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Polyaspartoyl·L-arginine inhibits platelet aggregation through stimulation of NO release from endothelial cells

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ABSTRACT

Polyaspartoyl·L-arginine (PDR) is an inhibitor of platelet aggregation *ex vivo* but *in vitro*. This study attempts to elucidate the target cell of PDR action and its action mechanism. PDR (1.7–170 μg/ml) significantly inhibited platelet aggregation *in vitro* in the presence of rat aortic endothelial cells (RAEC), NO synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) inhibited this effect, but it was ineffective in the RAEC absence. Correspondingly, PDR increased NO level in the supernatants of the platelet reactants in RAEC presence, but failed to influence NO level in RAEC absence, and these effects of PDR were more potent than those of L-arginine. Furthermore, PDR markedly elevated the intracellular level of L-arginine, and it (17–170 μg/ml) also augmented L-citrulline level in RAEC, argininosuccinate lyase (ASL) inhibitor succinate enhanced its effect on L-citrulline but L-NAME weakened it. 170 μg/ml of PDR slightly increased the L-aspartate level in RAEC, and succinate enhanced this effect. However L-arginine, L-aspartate or the combination of L-arginine and L-aspartate failed to change levels of these amino acids. In addition, PDR (170 μg/ml) stimulated the expression of argininosuccinate synthetase (ASS) protein. In conclusion, the endothelial cell is direct target cell of PDR's action; PDR facilitates the entry of L-arginine by serving as a carrier of L-arginine into RAEC; it also supplies aspartic acid and stimulates ASS expression, and then enhances the intracellular citrulline–NO cycle, thus increases the availability of L-arginine and NO synthesis. Therefore the effect of PDR on platelet aggregation is primarily attributed to its stimulation of NO synthesis in endothelial cells; PDR may be a better cardiovascular protective agent than L-arginine.

1. Introduction

Platelet activation and aggregation play a key role in the pathogenesis of thrombosis (Fitzgerald et al., 1986), which is directly associated with endothelial function. Adherence of platelets to a defective endothelial cell monolayer, at the site of injury, causes the release of potent vasoconstricting agonists such as thromboxane A_2 and serotonin from platelets activated with sub-endothelial stimuli, including collagen. To prevent these adverse effects, endothelial cells physiologically release vasodilatory and anti-aggregatory agents, e.g., nitric oxide (NO) and prostacyclin (Radomski et al., 1987; Az-ma et al., 1995). Platelets per se also produce NO (Radomski et al., 1990). NO is known to attenuate platelet activation (Brune and Hanstein, 1998; Mellgren et al., 1998) and inhibit platelet aggregation *in vitro* (Kurata et al., 1997) and *ex vivo* (Cheung et al., 1998). Studies have shown that endothelium-derived NO inhibits platelet adhesion to endothelial cells (Radomski et al., 1993), and clot formation in thromboelastography studies(Dambisya and Lee, 1996).

L-arginine, the sole physiological precursor, provides a guanidino nitrogen group for NO synthesis through nitric oxide synthase (NOS) (Ignarro et al., 1987); it inhibits platelet aggregation through platelet nitric oxide synthesis (Marietta et al., 1997). The arginine–NO pathway of the endothelial cell is involved in the regulation of platelet function. In endothelial cells, there may be a separate pool of L-arginine directed to endothelial NOS (eNOS), the formation of NO from L-arginine is dependent upon an adequate and continuous supply of L-arginine (Ahlers et al., 2004). Intracellular L-arginine can be obtained from exogenous sources via cationic amino acid transporter or by endogenous synthesis. L-citrulline, which is formed from L-arginine by the NOS reaction, can be recycled into L-arginine through the citrulline–NO cycle. In the presence of L-aspartate, this recycling is accomplished by the successive actions of argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) (Hattori et al., 1994; Hecker et al., 1990). It has been demonstrated that the citrulline–NO cycle may help to maintain a sufficient intracellular concentration of L-arginine for NO generation (Wu and Meininger, 1993; Hecker et al., 1990). ASS, the rate-limiting enzyme of the citrulline–NO cycle, has been found to be co-localized with eNOS in the caveolae of endothelial

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cells. Therefore, it is hypothesized that the regulation of ASS activity can manipulate NO synthesis via eNOS (Flam et al., 2001).

Polyaspartoyl·L-arginine (PDR), a synthesized L-arginine residue-rich compound with polyaspartate as the supporting molecular main chain, was recently reported to inhibit platelet aggregation *ex vivo* (Wang et al., 2004) but not *in vitro*(unpublished data), and reduced arterial thrombosis *in vivo* (Tang et al., 2003). Based on its behavior on platelet aggregation, the effects of PDR are most likely mediated by certain endogenous factors. This study attempted to define the target cell of PDR action and to explain its inhibition of platelet aggregation and anti-thrombotic effect by investigating (1) the influence of endothelial cells on the PDR's effect on platelet aggregation *in vitro*, (2) the effect of PDR on NO level in platelet reaction supernatant with or without rat aortic endothelial cells (RAEC), (3) the effects of PDR on intracellular concentration of L-arginine and related amino acids in RAEC, (4) the influence of some related enzymes on PDR's effects.

2. Materials and methods

2.1. Materials

PDR was synthesized by our colleagues at The Laboratories of Hydrone and Peptides in Capital University of Medical Sciences, and the light brown powder (purity is 98.8%) was dissolved in normal saline before use. Trypsin, EDTA-Na_2 and thrombin were Sigma Co. products; thrombin dissolved in normal saline before use. Medium 1640 was a GIBCO product; fetal calf serum was obtained from Tianjin Caihui Biochemical Product Factory; Penicillin G was a product of North China Pharmaceutical Corporation; Streptomycin was obtained from Dalian Meiluoda Pharmaceutical Factory; Cell lysis buffer was the product of Beyotime Biotechnology, China. Other chemicals and agents were obtained in the commercially available quality. Collagen in rat-tail was self-prepared as previously described (Wang et al., 2004). Sprague–Dawley rats were obtained from the Experimental Animal Center of Peking University.

2.2. Endothelial cell culture

Endothelial cells were obtained from rat aortas and subcultured as described by others (Centra et al., 1992). Briefly, Male Sprague–Dawley rats weighing 180–200 g were anesthetized with an overdose of sodium pentobarbital and the abdominal aortas of rats were rapidly removed and collected in medium 1640. Surrounding fat and connective tissue were cleaned off, and then aortas were cut longitudinally. The aortic endothelium were scraped with vertical ophthalmic forceps and the cells were collected into a T25 polystyrene flask, then cultured initially in medium 1640 containing 20% new born calf serum and 100 U/ml penicillin-100 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. The endothelial cells were allowed to grow undisturbed for 3–4 days and thereafter the media was changed once every 2 days for a total culturing period of 8 to 10 days. All monolayer were initially identified as endothelial cells by phase-contrast microscopy. The cell culture purity (98%) was assessed by staining for factor VIII antigen, as previously described (Jaffe et al., 1973). Confluent cells were passaged by trypsinization in D-Hank's containing 0.05% trypsin and 0.02% EDTA. Passage 4–6 cells were used in experiments. The incubation medium was changed to serum-free medium 1640 at 24 h before experiment.

2.3. Platelet aggregation activity in vitro

2.3.1. Preparation of washed platelets

After an overnight fasting, the blood from normal rabbits was collected in a plastic syringe containing 1/10 volume of 2% EDTA Na_2. Platelet-rich plasma was prepared by centrifuging the blood samples at room temperature for 10 min at 200 ×g. the washed platelets were prepared as the reported (Mikashima et al., 1987). Shortly, the upper layer was collected and diluted with the same volume of platelet-washed buffer and centrifuged at 1200 ×g for 10 min. The platelet pellets were then re-suspended and washed three times. The final pellets were suspended with platelet diluted buffer (1.17 mM $CaCl_2$, 0.1% BSA in the platelet washed buffer) and diluted to 2×10^8 platelets/ml, and stood at room temperature for use.

2.3.2. Pre-treatment of endothelial cells and platelets

The influence of endothelial cell on PDR's effect on platelet aggregation was examined as previously described (Igawa et al., 1990; Macdonald et al., 1988) with modification. In brief, monolayer RAEC in flasks were trypsinized and prepared into 2×10^6/ml of cell suspension with 1% serum-containing medium 1640 then pre-incubated with or without 100 μM of *N*-nitro-L-arginine methyl ester (L-NAME) for 24 h. Dispensed 50 μl cell culture medium, with or without endothelial cells, into each siliconized aggregate cuvettes, then incubated at 37 °C under an atmosphere of 5% CO_2. After a 4 h recovery incubation, the cuvettes were treated with NS or NS-containing agents (10 μg/ml of SNP, 170 μg/ml of L-Arginine, 1.7, 17 or 170 μg/ml of PDR) and stood for 30 min at 37 °C, then 200 μl of platelet suspension was then added into the cuvettes for platelet aggregation study.

2.3.3. Platelet aggregation assay

Platelet aggregation was performed on Chrono-log model 490 optical aggregometer as Born's method. The aggregation was induced using 0.2 IU/ml of thrombin. The percent inhibition of platelet aggregation was calculated according to the following formula: Inhibition (%)=$(A-A_1)/A\times100\%$, where A was the maximum light transmission of the vehicle group and A_1 was the maximum light transmission of each sample after treatment with the agents. After ending the test, the reaction mediums were centrifuged at 3000 ×g and the supernatant were immediately frozen and stored at −20 °C to determine the concentration of NO.

2.3.4. The determination of NO in the supernatant of platelet reaction mixtures

A sensitive fluorometric method for nitrite determination was used as previously described (Misko et al., 1993) to measure NO level in samples with minor modifications. Briefly, 100 μl of samples were placed into white opaque 96-well plates after thawing and centrifugation, then 10 μl of freshly prepared 2,3-diaminonaphthalene (0.05 mg/ml in 0.62 N HCl) was added and mixed immediately, then incubated for 15 min at room temperature. The reaction was terminated with 5 μl of 2.8 M NaOH and the plate was read on a Cary Eclipse luminescence spectrometer (excitation 360 nm, emission 440 nm). Standard curves were made daily with sodium nitrite, ranging from 0.04–10 μM, in Krebs–Henseleit buffer.

2.4. Intracellular amino acids' level in RAEC

2.4.1. Sample preparation

Confluent 4–6 passage endothelial cells, seeded into 6-well plates with 2×10^4 cells/ml, were used for the experiments. Each well was rinsed with serum free medium and equilibrated in the incubator (37 °C) for 30 min with 2 ml of Hank's balanced salt solution as other method (Su and Block, 1995). Then three sets of experiments were performed: in set 1 the cells were only treated with PDR and other agents; in set 2 the cells were pretreated with succinate (3 mM) for 30 min at 37 °C prior to PDR and other agents treatment; and in set 3 the cells were treated with L-NAME (100 μM) for 24 h at 37 °C prior to PDR and other agents treatment. The cells of each set were all treated with vehicle, L-arginine, L-aspartate, L-arginine plus L-aspartate or PDR for 30 min at 37 °C respectively, accompanied by A23187 (final concentration 1.0 μM) to observe the influences of PDR on eNOS

which is Ca^{2+} dependent. Succinate and L-NAME were applied to observe the influence of PDR on eNOS and the citrulline-NO cycle, respectively. The cell incubations were terminated by ice bath, the supernatants were immediately frozen and stored at −20 °C to determine the concentration of NO, its determination was performed as mentioned above (in *2.3.4*). To measure the intracellular AA levels, the monolayer cells were rinsed at least 5 times with cold PBS, collected with 200 μl of 96% methanol and were exposed to 3 cycles of freezing and thawing to lyse, then centrifuged at 10,000 ×g for 5 min at 4 °C. 100 μl of the supernatant was blown dry with nitrogen gas, then stored at −20 °C in order to measure the concentration of L-arginine and other amino acids. The cell residues were lysed again in lysis buffer and the supernatant was collected and stored at −20 °C for the measurement of protein after centrifuged at 10,000 ×g at 4 °C for 5 min. Protein concentrations were determined by Bradford method (Bradford, 1976) and used to normalize intracellular amino acid values.

2.4.2. Intracellular amino acids detection

Nitrogen gas-dried samples were thawed temporarily and the levels of L-arginine, L-citrulline and L-aspartate were determined by high-performance liquid chromatography according to published methods (Contreras et al., 1997; Sobrevia et al., 1998). Briefly, o-phthalaldehyde (OPA) solution was freshly prepared by dissolving 10 mg of OPA in 0.5 ml of methanol, and then 10 μl β-mercaptoethanol and 2 ml sodium tetraborate buffer (0.1 M, pH 9.4) were added. The test samples were dissolved in 200 μl mobile phase B, as mentioned below, containing 10 μM GABA as an internal standard. 30 μl of the sample was mixed with 30 μl OPA solution. After exactly 2 min, 20 μl of the mixture was immediately injected onto the spherisorb C18 ODS column(Waters 4.6×250 mm i.d. 5 μm) fitted with a security guard C18 ODS column (Phenomenex 4.6×30 mm i.d. 5 μm). Mobile phases consisting of 50 mM sodium acetate (pH=6.8): methanol: THF (Mobile phase A=82:17:1; Mobile phase B=22:77:1) were filtered through a 0.2 μm filter. Each component of the mobile phase was degassed ultrasonically before use. The following gradient systems were used: 0–1 min, isocratic with 5% mobile phase B; 1–8 min, linear gradient to 15% B; 8–14 min, linear gradient to 30% B; 14–19 min, linear gradient to 40% B; 19–20 min, increasing to 100% B; 20–34 min, isocratic with 100% B; linear reverse gradient to 5% B at 35 min. Between two consecutive samples, a 10 min wash-out was carried out with 5% B and then re-equilibrated. All separations were performed at 37 °C and at a flow-rate of 1.0 ml/ml using Agilent-1100 series HPLC. Fluorescent detection was accomplished by use of an excitation wavelength of 338 nm with emission detection at 450 nm. Amino acid concentrations were calculated from the peak areas by reference to the area of the internal standard GABA and normalized by protein contents. The limit of detection for all the amino acids measured was within the range of 10 pM.

2.5. Western blot analysis of argininosuccinate synthase

The 4–6 passage rat aortic endothelial cells were seeded into 6-well plates with 2×10^5 cells/ml and cultured until confluence. Endothelial cells were treated with vehicle, 1.7, 17 or 170 μg/ml of PDR for 24 h, respectively. After removal of media, cells were washed twice with ice-cold PBS, then lysed using cell lysis buffer. The lysates were collected by scraping from the plates and centrifuged at 10,000 ×g at 4 °C for 5 min, and the supernatants were stored at −20 °C for electrophoresis. For obtaining ASS protein control, 0.06 g of fresh rat liver was homogenized in 2 ml of cell lysis buffer and centrifuged at 10,000 *g* for 5 min at 4 °C and the supernatant was then collected for electrophoresis. Western blot was performed according to the procedure previously described (Towbin et al., 1979). Briefly, protein extracts were separated by electrophoresis (50 μg protein per lane) on a 12% SDS-polyacrylamide gel and transferred onto nitrocellulose transfer membranes (Osmonics, USA) at 0.8 mA/cm^2 for 2 h. Nonspecific activity was blocked in 5% fat-free milk in TBST (10 mM Tris–HCl, pH7.5, 150 mM NaCl, 0.1%Tween-20) for 1 h at room temperature. The membrane was then probed with a primary polyclonal mouse anti-ASS (1:1000) (Santa Cruz Biotechnology, USA) by incubation overnight at 4 °C, then washed in Tris buffer saline Tween (TBST, 50 mmol/l Tris/HCl, 150 mmol/l NaCl, 1% v/v Tween 20, pH 7.4), and incubated for 1 h in TBST/0.2% BSA containing horseradish peroxidase-conjugated goat anti-mouse antibody (1:200). Detection was performed by enhanced chemiluminescence (Santa Cruz Biotechnology, USA) and bands were then quantified by scanning densitometry (THERMAL IMAGING SYSTEM FTI-500, Pharmacia Biotech). Protein concentrations were determined by Bradford method. β-actin of rat aortic endothelial cells was used as a housekeeping protein, and determined following the same procedure mentioned above using a specific anti-actin mouse monoclonal antibody (1:1000) (Sigma–Aldrich, Madrid, Spain) and the horseradish peroxidase-conjugated goat anti-mouse antibody(1:200).

2.6. Statistical analysis

The results are expressed as mean±S.D. The difference between the treated groups and the control group was analyzed by Dunnet *t*-test. $P<0.05$ was considered to be a significant difference.

3. Results

3.1. Effect on platelet aggregation and NO synthesis in vitro

In washed rabbit platelets, PDR at the concentration of 1.7–170 μg/ml did not influence the platelet aggregation induced by thrombin and the NO level of the supernatant of reaction mixtures in the absence of RAEC, whereas in the presence of RAEC PDR at the

Table 1
Effects of PDR on platelet aggregation and NO level in platelet reactants with or without endothelial cells presence *in vitro* and the influence of NO synthase inhibitor L-NAME (mean±SD, $n=6$)

Groups	Inhibition % platelet aggregation			Nitrites (nmol/l)		
	EC (−)	EC (+)	EC+l-NAME	EC (−)	EC (+)	EC+l-NAME
Vehicle control	0±13.0	5.3±14.3	−6.2±27.1	41.0±20.8	43.9±15.4	37.0±21.0
PDR(μg/ml) 1.7	2.0±6.5	36.4±11.8[b, d]	6.8±8.0	40.9±30.5	108.2±13.4[b d]	57.0±19.5
17	−1.8±11.1	50.4±10.1[b d]	12.9±8.2	46.8±25.2	125.9±16.2b d[b d]	61.1±24.8
170	4.0±13.7	65.3±9.2[b d]	31.1±10.2[a]	45.2±30.0	145.7±19.1[b d]	77.4±15.5
L-Arginine 170	8.8±11.2	34.2±9.9[b d]	−1.5±.15.8	48.2±29.7	90.8±15.6[a c]	35.2±14.0
SNP 10 μg/ml	82.8±9.8 [b]	88.8±11.3[b]	79.0±9.7[b]	339.8±64.1[b]	321.9±94.0[b]	334.7±24.2[b]

EC (−): platelets without endothelial cells.
EC (+): platelets with endothelial cells.
EC+L-NAME: platelets with endothelial cells plus L-NAME.
[a]$P<0.05$, [b]$P<0.01$ compared *vs* vehicle control of the same treatment.
[c]$P<0.05$, [d]$P<0.01$ compared *vs* the corresponding treated group without RAEC.

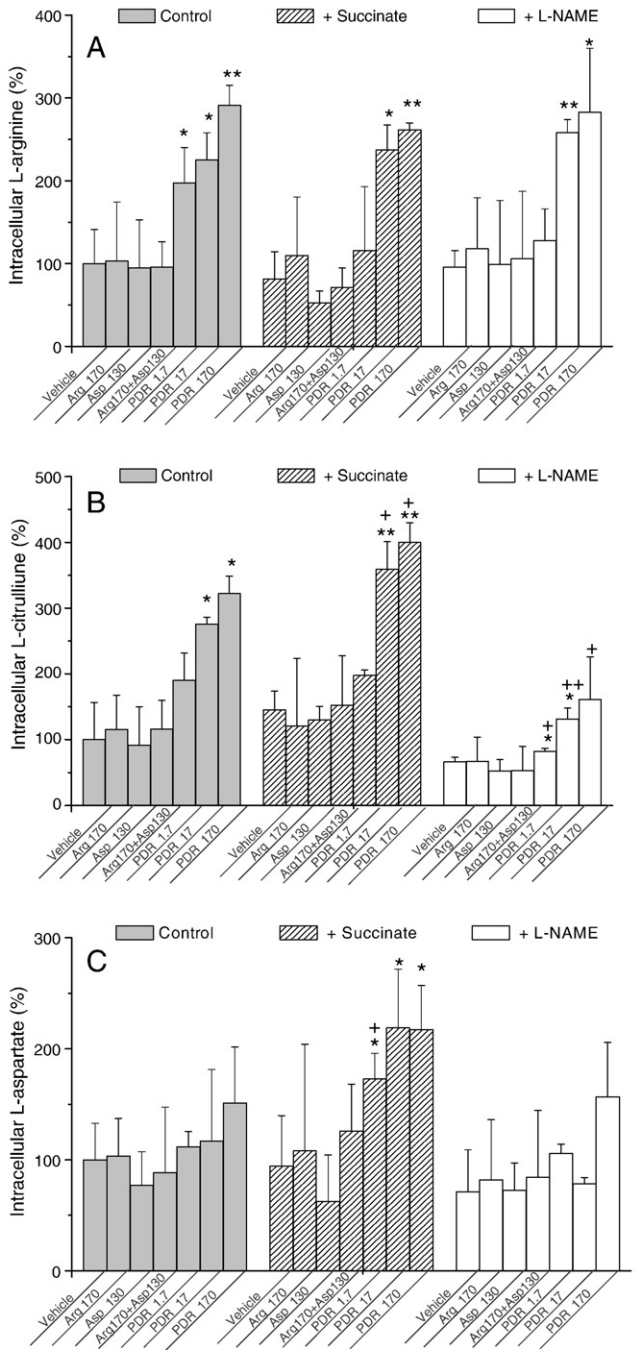

Fig. 1. The effects of PDR on intracellular amino acids level in cultured rat aortic endothelial cells (RAEC). The cells were pretreated with vehicle (Control), with succinate (+ succinate) for 30 min or with L-NAME (+L-NAME) for 24 h, and then were treated with vehicle or agents(μg/ml) for 30 min at 37 °C. L-arginine, L-citrulliune and L-aspartate in RAEC extracts were determined by HPLC respectively, and expressed as a percentage based on the mean value of vehicle group in the control, which was set at 100% (mean ± S.D., $n=3$). Panel A, B, C represented the L-arginine level, L-citrulliune level and L-aspartate level respectively. $^{*}P<0.05$; $^{**}P<0.01$ compared *vs* the intracellular amino acid of vehicle in the same set: $^{+}P<0.05$; $^{++}P<0.01$ compared *vs* intracellular amino acid of the same treated group in the control set.

same concentration range significantly inhibited the platelet aggregation and increased the NO level in the supernatant of reaction mixtures, the inhibition rates (%) on platelet aggregation for vehicle, 170 μg/ml of L-Arginine, 1.7, 17, 170 μg/ml of PDR treated group were 5.3 ± 14.3, 34.2 ± 9.9, 34.6 ± 11.8, 50.4 ± 10.1, 65.3 ± 9.2, respectively. Meanwhile, the NO levels (expressed by the concentration of nitrite) for vehicle, 170 μg/ml of L-Arg, 1.7, 17, 170 μg/ml of PDR treated group were 43.9 ± 15.4 nM, 90.8 ± 15.6 nM, 108.2 ± 13.4 nM, 125.9 ± 16.2 nM, 145.7 ± 19.1 nM, respectively. However, L-NAME (100 mM) markedly inhibited the effects on platelet aggregation and NO levels of these agents but SNP. The effects of L-arginine (170 μg/ml) on platelet aggregation and NO level were weaker than that of equal concentration of PDR. As expected, 10 μg/ml of SNP, a NO donor, significantly inhibited the platelet aggregation and enhanced the NO level of the supernatant of reaction mixture, L-NAME failed to influence its effects (Table 1).

3.2. Effects of PDR on intracellular amino acid Levels

The intracellular contents of L-arginine, L-citrulliune and L-aspartate are exhibited in Fig. 1. L-arginine, L-citrulliune and L-aspartate levels were expressed based upon their mean values of vehicle in set 1 which was set at 100%. The intracellular contents of L-arginine, L-citrulliune and L-aspartate were not significantly changed in the cells treated by L-arginine, L-aspartate or the combinatin of L-arginine and L-aspartate which was designed as the same proportion based on their contents in PDR. However 1.7, 17, 170 μg/ml of PDR significantly increased intracellular L-arginine level, which were 1.97 fold, 2.25 fold, 2.91 fold of vehicle control, respectively. Similarly PDR also evidently increased the level of intracellular L-citrulline, and its high concentration slightly increased the intracellular L-aspartate level. The Citulline-NO cycle inhibitor succinate did not influence the effect of PDR on intracellular L-arginine and L-citrulline levels, but markedly increased its effect on the intracellular L-aspartate level. NOS inhibitor L-NAME, significantly weakened the effect of PDR on the intracellular L-citrulline level.

3.3. Effect of PDR on NO release from RAEC

PDR increased NO level in the culture medium of RAEC in a concentration dependent manner, 170 μg/ml of it showed a significant increase. Both the NO-citrulline cycle inhibitor succinate and the NOS inhibitor, L-NAME, evidently attenuated the effect of PDR on the release of NO (Fig. 2).

3.4. Effect of PDR on argininosuccinate synthase protein expression

Western blot analysis using a polyclonal antibody against argininosuccinate synthetase to detect argininosuccinate synthase protein level. Samples containing ASS protein from rat liver tissue were used as a positive control according to another study (Xie and Gross, 1997) and a marked band corresponding to ASS was detected which corresponded to the molecular mass of one of the identical

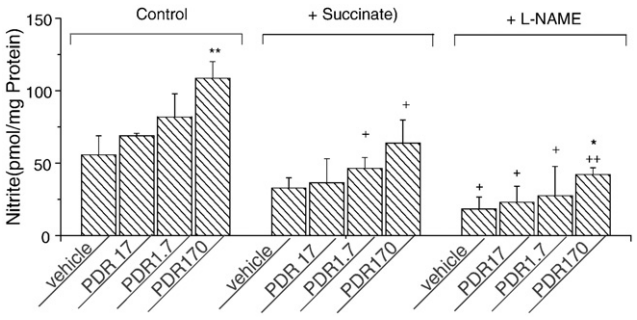

Fig. 2. The effects on NO level in cultured medium in rat aortic endothelial cells. The cells were pretreated with vehicle (set 1), succinate (set 2) or L-NAME (set 3), and then were treated with vehicle or agents (μM) as described in Fig. 3. The culture mediums were saved for NO measurement. Contents of nitrite were used to express the NO levels in the supernatants. Values are expressed as mean ± SD ($n=3$), $^{*}P<0.05$; $^{**}P<0.01$ compared *vs* the nitrite of vehicle in the same set. $^{+}P<0.05$; $^{++}P<0.01$ compared *vs* the nitrite of the corresponding treated in control set.

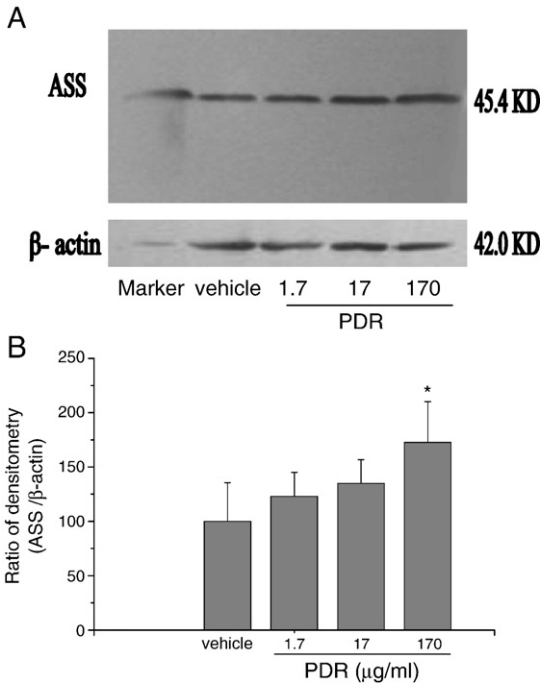

Fig. 3. Effect of PDR on ASS protein expression in cultured rat aortic endothelial cells. The cells were treated with PDR for the indicated periods and the cell extracts were subjected to Western blot analysis. A) The shown was one result of 3 separate Western blot experiments. Line 1 was loaded with liver extracts (2 μg of protein) as the ASS marker, Line 2–5 were loaded extracts (50 μg of protein) of cells. The bands of ASS and β-actin were identified using an anti-ASS mouse polyclonal antibody (1:1000) and an anti-actin mouse monoclonal antibody (1:1000), in which β-actin was used as a housekeeper. B) The statistic analysis results of ratio of ASS compared to β-actin were depicted as mean±S.D. (n=3), * P<0.05 *vs* vehiche groups.

subunits of the tetrameric ASS (45.4 kDa, as calculated from the amino acid composition). The cells treated by PDR exhibited a concentration dependent increase on the ASS protein level. 1.7–17 μg/ml of PDR showed a tendency to increase the ASS protein level, 170 μg/ml of PDR increased the ASS level significantly (Fig. 3).

4. Discussion

Since Furchgott and Zawadski first suggested the existence of endothelium-derived relaxing factor (Furchgott and Zawadzki, 1980), a large number of experimental studies have proven the important role that NO plays in the function of the cardiovascular system. L-arginine, the sole physiological precursor of NO, has a beneficial effect on endothelium dependent vaso-reactivity, as well as on the interaction between the vascular wall, platelets and leucocytes. Therefore, individuals with risk factors for atherosclerosis and patients with coronary artery disease or heart failure could benefit from therapy with L-arginine (Goumas et al., 2001).

PDR, an L-arginine residue-rich compound, has been reported to inhibit platelet aggregation *ex vivo* in rabbits or rats (Wang et al., 2004) and prevent arterial thrombosis in rats with raising NO level in serum (Tang et al., 2003). It did not, however, inhibit platelet aggregation *in vitro* (unpublished data). This study investigated the influences of PDR on platelet aggregation and on NO release *in vitro* in the presence and in the absence of RAEC. The results in this study showed that only when RAEC existed PDR inhibited platelet aggregation and increased NO synthesis, it is indicated that the endothelial cell is the intermediary target of PDR and the primary action of PDR is on endothelial cells but not on platelets. These effects of PDR were blocked or attenuated by L-NAME, suggesting its role of anti-platelet aggregation is attributed to its stimulating eNOS to enhance NO synthesis and release.

L-arginine was described to inhibit platelet aggregation *in vitro* at high concentration (Anfossi et al., 1999), in this study PDR up to 170 μg/ml did not inhibit platelet aggregation, but 170 μg/ml of L-arginine exhibited somewhat inhibitory effects in the absence of endothelial cells *in vitro*. However, in the presence of endothelial cells, PDR displayed a more potent inhibitory effect than L-arginine, suggested that the target of these two agents is slightly different, namely PDR only targets endothelial cells but L-arginine targets both platelets and endothelial cells.

In this study ASL inhibitor succinate evidently blocked PDR's effect on NO release in RAEC, indicating that PDR participates in the NO-citrulline cycle; NOS inhibitor L-NAME displayed more potent inhibitory effects on NO synthesis of PDR, suggesting the effect of PDR is primarily dependent on NOS.

To explore the details of PDR's effect on increasing the NO level, the levels of L-arginine, L-citrulline or L-aspartate in endothelial cells were determined. We failed to detect PDR's peak in RAEC extracts with HPLC, denoting that the entire molecule of PDR may not be able to enter into endothelial cells. The facts that levels of L-arginine and L-citrulline in RAEC were significantly raised by PDR, but were not changed by other agents such as L-arginine, L-aspartate or the combination of L-arginine and L-aspartate, suggested that free L-arginine or L-aspartate may not be easily taken in by endothelial cells, but that polyaspartoyl acid, acting as a carrier, may facilitate the uptake of L-arginine.

Several factors, like the intracellular compartmentalization of eNOS, L-arginine's uptake system and the multi-purposes of L-arginine, limit the intracellular L-arginine availability for eNOS *in vivo*. Accordingly L-arginine's oral or intravenous infusion dose that produced beneficial effects in the cardiovascular system was quite high, at a range of 6–30 g/day (Goumas et al., 2001; Kanno et al., 1992). The daily doses of L-arginine utilized in these studies exceeded the physiological uptake by 3–8 times (Visek, 1986). Based on this study, PDR may provide an easier absorbed and higher available L-arginine for eNOS compared with natural L-arginine, so the NO concentration produced by PDR on platelet aggregation is much higher than that of L-arginine.

Acting as the carrier molecule, containing L-arginine and L-aspartate components, PDR may improve the availability of L-arginine not only by providing the L-arginine component, but also by providing the L-aspartate component. This study showed that PDR slightly increased the intracellular aspartic acid level and the increase was enhanced when the citrulline–NO cycle was blocked by succinate, but other agents did not change the L-aspartate level in the presence of succinate (Fig. 1), indicating that the L-aspartate from PDR also participates in the citrulline-NO cycle with L-arginine, thus stimulating the recycle of L-arginine.

ASS was first identified as the rate-limiting enzyme of the urea cycle in the liver 56 years ago (Ratner and Petrack, 1951) and has more recently been recognized as a rate-limiting enzyme in the citrulline–NO cycle (Xie and Gross, 1997). It catalyses the reversible ATP-dependent condensation of citrulline with aspartate to form argininosuccinate, which is the immediate precursor of L-arginine, leading to the synthesis of NO in endothelial cells (Husson et al., 2003). In this study, PDR in 170 μg/ml significantly increased the expression of ASS in RAEC (Fig. 3), which also enhanced the citrulline–NO cycle and contributed to the increase of synthesis of NO.

In conclusion, this study demonstrated that the endothelial cell is the direct target cell of PDR's action on platelet aggregation; PDR facilitates the entry of L-arginine by serving as a carrier molecule of L-arginine into RAEC; it also supplies aspartic acid and stimulates ASS expression which then enhances the intracellular citrulline–NO cycle, leading to an increase in the availability of L-arginine and NO synthesis. The inhibitory effect of PDR on platelet aggregation is primarily attributed to its stimulation of NO synthesis in endothelial cells; PDR may be a much better cardiovascular protective agent than L-arginine.

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