal ratios for stance phase duration on Days 3 and 7 were $103.94\pm 10.06\%$ ($P=0.0013$) and $100.96\pm 8.89\%$ ($P=0.0142$), respectively (Figure 3C). In the analysis of stride length, there were no significant differences between the sham group and the control group, or between all prasugrel groups and the control group (Figure 3D).

Discussion

The role of the platelet P2Y₁₂ ADP receptor in cardiovascular and peripheral atherothrombosis in patients with PAD and the therapeutic potential of P2Y₁₂ antagonism for disease mod-

ification are of clinical interest. In the present study, we examined the effects of P2Y₁₂ deficiency and prasugrel treatment in a new model of thrombotic hindlimb ischemia. Both P2Y₁₂ deficiency and prasugrel administration attenuated blood flow reduction and yielded improvements in gait abnormalities in this model of limb ischemia with walking dysfunction. While P2Y₁₂ antagonists appear to be efficacious in reducing cardiovascular events in patients with PAD, their efficacy in controlling intermittent claudication in patients with PAD is less clear. Ticlopidine, the first-generation thienopyridyl P2Y₁₂ antagonist, demonstrated beneficial effects on the improvement of limb functions^{8,9} and the prevention of vascular complications^{8,11} in patients with intermittent claudication. However, other studies reported that ticlopidine and clopidogrel, the second-generation thienopyridine, had no clear beneficial effects on symptoms in PAD.^{7,10,12} One possible reason for these mixed results is that the antiplatelet effects of ticlopidine and clopidogrel may not have been sufficient to improve the limb ischemia in PAD. Of note, prasugrel has a more potent and consistent P2Y₁₂ inhibitory profile compared to clopidogrel.¹⁶ The present study showed a relationship between inhibition of platelet activation via ADP-P2Y₁₂ signaling and the symptoms in the thrombotic hindlimb ischemia model. Similar data were found

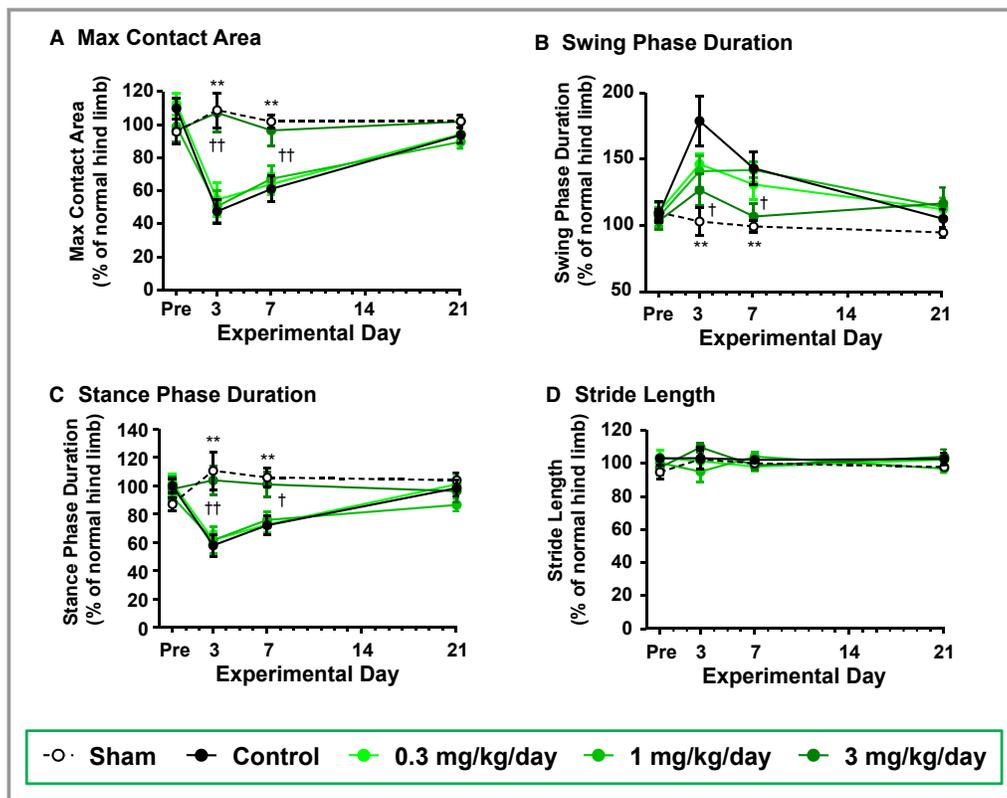


Figure 3. Effects of prasugrel on max contact area (A), swing phase duration (B), stance phase duration (C) and stride length (D) in mice with hindlimb injury. The gait parameters were measured by CatWalk and calculated as ratios (%) of the injured hindlimb value to the normal hindlimb value on Day -3 (pre) and on Days 3, 7, and 21 after the arterial injury. Data are presented as mean \pm SE ($n=10$). * $P<0.05$, ** $P<0.01$ vs control group (Student t test). $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs control group (Dunnett's test).

in P2Y₁₂ deficient mice. Taken together, these data suggest that prasugrel, by providing more optimal P2Y₁₂ blockade,¹⁶ could potentially reduce both cardiovascular and peripheral ischemic events in patients with PAD.

To date, PAD/CLI models such as multivessel ligation, vessel excision, and lauric acid injection have been widely used in nonclinical studies of PAD.^{17–19} Previous studies with these CLI models have reported improvements in blood flow, walking function, and/or gangrene of the ischemic limb, in response to a variety of antiplatelet agents such as thromboxane A₂ receptor antagonist,²⁹ 5-HT_{2A} receptor antagonists,^{30,31} phosphodiesterase 3 inhibitors,^{20,21} and P2Y₁₂ antagonists.^{19,32} However, in PAD patients, the complications of CLI are typically defined as severe rest pain and ischemic skin lesions,^{33,34} and many of the CLI animal models report severe necrosis at the periphery of the ischemic limb, presumably due to severe occlusion of the proximal arteries.^{19,35,36} Approximately 1% to 3% of PAD patients are clinically classified as having CLI, whereas 30% to 50% experience intermittent claudication and 50% to 70% are asymptomatic, with the latter group being classified as having “mild” PAD.⁵ Thus, the symptoms of the existing animal

models of PAD/CLI seem to be too severe compared to the symptoms experienced by the vast majority of PAD patients, and a PAD model that more closely reflects the clinical pathology of mild PAD is desirable. In the present study, we examined thrombotic hindlimb ischemia induced by the application of FeCl₃ to the femoral artery. We determined in this model that blood flow reduction and gait disturbances in the injured limb appeared to be secondary to platelet thrombus-induced vessel occlusion at the discrete site of FeCl₃ application, and no evidence of limb necrosis was found. While beyond the scope of the current study, some of the effects seen in this model may reflect neovascularization processes, including vasculogenesis, arteriogenesis, and angiogenesis, which would require further studies to evaluate. Alternatively, in contrast to the continuous recurrent leg muscle pain experienced by patients with mild PAD, our murine model of thrombotic hindlimb ischemia showed transient limb ischemia and walking dysfunction associated with an acute femoral artery thrombosis. However, limb ischemia without severe limb lesions observed in our model might be considered to reflect the situation in patients with mild PAD.⁵ Furthermore, the impact of P2Y₁₂ deficiency or

pharmacological antagonism in the present model were similar to clinical observations on the efficacy of ticlopidine in patients with intermittent claudication.^{8,9} Therefore, we believe our model mimics at least acute limb ischemia in patients with mild PAD. To our knowledge, the present study is the first to evaluate a thrombotic hindlimb ischemia model as a model of mild PAD.

The results of gait analysis using the CatWalk system showed that animals subject to our thrombotic hindlimb injury were ambulatory but had moderate difficulties in grounding and bearing weight on the injured limb. In previous studies, treadmill testing has been widely used for the evaluation of walking profiles in CLI animal models^{20,21} and demonstrated the difficulty of spontaneous walking due to the severity of the limb injury. Thus, forced walking on the treadmill was commonly used for functional evaluation in these animal models of CLI. The CatWalk system allows for the detection of subtle changes in natural walking.^{22,23} Such profiles proved of interest in gait evaluation in the current model. Intermittent claudication in patients with PAD is caused by the ischemic pain from insufficient blood flow in the iliac and femoral arteries.³⁷ If ischemic pain occurs in the hindlimb, grounding and weight bearing of the limb would be difficult. Our thrombotic hindlimb ischemia model showed a decrease in contact area with the floor and contact duration for the ischemic limb. In particular, maximum contact area and stance phase duration of the ischemic limb were reduced, and swing phase duration of the ischemic limb was increased, while stride length of the ischemic limb did not alter in our model. Vrinten and colleagues showed that paw placement-related parameters of the injured limb such as contact area and contact intensity were reduced in their chronic constriction injury model, a neuropathic pain model.²⁴ Their model also showed prolongation of swing phase duration and shortening of stance phase duration in the injured limb.²⁴ Parvathy and colleagues evaluated gait disturbances in an inflammatory pain model.²⁵ Similar to the neuropathic pain model, grounding and weight bearing of the injured limb were reduced in their complete Freund's adjuvant-induced arthritis model. However, the stride length of the injured limb did not change significantly compared to that of the normal limb in their model.²⁵ Thus, the changes in gait in our model were similar to those in these 2 pain models. Analgesic treatment could be a possible future approach to determine whether the gait abnormalities in the present thrombotic limb ischemia model are caused by pain. Alternatively, it could be argued that the changes in gait are caused by acute femoral nerve injury due to the FeCl₃ application. However, to the best of our knowledge, prasugrel shows selective P2Y₁₂ inhibition and has no direct neuroprotective effect. Thus, prasugrel is likely to inhibit the gait disturbances via the inhibition of thrombotic ischemia. Our

results demonstrated the usefulness of using the CatWalk system for the study of moderate gait abnormalities associated with limb ischemia.

In the present study, we used laser speckle blood flow imaging, which is a noninvasive blood flow measurement technique utilizing principles similar to those of laser Doppler imaging,³⁸ to evaluate hindlimb ischemia. However, laser speckle imaging captures blood flow images in a much shorter time but has relatively low spatial resolution compared with laser Doppler imaging. Since there are no reports that compare blood flow in the murine hindlimb by each technique, further study is required to determine the differences, if any, in results obtained by each technique.

In conclusion, acute femoral artery thrombosis resulted in hindlimb ischemia and moderate gait abnormalities in a murine model. Our data further suggest a possible role of P2Y₁₂ in the complications with thrombotic limb ischemia and the therapeutic potential of P2Y₁₂ antagonism in PAD.

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Disclosures

Jakubowski is an employee and minor shareholder of Eli Lilly and Company. Other authors are employees of Daiichi Sankyo Co., Ltd.

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