Panax Notoginseng Saponins Restrains Ischemia-reperfusion-induced Rat Mesenteric Microcirculatory Disturbance

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ABSTRACT

Objective: To investigate the effect of Panax notoginseng saponins (PNS) on ischemia reperfusion (I/R) induced rat mesenteric microcirculatory dysfunctions.

Methods: Male Wistar rats weighting 200–250 g were subjected to 10 min ligation of the superior mesenteric artery and vein, followed by 60 min reperfusion. PNS (5 mg/kg/hr) was continuously administrated starting from 10 min before ischemia or 10 min after reperfusion until 60 min after reperfusion via left jugular vein. Leukocytes adhesion, mast cell degranulation, endothelial peroxidation, and albumin leakage of rat mesenteric venules were observed. Serum myeloperoxidase (MPO) level, intercellular adhesion molecule-1 (ICAM-1) expression and Src phosphorylation were examined.

Results: PNS ameliorated leukocyte adhesion and mast cell degranulation, while with no obvious effects on endothelial peroxidation and albumin leakage. In addition, PNS inhibited serum MPO increase, intestinal ICAM-1 expression and Src phosphorylation induced by I/R.

Conclusions: PNS ameliorated I/R-induced leukocyte adhesion and mast cell degranulation, the former is related to its inhibition of Src phosphorylation and ICAM-1 expression.

Key words: ICAM-1, Src, leukocytes adhesion, mast cell degranulation, panax notoginseng

INTRODUCTION

Intestinal ischemia and reperfusion (I/R) is a challenging and life-threatening clinical problem which occurs in a number of clinically relevant pathophysiologic processes, such as mesenteric artery embolism, intestinal transplantation, cardiopulmonary bypass and abdominal aortic aneurysm surgery, and traumatic or hemorrhagic shock\textsuperscript{[1-2]}, with high morbidity and mortality\textsuperscript{[3-4]}. Intestinal I/R not only causes the injury of local tissue, but also induces translocation of intra-intestinal bacteria and toxins and then activates reticuloendothelial system reaction, leading to release of massive inflammatory factors and even multiple system organ failure\textsuperscript{[5-7]}. I/R exerts multiple insults in microcirculation, frequently accompanied by enhanced adhesion of leukocytes, mast cell degranulation, production of oxygen radicals, and albumin leakage\textsuperscript{[8]}. I/R increased expression of intercellular adhesion molecule 1 (ICAM-1) in endothelial cells and CD11b/CD18 in leukocytes\textsuperscript{[9]}, leading to leukocytes adhesion to endothelium\textsuperscript{[10]}. The adherent leukocytes then release inflammatory factors, such as tumor necrosis factor (TNF-\alpha), which induce activation of Src kinase\textsuperscript{[11]}. Src kinase subsequently activates nuclear factor-kappa B (NF-kB), which also can induce ICAM-1 expression and leukocytes activation, finally exacerbate the damage of microvessel and surrounding tissue\textsuperscript{[12]}. Panax notoginseng (PN) is the dried root of the Chinese traditional herb Panax notoginseng (Burk) F.H. Chen. Panax notoginseng saponins (PNS) constitutes the major effective components of PNS\textsuperscript{[13]}, which has been used clinically and widely in the treatment of microcirculation disturbance related diseases\textsuperscript{[14-16]}. Our previous studies demonstrated that PNS could inhibit the leukocytes adhesion to venules, degranulation of mast cell, and depress the expression of adhesion molecular CD11b/CD18 induced by LPS\textsuperscript{[17,18]}.
Furthermore, compound herbal medicine (such as Myakuryu and Herbal cardiotonic pills) containing PN attenuated leukocyte adhesion induced by I/R\(^\text{19,20}\). However, the effects of PNS on I/R-induced microcirculation disturbance remains unclear. Therefore, this study aimed to investigate whether PNS has beneficial effects on microcirculatory disturbance induced by superior mesenteric artery I/R, and the possible mechanism.

**METHODS**

**Reagents**

PNS was purchased from Feng-Shan-Jian Medical Company (Kun Ming, Yun Nan, China). Dihydrorhodamine-123 (DHR) was obtained from Molecular Probes (Eugene, OR, USA). Fluorescein isothiocyanate (FITC)-albumin and toluidine blue were obtained from Sigma (St Louis, MO, USA). Antibodies against Src, phosphor-Src (p-Src) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against ICAM-1 was obtained from BD Biosciences (San Jose, CA, USA). Rat MPO ELISA Kit was obtained from GBD Ltd. (California, CA, USA).

**Animals**

Male Wistar rats weighing 200-250 g were obtained from the Animal Center of Peking University Health Science Center (Beijing, certificate NO. SCXK 2006-0008). The rats were given standard laboratory rodent chow (Animal Center of All Animals Peking University Health Science Center) and tap water and were housed at 24 ± 1 °C and relative humidity of 50 ± 1% with a 12/12 hr light/dark cycle. Before the experiment, the rats were fasted for 12 hr, while allowed free access to water. All animals were handled according to the guidelines of the Peking University Health Science Center Animal Research Committee, and the surgical procedures and experimental protocol were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (LA2010-001).

**Surgical protocols**

Surgical procedure in the present study was almost the same as our previous study\(^\text{21}\). After rats were anesthetized, the left jugular vein was cannulated for the infusion of PNS or saline. After the abdomen was opened, an ileocecal portion of the anterior mesenteric artery and the corresponding vein were selected for study. After 10 min of basal observation of the hemodynamics in the rat mesenteric microvasculature, I/R was accomplished by ligating the feeding branch of the anterior mesenteric artery and the corresponding vein simultaneously with a snare created by 2-0 silk suture for 10 min and subsequent release of the blood flow for 60 min. Red blood cell velocity in the vessels during the ischemia was not zero because of the possible collateral perfusion to the observed area. Thus both artery and vein were ligated to stop blood supply and induce venule congestion to enhance ischemia. A previous study showed that 10 min ischemia followed by reperfusion was long enough to induce mesenteric microcirculatory disturbance with minimum intestinal tissue injury\(^\text{22}\). Sham-operated rats without I/R were used as control.

**Experimental group**

Rats were randomly distributed into Sham group, I/R group, PNS group, PNS+I/R group and I/R+PNS group. In I/R group, the vehicle saline (2 mL/hr) was infused via the left jugular vein catheter starting from 10 min before the ischemia and sustained until the end of the observation. The animals of the sham-operated group (Sham group) received the same infusion as those in I/R group. The animals of the PNS group received only PNS (5 mg/kg/hr) until the end of the observation, without I/R surgery. In drug pretreatment group (PNS+I/R group), PNS (5 mg/kg/hr) was continuously infused via the left jugular vein catheter starting from 10 min before ischemia until the end of the observation. In drug post-treatment group (I/R+PNS group), PNS (5 mg/kg/hr) was continuously infused via the left jugular vein catheter at the same doses as those in pre-treatment group but starting from 10 min after reperfusion until the end of the observation.

**Intravital microscopy**

The number of leukocytes adhered to the venules was determined off-line during play-back of videotaped images. Leukocytes adhered to the venules were identified as cells that attached to the same site for more than 10 s judging from the replayed video images. The number of adherent leukocytes was counted along venules (30-50 µm in diameter, 200 µm in length) selected from the recorded images recorded and expressed as the number per 200 µm of venule length\(^\text{21}\).

In another set of experiments, the oxidant-sensitive fluorescent probe DHR was added to the mesenteric superfusate (10 µM) to estimate oxidant stress in venular walls, as described previously\(^\text{21}\). DHR fluorescence intensity was monitored with an image processor. Gray scale values were measured on the venular wall (I\(_{W}\)) and in the background (I\(_{B}\)). The DHR fluorescence intensity on the venular wall was expressed as the difference between I\(_{W}\) and I\(_{B}\). Since the
baseline fluorescence intensity varies depending on the animal, the ratio of the fluorescence intensity of each time point to the baseline fluorescence intensity was calculated as DHR fluorescence ratio.

To quantify albumin leakage across mesenteric venular walls, the rats were intravenously injected with 50 mg/kg of FITC-labeled bovine serum albumin 10 min before each experiment, as described previously[21]. Fluorescence signal (excitation wavelength at 420 to 490 nm, emission wavelength at 520 nm) was acquired using a silicon-intensified target camera (C-2400-08; Hamamatsu Photonics, Hamamatsu, Japan). The fluorescence intensities of FITC-albumin in the venules (IV) and in the perivenular interstitium (IP) area were measured with Image-Pro Plus 5.0 software. Albumin leakage was estimated by dividing IP by IV, and the dynamic alteration of albumin leakage was expressed as the ratio of albumin leakage at different time points to that of the baseline.

**Mast cells degranulation**

Mast cells were identified by vital staining with topical application of 0.1% toluidine blue to the mesentry 60 min after the I/R. The numbers of both nondegranulated and degranulated mast cells were scored from the CCD video images, and the ratio of the number of degranulated mast cells to the total number of mast cells evaluated was calculated and presented as the degranulated mast cell ratio[21].

**MPO level in serum**

After 60 min of reperfusion, blood was taken from the abdominal aorta of rat in each group and anticoagulated with heparin. The blood was centrifuged at 1300 × g for 15 min at room temperature, then the serum was collected for MPO detection[23]. The level of MPO was measured as an indicator of leukocyte activity using Rat MPO ELISA Kit (GBD Ltd., California, CA, USA).

**Western blotting analysis**

Intestinal tissues were removed and harvested 60 min after reperfusion. Whole protein of tissues was homogenized in lysis buffer containing the protease inhibitor. Equivalent amount of protein was loaded on Tris-glycine SDS-PAGE for separation. Protein on the gel was blotted onto polyvinylidene fluoride membranes and incubated with blocking buffer (Tris-buffered saline containing Tween 20 and (TBST) 3% BSA) for 1 hr at room temperature. The membranes were then incubated overnight at 4 °C with antibodies against Src (1:1000), phospho-Src (1:1000), ICAM-1 (1:1000), GAPDH (1:2000), respectively, in diluent buffer (3% BSA in TBST). Following washing, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) respectively at room temperature for 1 hr. Blots were developed using the enhanced ChemiLucent Detection System Kit (Millipore, Temecula, CA, USA), and protein bands were visualized on X-ray film. Semiquantification of the protein was performed using Image-Pro Plus 5.0 software (Bethesda, MD, USA). The GAPDH western blotting was performed for each membrane as a loading control.

**Statistical analysis**

All parameters were expressed as mean ± standard deviation (SD). Statistical analyses were performed using statistical analysis software (GraphPad Prism, version 5.0). For comparison of one condition a one-way analysis of variance (ANOVA) with Tukey post hoc test was used. For comparison of >2 conditions a repeated measures ANOVA with Bonferroni post hoc test was used. A probability less than 0.05 was considered to be statistically significant.

**RESULTS**

**The effect of PNS on I/R-induced leukocytes adhered to the venular walls**

Leukocytes adhesion was evaluated after 60 min of reperfusion in each group (Figure 1). Figure 1A showed the representative pictures of each group at baseline, 10 and 60 min after reperfusion. Before ischemia, no adherent leukocyte was visible in each group (a1-e1). After I/R, numerous adherent leukocytes occurred at 10 min reperfusion (c2), and extremely increased at 60 min reperfusion (c3). Pre-treatment of PNS showed less adherent leukocytes than I/R group at 10 and 60 min reperfusion (d2, d3), the latter with a sharper contrast. Post-treatment of PNS at 10 min reperfusion resulted in a pronounced decrease in the number of adherent leukocytes (e3).

The time course of changes in the leukocyte adhesion was demonstrated in Figure 1B. The adherent leukocyte number was significantly increased from 1 min and further up to 60 min after reperfusion. Pre- or post-treatment with PNS significantly reduced I/R-induced leukocyte adhesion at 10 min and 30 min after reperfusion.

**The effects of PNS on I/R-induced Mast cell degranulation**

Mast cell degranulation was evaluated in perivascular interstitial after 60 min of reperfusion in each group (Figure 2). Figure 2A showed the representative pictures of mast cell degranulation in each group. In sham and PNS group, the mast cell degranulated with a spontaneous occurrence (a, b). After I/R, the number of degranulated mast cells were obviously increased contrasted to sham group (c). Pre- or post-treatment with PNS resulted in less degranulated mast cells number than I/R group (d, e).

The statistical analysis of mast cells degranulation rate is shown in Figure 2B. Compared with Sham group, I/R induced an increase in mast cell degranulation rate significantly. While Pre- or post-treatment with PNS significantly inhibited the I/R-induced degranulation of mast cell.

**The effect of PNS on I/R-induced DHR fluorescence change on rat mesenteric venular wall**

I/R induced an increase in DHR fluorescence change significantly on rat mesenteric venular wall. While either pre or post-treatment of PNS had no significant effect on the change of DHR fluorescence induced by I/R (Figure 3A).
The effect of PNS on I/R-induced FITC-labeled albumin leakage from rat mesenteric venular wall

I/R induced an increase in FITC-labeled albumin leakage significantly from the rat mesenteric venules. While either pre or post-treatment of PNS had no significant effect on the albumin leakage induced by I/R (Figure 3B).

The effect of PNS on I/R-induced serum MPO level change

Figure 4 showed the rat serum MPO level. Compared with Sham group, after I/R, rat serum MPO level was significantly increased, whereas pre- or post-treatment with PNS significantly inhibited the I/R-induced augment in serum MPO level.

The effect of PNS on I/R-induced rat intestinal ICAM-1 expression

Figure 5 showed the representative images (5A) and quantitative analysis (5B) of ICAM-1 expression in rat intestine. Western blotting analysis demonstrated that, compared with Sham group, I/R induced an increase in expression of ICAM-1 significantly, whereas pre- or post-treatment with PNS inhibited the I/R induced expression of ICAM-1.

The effect of PNS on I/R-induced rat intestinal Src phosphorylation

Figure 6 showed the representative image (6A) and quantitative analysis (6B) of Src phosphorylation in rat intestine. Western blotting analysis demonstrated that, compared with Sham group, I/R induced an increase phosphorylation of Src significantly, whereas pre- or post-treatment with PNS inhibited the I/R induced Src phosphorylation.

DISCUSSION

The present study demonstrated that PNS could attenuate the number of adherent leukocytes to venule wall and mast cell degranulation in rat mesentery induced by I/R, regardless of pre- or post-treatment. Moreover, PNS also reduced serum MPO level, intestinal ICAM-1 expression and Src phosphorylation induced by I/R.

Microvascular endothelial cells express few adhesion molecules in the physiological condition. After I/R, adhesion molecules, such as CD11b/CD18 expressed by leukocytes and ICAM-1 expressed by endothelium initiate leukocytes adhesion to endothelium\(^{[9,10]}\). The adherent leukocytes released reactive oxygen species (ROS), inflammatory factors (such as leukotriene B4, IL-1, IL-6 and TNF-\(\alpha\)) and enzymatic
granule, which further injure endothelium and basement membrane. Some of the adherent leukocytes immigrate outside the microvessel and induce surrounding tissue damage. Attenuating the adhesion of leukocytes to endothelium is one of the critical steps for ameliorating microcirculation disturbance induced by I/R. In this study, pre- or post-treatment of PNS significantly decreased I/R-induced leukocyte adhesion. In addition, we also found that

Figure 2. The effects of PNS on I/R-induced Mast cell degranulation. A: Representative images showing degranulated mast cells in the mesentery after I/R. a: Mast cells of Sham group; b: Mast cells of PNS group; c: Mast cells of I/R group; d: Mast cells of PNS + I/R group; e: Mast cells of I/R + PNS group. V: Rat mesenteric venule; Arrows: degranulated mast cells; Bar = 50 µm. B: Quantitative evaluation of mast cell degranulation along venules. Sham: Sham group; PNS: treatment with PNS at 5 mg/kg/hr alone group; I/R: ischemia 10 min and reperfusion 60 min group; PNS+I/R: pre-treatment with PNS at 5 mg/kg/hr, 10 min before ischemia group; I/R+PNS: post-treatment with PNS at 5 mg/kg/hr, 10 min after reperfusion group. Values are mean ± SD of 6 independent experiments. *p < 0.05 vs sham group; #p < 0.05 vs I/R group.

Figure 3. A: The effect of PNS on I/R-induced DHR fluorescence change on rat mesenteric venular wall. B: The effect of PNS on I/R-induced FITC-labeled albumin leakage from rat mesenteric venular wall. Sham: Sham group; PNS: treatment with PNS at 5 mg/kg/hr alone group; I/R: ischemia 10 min and reperfusion 60 min group; PNS+I/R: pre-treatment with PNS at 5 mg/kg/hr, 10 min before ischemia group; I/R+PNS: post-treatment with PNS at 5 mg/kg/hr, 10 min after reperfusion group. Values are mean ± SD of 6 independent experiments. *p < 0.05 vs sham group.
pre- or post-treatment with PNS could inhibit increased ICAM-1 expression in rat intestine evoked by I/R. The enhancement of ICAM-1 expression is essential for leukocyte adhesion\cite{28}. Therefore, inhibition of ICAM-1 expression might contribute to the inhibitory effect of PNS on leukocyte adhesion.

Our most interesting result is that both pre- and post-treatment with PNS inhibited the phosphorylation of Src kinase in rat intestine induced by I/R. Src activation plays a critical role in I/R injury\cite{29}. Inflammatory factors, such as TNF-\(\alpha\), induce phosphorylation of Src, leading to activation of NF-\(\kappa\)B and expression of ICAM-1\cite{11,12}. ICAM-1 clustering in turn facilitated activation of Src and neutrophil migration\cite{30}. Therefore, our results showed that the inhibitory effect of PNS on ICAM-1 expression induced by I/R might be relevant to depression of Src kinase activity.

MPO is the most abundant protein in neutrophils and is also present in monocytes. In neutrophils, it is stored in azurophilic granules and released during phagocytosis. It is well established that MPO-derived oxidants damage cells and tissue. Hypochlorous acid, the major strong oxidant produced by neutrophils, oxidizes proteins, lipids, lipoproteins, and nucleic acids with a multitude of pathological consequences\cite{31}. MPO is also one kind of vascular inflammatory factors, and has been advocated as a prognostic marker of cardiovascular disease\cite{32}. In the present study, PNS significantly inhibited the increase of serum MPO induced by I/R, which suggest pre- and post-treatment with PNS might have an effect on inhibiting the activation of leukocytes.

In addition, we found that pre- or post-treatment of PNS inhibited I/R-induced degranulation of interstitial mast cell. Inflammatory factors, such as TNF-\(\alpha\) and platelet activating factor, induce mast cell degranulation during I/R\cite{33}. In mesentery venules, degranulated mast cell evokes leukocyte adhesion to the venular endothelium on one hand\cite{34}, on the other hand, the activated mast cells release cytokine, histamine and vasoactive substance, which attack vessel from outside. The inhibitory effect of PNS on mast cell degranulation may also contributed to ameliorating microcirculation disturbance.

In our experiment, PNS could not diminish the peroxide production of venular wall. ROS produced during I/R interact with multiple cellular components, such as membrane phospholipids, enzymes, proteins and nucleic acid\cite{35}, leading to damage of vascular endothelium and basement membrane, increase of microvascular permeability and
leakage of plasma albumin[36,37]. Our study also demonstrated that PNS treatment could not inhibit venular albumin leakage induced by I/R. The pathophysiological changes of I/R is a complicated process involving diverse insults including those in neuroendocrine system[38,39] and, sometimes, even zone specific[40,41]. Therefore, the role of PNS in I/R-injured mesentery and intestine merits further exploration.

In the present study, the dose of PNS used is the equivalent clinical dose of PNS in Xueshuantong injection, which was also determined based on the previous studies in animal models on the role of PNS in LPS-induced mesenteric injury[17,18].

In conclusion, PNS ameliorated I/R induced mesenteric leukocyte adhesion and mast cell degranulation. The inhibitory effect of PNS on leukocyte adhesion may be related to its inhibition of Src phosphorylation and ICAM-1 expression.

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CONFLICT OF INTEREST

None declared.

REFERENCES


11. Huang S, Dudez T, Scerri I, Thomas MA, Giepmans BN, Suter S, Chanson M. Defective activation of c-Src in cystic fibrosis airway epithelial cells results in loss of tumor necrosis factor-alpha-induced...


