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Polyaspartoyl·L-arginine enhances prostacyclin synthesis in rat aortic endothelial cells

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ABSTRACT

Nitric oxide (NO) and prostacyclin (PGI_2) are two of the most important vasodilators produced by endothelial cells, the regulation of NO on PGI_2 production has not been fully clear yet. Polyaspartoyl·L-arginine (PDR) is an L-arginine residue-rich compound with inhibitory effects of platelet aggregation and thrombosis. This study investigated its effects on NO production in rat aortic endothelial cells (RAECs) and observed the influence of NO on PGI_2 level in RAECs. NO concentration in the medium of RAECs was assessed with fluorometric method; 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, in the medium of RAECs was measured with radioimmunoassay kits; Protein level of PGI_2 synthase in RAECs was determined by Western blot analysis. PDR (17.0~170 μg/ml, equal to 0.5 μM–5 μM) enhanced NO level in culture medium of RAEC with concentration-dependent manner ($P<0.01$); L-arginine (170 μg/ml, equal to 1000 μM) and 1.70 μg/ml (0.05 μM) of PDR slightly increased NO level ($P>0.05$). Interestingly PDR (1.70–500 μg/ml), L-arginine (17.0–170 μg/ml) significantly elevated PGI_2 levels in medium of RAECs ($P<0.01$), NO synthase inhibitor, N^G-nitro L-arginine methyl ester (L-NAME), markedly inhibited the elevated PGI_2 levels by PDR and L-arginine. NO donor, sodium nitroprusside(SNP)(1–500 μM), showed the most powerful effects of increasing PGI_2 level in RAECs, which was not influenced by L-NAME. Cyclooxigenase(COX) inhibitor, indomethacin, significantly reduced elevated PGI_2 level by both PDR and SNP in RAEC medium. PDR (170 μg/ml) increased the expression of PGI_2 synthase, L-NAME partly inhibited this effect. In conclusion, PDR enhances PGI_2 synthesis in RAEC, which is attributed to its effect of NO production; the stimulating effect of PDR on PGI_2 synthesis may be mediated via COX and PGI_2 synthase.

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1. Introduction

Endothelial cells produce vasodilators and vasoconstrictors, the most important vasodilators include nitric oxide (NO), prostacyclin (PGI_2) and endothelium derived hyperpolarizing factor (EDHF). NO and PGI_2 are platelet aggregation inhibitors either. NO is synthesized from L-arginine by a family of enzymes. PGI_2 is the predominant metabolite of arachidonic acid metabolism by cyclooxygenase (COX) in vascular tissues (Zhao et al., 1996). Treatment of cultured bovine aortic endothelial cells (BAECs) with glyceryl trinitrate, sodium nitroprusside (SNP) or other NO donor dramatically enhanced PGI_2 release from BAEC by independent of cGMP way (Salvemini et al., 1996). In A23187 treated bovine microvessel endothelial cells, both NO and PGI_2 production were increased several fold in a cGMP-independent manner, whereas the inhibition of NO production with N^G-nitro L-arginine methyl ester (L-NAME) attenuated PGI_2 release by half (Davidge et al., 1995). About half of the elevated production of PGI_2 in shear stressed cells was due to NO-dependent signaling, indicating that hemodynamic control of these two dilatory molecules is partially coupled (Wang and Diamond, 1997). However several contrary data have been reported yet. For example, Doni et al. reported that exogenous administration of NO exerted a dose-dependent inhibition on the bradykinin-stimulated release of PGI_2 from bovine endothelial cells (Doni et al., 1988); arachidonic acid-induced PGI_2 synthesis was enhanced when NO synthesis was inhibited. NO inhibited arachidonic acid-induced relaxation and PGI_2 release in the coronary circulation. A NO donor S-Nitroso-acetyl-DL-penicillamine decreased PGI_2 production in cultured endothelial cells (Marcelín-Jiménez and Escalante, 2001). Matthews et al found that sodium nitropruside (SNP), glyceryl trinitrate (GTN) or 3-morpholino-sydnonimine (SIN-1) significantly inhibited bradykinin-stimulated PGI_2 release from the bovine aortic endothelial cell line (Matthews et al., 1995). These inconsistent data indicated that NO regulation on PGI_2 generation remains to be a controversial issue.

Polyaspiartoyl L-arginine (PDR) is an L-arginine rich-compound (average molecular weight: 33,200 Da, Fig. 1), we reported its platelet aggregation inhibitory effect *ex vivo*(Wang et al., 2004) and anti-thrombotic effect *in vivo*, the latter companied plasma NO increase (Tang et al., 2003). Recently we observed the enhancing effect of PDR on NO synthesis in rat aortic endothelial cells (RAECs)(Tang et al., 2008). Based on above data, we attempt to investigate the regulative

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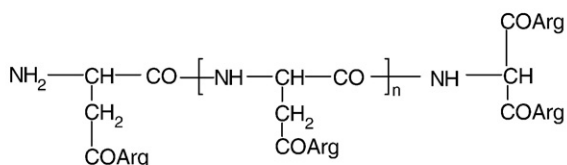

Fig. 1. Structure of polyaspartoyl·L-arginine (PDR).

effect of PDR on PGI_2 release from endothelial cells in this study, to reveal its mechanism of anti-thrombotic effect.

2. Material and methods

PDR was synthesized by our colleagues in Beijing Key Laboratories of Hydrone and Peptides in Capital University of Medical Sciences and the light brown powder was dissolved in phosphate buffered saline (PBS) before use. 6-keto-$PGF_{1\alpha}$ immunoassay kits were products of Radioimmunology Institute of People's Liberation Army General Hospital, Beijing; L-NAME, tumor necrosis factor-α(TNF-α), Arachidonic acid and indomethacin were all products of Sigma. 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. Sprague–Dawley rats were obtained from the Experimental Animal Center of Peking University.

2.1. Rat aortic endothelial cell culture

Endothelial cells were obtained from rat aortas and subcultured as described previously (Centra et al., 1992). Briefly, Male Sprague–Dawley rats weighing 180–200 g were anesthetized with overdose of sodium pentobarbital and the abdominal aortas of rats were rapidly removed and collected in 1640 medium. Surrounding fat and connective tissue were cleaned off, and then aortas were cut longitudinally. Aortic endothelium was scraped with vertical ophthalmic forceps and endothelial 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 described by others (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.

2.2. Determination of NO in RAEC culture medium

Passage 4–6 cells were seeded into 24 well plates with 1×10^5 cells/ml and cultured with medium 1640 containing 10% serum at 37 °C for 48 h, then pre-incubated with or without L-NAME (1 mmol/L), removed the culture medium and rinsed the cells with serum free medium 1640 after 16 h incubation. Then cells were pre-treated with PBS containing 0.9 mmol/L $CaCl_2$ and 0.5 mmol/L $MgCl_2$ for 30 min, and treated with L-arginine (170 µg/ml) and PDR(1.7–170 µg/ml) for 30 min. The reaction was terminated by ice-bathing, samples of reaction solution were collected and stored at −20 °C for NO measurement. Cells were lysed in lysis buffer and supernatants were 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 according to Bradford method (Bradford, 1976) and used to normalize NO values.

A sensitive fluorometric method for nitrite determination was used as previously described (Misko et al., 1993; and Kleinhenz et al., 2003) 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 to wells, mixed slightly, and 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.

2.3. Determination of 6-keto-$PGF_{1\alpha}$ in media of rat aortic endothelial cells

4–6 passage rat aortic endothelial cells were seeded into 96-well plates with 1×10^5 cells/ml, cultured for 48 h to confluence, and pre-incubated for 24 h with or without L-NAME and removed media. Hank's balanced salt solution was added as reaction medium after rinsing with serum free medium, arachidonic acid (30 µM) and then A_{23187}(1 µM) were added to wells. Then cells were treated with vehicle or L-arginine, SNP or PDR with or without NOS inhibitor or COX inhibitor, and equilibrated in the incubator (37 °C) for 30 min. Culture media were saved for the determination of 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, which was detected with immunoassay kits (Radioimmunology Institute of People's Liberation Army General Hospital, Beijing). Shortly, Samples, standard solution or buffer were incubated with or without anti-serum at 37 °C for 6 h, then treated with ^{125}I-6-keto-$PGF_{1\alpha}$ at 4 °C for 12 h, and reacted with precipitating agent for 20 min at 25 °C, the cpm of precipitation was counted and the value of 6-keto-$PGF_{1\alpha}$ 0was determined according to standard curve.

2.4. Western blot analysis of PGI_2 synthase

RAECs were seeded into 6-well plates with 2×10^5 cells/ml and cultured until confluence, cells were pre-treated with arachidonic acid (30 µmol/ml) for 30 min, then were treated with vehicle, PDR (1.7–170 µg/ml), 10 ng/ml of tumor necrosis factor-α (TNFα) or 170 µg/ml of PDR with L-NAME for 24 h, respectively, L-NAME was added at 24 h before arachidonic acid addition. After removal of media, cells were washed twice with ice-cold PBS, then lysed using cell lysis buffer. Lysates were collected by scraping from plates, and then centrifuged at 10,000×g at 4 °C for 5 min. Western blot was performed according to the other's procedure (Towbin et al. 1979). Briefly, proteins (30 µg) were loaded on a 12% of SDS-polyacrylamide gel for electrophoresis, then transferred onto nitrocellulose transfer membranes (Osmonics, USA) at 0.8 mA/cm2 for 2 h. Membranes were blocked at room temperature for 1 h with blocking solution. Membranes were then incubated overnight at 4 °C with anti-PGI_2 synthase rabbit polyclonal antibody at 1:150 (Santa Cruz Biotechnology, USA) in blocking solution. After two 10 min washing in TBST, membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated antirabbit secondary antibody at 1:500 in blocking solution.

Detection was performed by enhanced chemiluminescence (ECL) using a Western Blotting Luminol Reagent (Santa Cruz Biotechnology, USA) according to the manufacturer's instructions. Bands were then quantified by scanning densitometry (THERMAL IMAGING SYSTEM FTI-500, Pharmacia Biotech). Protein concentrations were determined using the Pierce Micro BCA protein assay system (Pierce, Rockford, Illinois, USA).

2.5. Statistical analysis

The data are expressed as mean±S.D. Statistical evaluation was performed using Dunnet *t*-test to compare the differences between

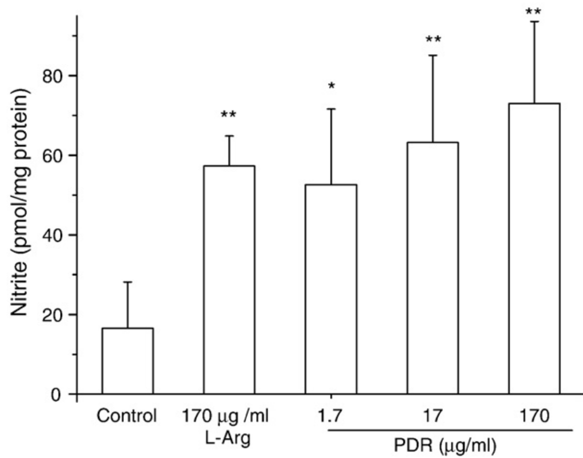

Fig. 2. The effect of PDR on NO production of RAECs. Cells were pre-incubated with vehicle or agents at 37 °C for 30 min, and then terminated the reaction by ice-bath, NO in reaction medium was tested with 2,3-diaminonaphthalene fluorescence method and calibrated with protein content (n=4). *P<0.05, **P<0.01 compared *vs* control.

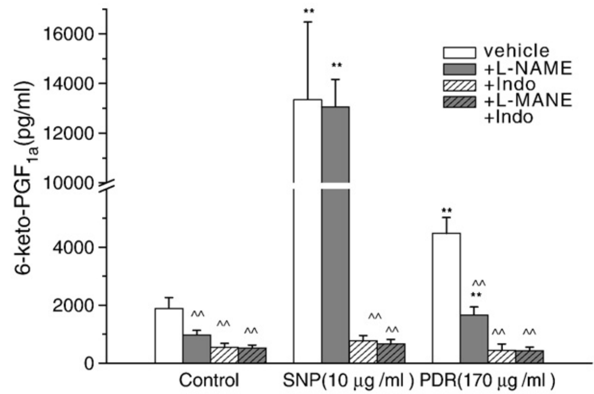

Fig. 4. Influence of COX inhibitor or NOS inhibitor on PGI_2 released from RAECs. Endothelial cells were incubated without or with L-NAME (100 μM) for 24 h, or with indomethacin 30 min, or first with L-NAME, then with indomethacin (L-NAME+ indomethacin). Cells were then pre-treated with arachidonic acid (30 μM) and A_{23187} (1 μM) respectively, and then treated with vehicle, SNP or PDR. Then 6-keto-$PGF_{1\alpha}$ in medium was detected as Fig. 3. Values were expressed as mean±SD (n=4). ^^P<0.01 *vs* corresponding control; **P<0.01compared *vs* vehicle.

treated groups and control groups. P<0.05 was considered to be statistically significant.

3. Results

3.1. Effect on NO release from endothelial cells

The data of effects of PDR on NO release were summarized in Fig. 2. NO level in medium of RAECs treated with PDR was enhanced with concentration-dependent way, L-arginine (170 μg/) enhanced NO level either, but its effect is inferior by comparison with that of the same concentration of PDR, indicated that PDR stimulated NO release from RAEC.

3.2. Effect on 6-keto-$PGF_{1\alpha}$ level in the culture medium of RAECs

Compared with vehicle control PDR concentration-dependently enhanced 6-keto-$PGF_{1\alpha}$ level in culture medium, L-arginine showed a less potent effect than PDR. NO donor SNP, which directly give off NO, exhibited powerful effect of increased 6-keto-$PGF_{1\alpha}$ level. L-NAME significantly blocked stimulating effects of PDR and L-arginine on PGI_2, but it did not influence PGI_2 level in SNP treated cells. In addition, basal PGI_2 level was also blocked by L-NAME (Fig. 3). These results indicated that NO can enhance PGI_2 production; the inhibition of NO synthesis decreased PGI_2 production.

3.3. Influence of different enzyme inhibitors on PGI_2 elevation

In this assay we observed the influences of NOS inhibitor and COX inhibitor on 6-keto-$PGF_{1\alpha}$ level in the medium of RAECs. In the absence of PDR L-NAME and indomethacin reduced 6-keto-$PGF_{1\alpha}$ level in medium by 50.5% and 76.2%, respectively, compared with vehicle control, the combined use of the two inhibitors did not further reduce its level compared with indomethacin alone. In the presence of PDR, L-NAME and indomethacin reduced 6-keto-$PGF_{1\alpha}$.level in RAEC medium by 62.8% and 91.4%, respectively, compared with its vehicle control. Differently the effect of SNP on PGI_2 was significantly inhibited by indomethacin but by L-NAME. The combined use of the two inhibitors did not further reduce its level compared with indomethacin alone. These results suggest that the effect of PDR on PGI_2 release is related with both NOS and COX (Fig. 4), which is different from SNP.

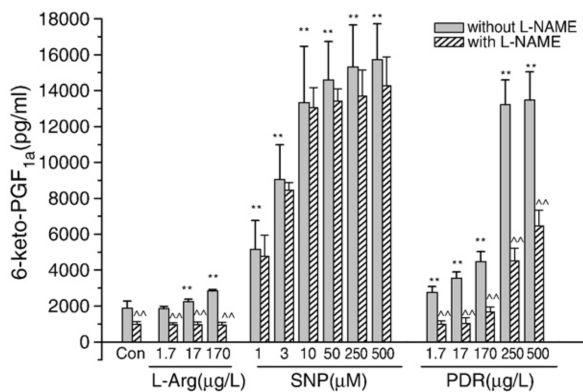

Fig. 3. Effect of PDR on PGI_2 released from cultured RAECs. Confluent cells were incubated for 24 h with or without L-NAME and culture media were changed into reaction medium. Arachidonic acid (30 μM) and then A_{23187} (1 μM) were added and mixed, then cells were treated with vehicle or agents for 30 min in the incubator (37 °C). 6-keto-$PGF_{1\alpha}$ in reaction media was detected with immunoassay kits. Values are expressed as mean±SD (n=4). **P<0.01 *vs* control; ^^P<0.01compared *vs* group without L-NAME.

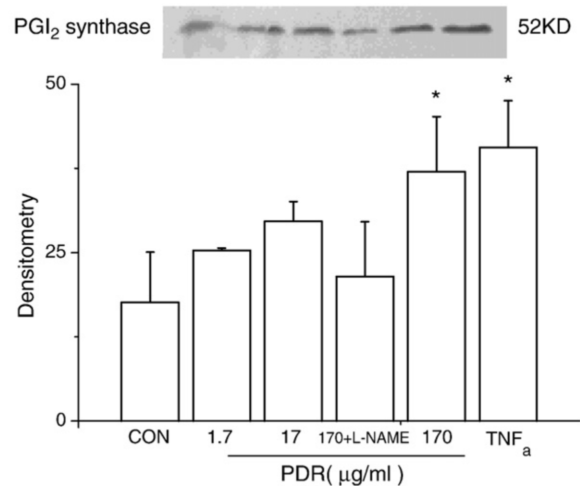

Fig. 5. Effect of PDR on the expression of PGI_2 synthase in RAECs. RAECs were treated as other part. Western blot was performed. Proteins were detected using enhanced chemiluminescence as detection reagents and quantitated by densitometry. The upper: the band of PGI_2 synthase by Western blot assay. The lower: the data of the band quantitated by densitometry (n=3) values are expressed as mean±SD. *P<0.05 compared *vs* control.

3.4. Effect on PGI_2 synthase protein level in RAECs

We investigated the effect of PDR on the expression of prostacyclin synthase with western blot analysis. TNFα was used as a positive control. Compared with vehicle control the treatment with PDR enhanced PGI_2 synthase protein level, and 170 μg/ml of it exhibited significant effect. TNFα increased PGI_2 synthase expression with a slight higher level compared with170 μg/ml of PDR. L-NAME reduced the effect of PDR on PGI_2 synthase protein level (Fig. 5). This result manifested that PDR stimulates PGI_2 synthase expression in rat aortic endothelial cells; this effect was partly decreased when NO production was inhibited.

4. Discussions

PDR inhibits thrombosis *in vivo* (Tang et al., 2003) and platelet aggregation *ex vivo* but it fails to be effective *in vitro* platelet aggregation (Tang et al., 2008), indicating the direct target of its effect is not on platelets although platelet has own L-arginine-NO-cGMP pathway. The results in this study showed that PDR significantly and concentration-dependently increased NO level in medium of cultured RAECs, suggesting it enhanced NO production in RAECs, and this action may be the major mechanism of its inhibiting platelet aggregation and thrombosis. We recently reported PDR significantly elevated L-arginine level in RAECs (Tang et al., 2008), which may be its key factor of increasing NO release.

PGI_2 is a platelet aggregation inhibitor and vasodilator synthesized and released by endothelial cells as NO. The regulative effect of NO on PGI_2 synthesis described by different research groups is inconsistent. Davidge et al. and other authors suggested NO up-regulated PGI_2 production (Davidge et al., 1995; Wang and Diamond et al., 1997; Cuzzocrea and Salvemini, 2007; Vassalle et al., 2003). Due to the enhanced effect of PDR on NO synthesis, we investigated its influence on PGI_2 production in endothelial cells in this study. Simultaneously we also observed the effects of a NOS substrate (L-arginine) and a NO donor (SNP) on PGI_2. PDR, an L-arginine-rich big molecule, is a agent different from NO donor, which produces NO through NOS, thus NOS inhibitor can use as a tool to compare different agents' peculiarity in studying the effect of NO on PGI_2 production. All three agents enhanced PGI_2 production in endothelial cells in this study, among them SNP displayed most potent effect, PDR exhibited more potent effect compared with L-arginine, and the effects of L-arginine and PDR significantly reduced by NOS inhibitor L-NAME (by more than 50%). As we anticipated the effect of SNP was not influenced by NOS inhibitor. The findings were consistent with the data described by authors mentioned above(Davidge et al., 1995; Wang and Diamond, 1997; Cuzzocrea and Salvemini, 2007; Vassalle et al., 2003), and suggested that NO indeed stimulated PGI_2 production in RAECs.

The elevated levels of NO can lead to the activation of constitutive cyclooxygenase (COX_1), and enhanced PGI_2 production in bovine microvessel endothelial cells (Davidge et al., 1995); NO activates the COX enzymes, an event leading to overt production of PGs, suggesting that COX enzymes represent important endogenous 'receptor' targets for modulating the multifaceted roles of NO (Cuzzocrea and Salvemini, 2007). In this study we did not investigate COX activity and protein expression but observed the influence of indomethecin, a COX inhibitor, on PGI_2 synthesis, it suppressed PDR's stimulating effect on PGI_2 by 91%, suggesting NO from PDR enhanced PGI_2 production via COX. Furthermore we observed that PDR (170 μg/ml) significantly up-regulated the expression of PGI_2 synthase which was partly inhibited by L-NAME (about 43%), indicated that the increase of PGI_2 synthase expression may also be contributed to PDR on the stimulation of PGI_2 production (Fig. 5).

Oppositely some authors demonstrated NO down-regulated PGI_2 production in endothelial cells(Doni et al., 1988; Marcelín-Jiménez and Escalante 2001; Matthews et al., 1995; Takeuchia et al., 2004).

The contrary data from different authors has not been explained clearly, it may be attributed to the states of endothelial cells and associated enzyme proteins under different experiment conditions. NO is synthesized by NOS, also NOS can synthesize O^{2-} which represents an enzymatic malfunction, this can occur when NOS uncouples under endothelial dysfunction and other factors including limitation of L-arginine supply and alterations in the heat shock protein 90. An uncoupled eNOS could be a consequence of prolonged oxidative stress(Maier et al., 2000; Landmesser et al., 2003; Stuehr et al., 2004), O^{2-} and NO react to produce $ONOO^-$, which represents an unstable and reactive molecule(Beckman and Koppenol, 1996). Under physiological conditions, $ONOO^-$ levels are kept in the nanomolar range by cellular antioxidant systems to avoid damages of cellular macromolecules. However, with increasing levels of O^{2-} and NO and the involvement of metal catalysis, specific oxidations at proteins can occur (sulfenic acid formation, sulfoxidation, and zinc finger oxidation) which lead to posttranslational protein modifications and activity changes. In presence of low levels of $ONOO^-$ ($IC_{50} \approx 50$ nM) a Tyr-residue at the active site of PGI_2 synthase becomes nitrated and inhibits the active site (Schmidt et al., 2003).

After checking the concentration of NO donor used by different authors we suppose that the concentration of NO may be an important factor inducing PGI_2 regulative direction, when NO in lower level (NO donor concentrations <200 μmol/L) up-regulates PGI_2 synthesis and release; and high level NO (NO donor concentration≥ 500 μmol/L) down-regulates PGI_2 synthesis and release, Excessive NO likely causes eNOS uncoupling and increases $ONOO^-$ generation, thus inactivates PGI_2 synthase, then lowers PGI_2 release. In order to confirm this view we used a wider concentration range of SNP(1–500 μmol/L) and PDR (1.7–500 μg/ml) to study the effect of NO on PGI_2 synthesis, unfortunately the results fail to support our view, in all concentrations used by us both SNP and PDR enhanced PGI_2 synthesis. So the inconsistent data of NO on PGI_2 regulation may be due to other reason such as the state, the origin or other factors of endothelial cells, further experiment data will be awaited to expound it.

In conclusion, PDR enhances PGI_2 synthesis in RAEC, which is attributed to its effect of NO production; the stimulating effect of PDR on PGI_2 synthesis may be mediated via COX and PGI_2 synthase. These findings suggest the enhancing NO and PGI_2 may be the major action mechanism of PDR on thrombotic inhibition.

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