Inhibition of platelet aggregation by polyaspartoyl *L*-arginine and its mechanism[1]

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KEY WORDS platelet aggregation; bleeding time; platelet adhesiveness; polyaspartoyl *L*-arginine; nitric oxide; cyclic AMP; Thromboxane B_2; 6-ketoprostaglandin F1 alpha

ABSTRACT

AIM: To observe the oral anti-platelet efficacy and the potential action mechanism of polyaspartoyl *L*-arginine (PDR), a new *L*-arginine rich compound. **METHODS:** Platelet aggregation was conducted by Born's method; bleeding time was determined using tail's bleeding time in mice; platelet adhesion was carried out with glass bottle method; nitric oxide (NO) was tested with Griess' method; and cAMP, thromboxane B_2 (TXB_2) and 6-keto-$PGF_{1\alpha}$ were assessed with commercial kits. **RESULTS:** The inhibition by PDR (15-60 mg/kg ig or 10 mg/kg iv) of platelet aggregation induced by adenosine diphosphate (ADP), collagen or thrombin at 1 h after oral administration or at 20 min after iv injection for rats ($P<0.01$), and its (15 mg/kg, ig) inhibition of ADP-induced platelet aggregation for rabbits during 6 h after administration were observed. PDR (15-60 mg/kg) prolonged the bleeding time of mice ($P<0.05$) and (30 mg/kg) increased NO concentration in plasma. On the other hand PDR did not change the contents of cAMP in platelet and TXB_2 or 6-keto-$PGF_{1\alpha}$ in plasma. **CONCLUSION:** PDR is a novel, oral effective platelet aggregation inhibitor and its action mechanism possibly related to increasing NO generation.

INTRODUCTION

There have been several anti-platelet agents for long term use to prevent thrombosis in clinic, among which acetyl salicylic acid (ASA) has been most widely used and exhibited confirmed efficacy in the prevention of thrombotic complications associated with cardiovascular diseases. However the use of ASA is limited by some deficiencies such as high dose related anti-platelet action lesion and the aggravation of asthma, *etc*. Though a lot of attempts have been given to develop orally effective novel platelet aggregation inhibitor, for example TXA_2 synthase inhibitor, TXA_2 receptor antagonist, or GPIIb/IIIa antagonists, *etc*, no platelet inhibitor superior to ASA has ben found so far[1,2]. Therefore, orally effective novel anti-platelet agents with less side-effect remain to be awaited.

As a key molecule in the regulation of vascular diseases and homeostasis endothelium-derived nitric oxide (NO) has been well recognized. In blood vessel endothelial cells NO is formed from *L*-arginine and oxygen with the low output endothelial NO synthase (eNOS) as the catalyst[3]. Furthermore in platelet there is also a constitutive calcium-dependent NO synthase which is activated by collagen-induced platelet aggregation and leads to NO synthesis from *L*-arginine. NO in turn in-

[1] This work was supported by National New Drug Developed Funds, National Economy and Trade Ministry Funds and North China Pharmaceutical Groups New Drug R & D funds.
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Received 2002-11-25 Accepted 2003-07-04

creases cyclic 3',5'-guanosin monophosphate (cGMP) level and down-regulates platelet aggregation. *L*-arginine reduces platelet aggregation response both in hypercholesterolemic rabbits[3] and healthy men after orally administration[5], and decreases platelet aggregation in human subjects by infusion, but its effective dose is very high (30 g per person≈0.5g/kg), which induces hypotension meanwhile[6].

So far there has not been any anti-platelet agent which enhances NO level except *L*-arginine in clinic. Therefore, polyaspartoyl-*L*-arginine (PDR, Fig 1, M_r 33 200) in which the *L*-arginine residue was abundant, was designed and synthesized to increase *L*-arginine related potency and availability. In the present study the effect of PDR on platelet function and its possible action mechanism were investigated.

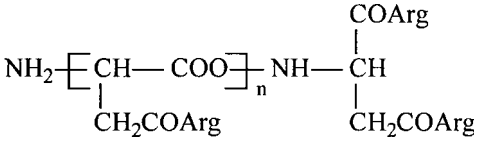

Fig 1. Structure of polyaspartoyl *L*-arginine (PDR).

MATERIALS AND METHODS

Animals Male Wistar rats (Grade II, Certificate No scxk-11-10-0006) were ordered from Experiment Animal Institute of Chinese Academy of Medical Sciences. Male SD rats (Grade SPF, Certificate No scxk 11-00-0008) and male ICR mice (Grade II, Certificate No scxk-11-00-0008) were supplied by Experiment Animal Center of Peking University; Male New Zealand white rabbits (Certificate No 2000-017) were purchased from Chinese Institute for Certified Drug and Biological Products, Beijing, China.

Drugs and reagents PDR synthesized by Sino-Germany United Laboratory, School of Pharmaceutical Sciences, Peking University was dissolved in de-ionized water for oral administration or in normal saline for intravenous injection. ASA produced by Astra Pharmaceutical Co, Wuxi, China was prepared freshly to an aqueous solution (1.5 g/L). Dipyridamol (DPD) was obtained from Tianjing Lisheng Pharmaceutical Company and suspended in water. Adenosine diphosphate (ADP) and thrombin purchased from Sigma Co were dissolved in normal saline. Collagen solution was freshly prepared from rat skill as described in literature[7], collagen protein content was determined by Coomassie's method, ^{125}I-assay kit for thromboxane B_2 (TXB_2) or 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) were obtained commercially from Dongya Biotech Institute, Beijing. [^{3}H]cyclic-adenosine monophosphate (cAMP) and related reagent were purchased from China Institute of Atomic Energy, Beijing.

Determination of bleeding time of mice Male ICR mice (20-24 g) were administered orally with PDR 15, 30, and 60 mg/kg, ASA 30 mg/kg or vehicle control. At 1 h after the administration 3 mm of the tail tip of the animal was cut off and blood drop was dripped on filter paper. The duration of bleeding was recorded[8].

Platelet aggregation *ex vivo* study in rats Male Wistar rats weighing 250-300 g were dosed orally or intravenously with PDR, *L*-arginine, ASA, or control. Blood samples from the rats anesthetized with sodium pentobarbital were collected at 1.5 h after oral dose or at 20 min after iv injection, and mixed with 0.1 volume of tri-sodium citrate (129.2 mmol/L) and PRP or PPP was prepared as previous method[9]. PRP was adjusted with PPP to about 2×10^{11}-3×10^{11} platelets/L. The platelet aggregation induced by ADP (final concentration: 1-5 mol/L); thrombin (final concentration: 60 to 70U/L) or collagen (final concentration: 40-50 mg/L) was recorded on a dual sample aggregation meter (Sienco. DP-247E) according to Born's method[10]. The anti-platelet efficacy was evaluated by comparing maximum aggregation response of PDR or ASA groups with that of control group.

Time course of *ex vivo* platelet aggregation in rabbits Male New Zealand rabbits weighing 2.5-3.5 kg were administered with placebo capsules or PDR capsules (30 mg/kg, ig). The arterial blood was collected at 0, 1, 2, 4, and 6 h after administration and mixed with 0.1 volume of sodium citrate (129.2 mmol/L). ADP-induced platelet aggregation was conducted as described above.

Assay of TXB_2 and 6-keto-$PGF_{1\alpha}$ in rat plasma Male Wistar rats weighing 250-350 g were anesthetized at 2 h after the administration of PDR, ASA, or control. Blood samples were collected and mixed with 0.1 volume of sodium citrate (129.2 mmol/L). The concentration of TXB_2 and 6-keto-$PGF_{1\alpha}$, the stable metabolites of TXA_2 and PGI_2, in plasma were determined with RIA kits, respectively.

Assay of cAMP in platelet and NO in plasma Blood samples were collected from anesthetized rats and mixed with 0.1 volume of 2 % edetate-Na_2. PRP was prepared and platelet pellets were obtained from

exact 1.0 mL of PRP by centrifugation at 1300×*g* for 10 min, the supernatant was saved for NO assay. cAMP in platelet was extracted by the method in the literature[11], and the content was determined with radio-immunological assay, NO concentration in the supernatant was assessed as Griess method with kits.

Statistical analysis Data were expressed as mean±SD. The difference between groups was analyzed by Student's unpaired, two-tailed *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

The bleeding time of the mice treated with PDR 15, 30, or 60 mg/kg was significantly prolonged ($P<0.05$) and this effect of PDR is similar to that of ASA (Fig 2).

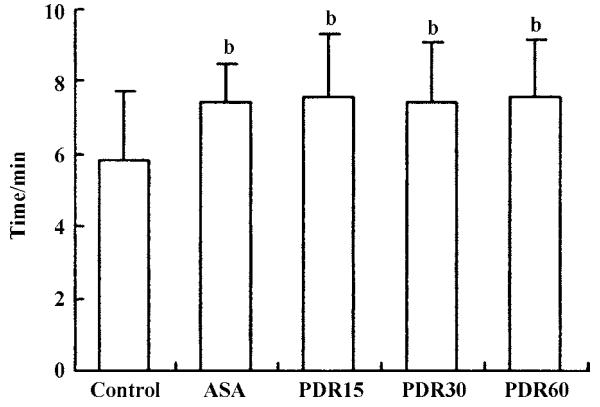

Fig 2. Effect of PDR on bleeding time in mice. Control (H_2O, n=13), ASA 60 mg/kg (n=10); PDR 15 (n=10), 30 (n=13), or 60 mg/kg (n=10) were given ig and the bleeding time was represented by the duration of tail dripping blood. $^bP<0.05$ *vs* control.

At 1.5 h after the administration (30 or 60 mg/kg), PDR significantly inhibited rat platelet aggregation induced by ADP, collagen, or thrombin ($P<0.01$), PDR 15 mg/kg inhibited collagen- or thrombin-induced but not ADP-induced platelet aggregation. At the same dose (30 mg/kg) the effect of PDR was similar to ASA (Tab 1). Twenty min after intravenous injection, PDR 10 mg/kg significantly decreased aggregation response ($P<0.01$), but the same dose of *L*-arginine did not show evident influence on aggregation (Tab 2). In the time course *ex vivo* study of rabbits the initial anti-aggregation effect was observed at 1 h after treatment with

Tab 1. Effect of PDR on *ex vivo* platelet aggregation in rats. n=10. Mean±SD. $^cP<0.01$ *vs* control.

Group	Dose/ mg·kg^{-1}	Max aggregation rate/% ADP	Collagen	Thrombin
Control	-	81±11	64±13	70±10
ASA	30	28±18^c	12±14^c	7±7^c
PDR	15	66±23	27±23^c	16±18^c
	30	23±15^c	10±10^c	11±10^c
	60	17±13	11±9^c	15±12^c

Tab 2. Effect of PDR and *L*-arginine on ADP- induced platelet aggregation. n=10. Mean±SD. Dose=10 mg/kg (iv). $^cP<0.01$ *vs* control.

Groups	Aggregation rate/%
Control	75±8
L-Arg	73±23
PDR	46±12^c

PDR (po) 15 mg/kg, the effect reached its peak value at 2 h after treatment, and the aggregation activity restored at 6 h after treatment (Fig 3).

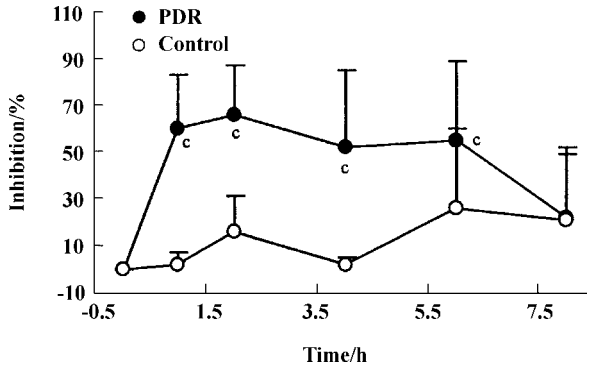

Fig 3. Effect of PDR on ADP-induced platelet aggregation-time course study in rabbits. PDR 15 mg/kg or control were ig. n=8. Mean±SD. $^cP<0.01$ *vs* control.

After administration of PDR 30 mg/kg, the concentration of NO in plasma was significantly increased and the content of cAMP in platelets and the concentrations of 6-keto-$PGF_{1\alpha}$ in plasma were slightly enhanced (Tab 3) , but the concentration of TXB_2 in the plasma was not changed (Tab 4).

Tab 3. Effect of PDR on cAMP in platelet and NO in plasma of rats. Agents were ig at 1 h before the blood collected; dose=30 mg/kg. *n*=10. Mean±SD. $^{b}P<0.05$ *vs* control.

Groups	cAMP/nmol·L^{-1} PRP	NO/μmol·L^{-1}
Control	32±12	41±19
PDR	49±21	65±19^{b}
Dipyridamol	45±22	63±15^{b}

Tab 4. Effect of PDR on TXB_2 and 6-keto-$PGF_{1\alpha}$ in plasma of rats. Agents were ig at 1 h before the blood collected, dose=30 mg/kg. *n*=10. Mean±SD. $^{c}P<0.01$ *vs* control.

Group	TXB_2/μg·L^{-1}	6-keto-$PGF_{1\alpha}$/μg·L^{-1}
Control	0.43±0.08	0.43±0.25
PDR	0.38±0.05	0.70±0.55
ASA	0.23±0.06^{c}	0.14±0.09^{c}

DISCUSSION

PDR is an *L*-arginine rich compound. The present work demonstrated that PDR inhibited platelet aggregation of rats or rabbits, prolonged the bleeding time of mice, suggesting that this compound inhibit platelet function. Platelet function is regulated through various factors, for instance, arachidonic acid metabolism, cyclic nucleotides level, calcium ion concentration in it and its membrane receptor activation, *etc*[2]. Our preliminary observations including the significant increase of NO concentration in plasma, slight increase of 6-keto-$PGF_{1\alpha}$ concentration in the plasma and cAMP content in the platelets, and no influence on TXB_2 suggested that the anti-aggregation of PDR may be a combination result of a series of factors, and the influence of *L*-arginine NO-cGMP pathway may be more important.

Based on the findings that *L*-arginine exerted its anti-platelet aggregation efficacy in healthy men or pregnant women through a nitric oxide-dependent synthesis of cGMP[6,12,13] and endogenous NO release modulated mural platelet thrombosis[14], the abundant *L*-arginine residues in PDR which may release *L*-arginine and generate NO via the stimulation of vascular endothelial cell or platelet was given partial speculation to understand the action mechanism of PDR.

An observation in our data is that at the same dose (10 mg/kg iv) PDR but not *L*-arginine significantly inhibits platelet aggregation. *L*-Arginine inhibits platelet aggregation only at high dose (about 500 mg/kg infusion)[6]. These results suggest that polyaspartic acid moiety in PDR may contribute to its effect and the availability of *L*-arginine in PDR has been enhanced. In addition, PDR may be safe (its maximum tolerant dose is more than 5 g/kg in mice ig).

In conclusion, PDR is a novel, oral effective, and safe platelet aggregation inhibitor, and its action mechanism may be related to *L*-arginine NO-cGMP pathway. Its effect on thrombosis, safety, and detailed action mechanism deserve to be investigated further.

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