

Protosappanin A protects against atherosclerosis via anti-hyperlipidemia, anti-inflammation and NF- κ B signaling pathway in hyperlipidemic rabbits

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ARTICLE INFO	ABSTRACT
<p>Article type: Original article</p> <p>Article history: Received: Sep 6, 2016 Accepted: Sep 28, 2017</p> <p>Keywords: Anti-hyperlipidemic Anti-inflammatory Atherosclerosis NF-κB Protosappanin A</p>	<p>Objective(s): Protosappanin A (PrA) is an effective and major ingredient of <i>Caesalpinia sappan</i> L. The current study was aimed to explore the effect of PrA on atherosclerosis (AS).</p> <p>Materials and Methods: Firstly, the experimental model of AS was established in rabbits by two-month feeding of high fat diet. Then, the rabbits were randomly divided into five groups and treated with continuous high lipid diet (model control), high lipid diet containing rosuvastatin (positive control), 5 mg/kg PrA (low dose) or 25 mg/kg PrA (high dose).</p> <p>Results: Our results showed that PrA markedly alleviated AS as indicated by hematoxylin/eosin (HE) staining. PrA also reduced hyperlipidemia (as demonstrated by the serum levels of total blood cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL)) in a time and dose-dependent manner, and decreased inflammation (as indicated by the serum levels of matrix metalloproteinase-9 [MMP-9], interleukin-6 [IL-6] and tumor necrosis factor-α [TNF-α]). Moreover, PrA significantly inactivated nuclear factor kappa B (NF-κB) signaling as indicated by nuclear NF-κB p65 protein expression, as well as the mRNA expression and serum levels of downstream genes, interferon-γ (IFN-γ) and interferon-gamma-inducible protein 10 (IP10).</p> <p>Conclusion: This study proved that PrA might protect against atherosclerosis via anti-hyperlipidemia, anti-inflammation and NF-κB signaling pathways in hyperlipidemic rabbits.</p>

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Introduction

Chinese herb *Caesalpinia sappan* L., as a member of the leguminous plant family, exhibits therapeutic potential for burning sensations, leprosy, skin diseases, dysentery and diabetic complications in China and other Asian countries (1-4). Protosappanin A (PrA), is an important active ingredient isolated and identified from *Caesalpinia sappan* L. (1). PrA, as an immunosuppressive ingredient, plays an important role in prolonging graft heart survival (5). Moreover, it has been proved that PrA suppresses CD4⁺/CD8⁺ ratio of peripheral T cell, and inhibits expression of perforin and granzyme B (secreted by T cells to kill donor cells) (6).

Atherosclerosis (AS) mainly occurring in the large or medium arteries has been called the first killer of human health because of the significant increasing morbidity and mortality (7). AS is also known as an important risk factor for ischemic cardiac-cerebral vascular diseases (8). AS animal models have been established through various strategies, including high-fat-diet method, intima-injury method, immune-

stimulation method, etc (9-11). The causes and mechanisms of AS have not been fully understood. Hyperlipidemia, which is characterized by the increased total blood cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and decreased high-density lipoprotein (HDL) (12), is a well-known cause for AS (13). Moreover, inflammation plays a vital role in the occurrence, development and complications of hyperlipidemia and AS (14). Interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) are key cytokines that mediates effector pathways in atherosclerotic vessels (15-18). Matrix metalloproteinase-9 (MMP-9) is critical for the formation and rupture of the unstable AS plaques (19-21). As an ubiquitous transcription factor, nuclear factor kappa B (NF- κ B) has been implicated in survival of transplantation, lymphocyte activation and immunological processes (22). Activated NF- κ B is observed in atheromatous areas of the AS lesion, indicating the involvement of NF- κ B in the pathogenesis of AS (23). Interferon- γ (IFN- γ) and IFN- γ -inducible protein 10 (IP10), downstream effectors of NF- κ B signaling (24), may promote the development of AS (18, 25).

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In this current study, we established AS rabbit models by high-fat-diet method and then PrA-administration experiments were performed. We proved that PrA significantly alleviated the occurrence of AS and hyperlipidemia, and decreased levels of several inflammatory cytokines. Notably, PrA significantly decreased NF- κ B signaling. Taken together, PrA might protect against AS via anti-hyperlipidemia, anti-inflammation and through NF- κ B signaling pathways in hyperlipidemic rabbits. The study suggested that PrA might be used for the treatment of AS.

Materials and Methods

Grouping of animals

Sixty healthy male New Zealand rabbits (2.5±0.4 kg) were obtained from Shanghai SLRC Experimental Animal Co LTD (Shanghai, China) and were housed in a room under controlled temperature (21±1°C) with free access to water. All the rabbits were fed with high fat diet containing 1% high cholesterol for 60 days (10), and the food intake was limited to 150 g/d. Rabbits were then randomly divided into five groups with each group containing 12 rabbits: Group 1 was healthy control and offered continued normal diet (Mock); Group 2 was model control and offered continued high fat diet; Group 3 was the positive control and fed with high fat diet containing 0.5 mg/kg rosuvastatin (26) once every day; Group 4 (PrA low dose) and Group 5 (PrA high dose) were fed with high fat diet containing 5 mg/kg PrA and 25 mg/kg PrA (5), also once every day, respectively. The serum levels of total blood cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were measured at 28 days and 42 days after treatment. After 42 days, the following assays were performed.

Hematoxylin/eosin (HE) staining of the large or medium arteries

Tissue blocks of large or medium arteries were resected, fixed in 10% formalin for 48 hr, embedded in paraffin, sectioned and stained with hematoxylin/eosin (HE). The degree of AS was analyzed under a light microscope (magnification, ×100).

Measurement of TC, TG, LDL, HDL and inflammatory cytokines in serum

At 28 days and 42 days after the rabbits were treated with the corresponding drugs, blood was collected from the ear vein after overnight fasting. The serum levels of TC and TG were detected by enzyme method using TC and TG Detection Kit (JRDun Biotechnology, Co, Ltd), respectively. The serum levels of LDL and HDL were detected by selective precipitation method using LDLC and HDLC Kit (JRDun Bio), respectively. The serum concentrations of MMP-9, IL-6, TNF- α , IFN- γ and IP10 were measured

by Enzyme linked immunosorbent assay (ELISA) with the kits purchased from JRDun.

Quantitative real-time PCR (qRT-PCR) analysis

The mRNA levels of IFN- γ and IP10 from the arteries were quantified by qRT-PCR. Total RNA was isolated from arteries tissues using Trizol Reagent (Invitrogen, Japan). cDNA was synthesized from 5 μ g of total RNA using AMV reverse transcriptase (Fermentas, USA). qRT-PCR reactions were performed in a 25 μ l total volume and on Roche Light Cycler® 480II System (Roche Diagnostics Ltd., Switzerland) using SYBR® Green 10 × Supermix (Thermo, #K0223), with GAPDH as an internal control. Primer pairs for indicated genes were designed using the Primer Express Software (Applied Biosystems, Shanghai, China) and listed in Table 1. The PCR procedure was: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 45 sec; one cycle of 95 °C for 15 sec, 60 °C for 1 min; one cycle of 95 °C for 15 sec, 60 °C for 15 sec. All PCR reactions were performed in triplicate and the relative expression levels of different groups were calculated by normalizing to the mRNA expression level of GAPDH using $2^{-\Delta\Delta CT}$ method (27).

Western blot analysis

Fresh arteries tissues was frozen in liquid nitrogen and the nuclear extract was prepared as previously described (28). The protein concentration was quantified using BCA kit (Thermo Fisher Scientific Inc, USA). A total of 80 μ g protein for each sample was loaded on SDS-PAGE gel and transferred to PVDF membranes. The blots were blocked with 5% non-fat milk and incubated overnight with different primary antibodies, which were as follows: NF- κ B, Abcam, Ab32360, 1:1500 dilution; H3, Abcam, Ab4729, 1:1000 dilution. Blots were then incubated for 1 hr at 37 °C with goat anti-mouse or anti-rabbit secondary antibody (Beyotime, Shanghai, China) and Intensities were measured using enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China). H3 was used as a loading control. Quantitation of the results was performed by Image J software.

Statistical analysis

Experiments were performed with 12 rabbits per group. Values were expressed as means±standard errors (SD). Statistical analysis was determined by ANOVA using Graphpad Prism 6.0 software. Differences were considered statistically significant at a value of $P < 0.05$.

Results

PrA obviously alleviated high-fat-diet-induced AS

In order to identify the experimental model of AS and explore the effects of PrA on AS, HE staining was performed. In the model group, the endarterium was thicker with accumulation of multiple foam cells, the medial layer was thinner, and multiple immersed cells

Table 1. Primers used in qRT-PCR analysis of IFN- γ and IP10

Gene	Primer sequence	Species	Amplicon (bps)
IFN- γ	Forward: 5'-GACTCTCGTTTCAACTTCTTC-3' Reverse: 5'-CCTTTAGGTGTTCTGTTTCTC-3'	Oryctolagus	148
IP10	Forward: 5'-TGCCACACTTCCCTTCTTC-3' Reverse: 5'-ACGTAGCAGCTTGGTGTAG-3'	Oryctolagus	172
GAPDH	Forward: 5'-CTCCTGCGACTTCAACAGTG-3' Reverse: 5'-TGAGGGCTTACTCCTTGG-3'	Oryctolagus	172

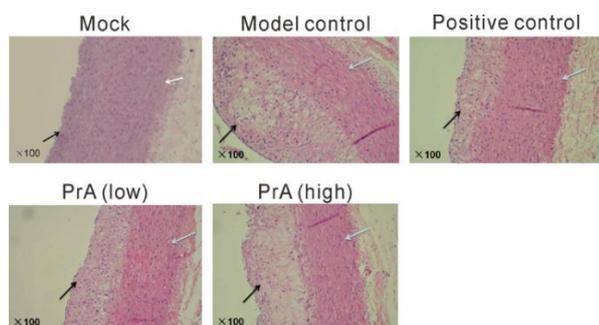


Figure 1. Histological analysis by hematoxylin/eosin staining. Rabbits were then randomly divided into five groups (n=12): Mock, rabbits were offered continued normal diet; Model control, rabbits were offered continued high fat diet; Positive control, rabbits were fed with high fat diet containing 0.5 mg/kg rosuvastatin once every day; PrA low, rabbits were fed with high fat diet containing 5 mg/kg PrA once every day; PrA high, rabbits were fed with high fat diet containing 25 mg/kg PrA once every day. After 42 days, large or medium arteries were collected and subjected to HE staining. The dark arrow represents end arterium, and the white arrows represents medial layer (magnification, $\times 100$)

were observed between endarterium and the medial layer. This suggested that the experimental model of AS was successfully established in rabbits through high fat diet feeding (10). In the Rosuvastatin treated group, as a positive control, the degree of AS was mitigated as indicated by the thinner endarterium and decreased foam. PrA alleviated the degree of AS and high dose of PrA had better effects than low dose of PrA (Figure 1).

Effects of PrA on the serum levels of TC, TG, LDL and HDL

In order to determine the influence of PrA on hyperlipidemia, serum biochemical indices, TC, TG, LDL and HDL were detected (Figure 2). Compared to healthy control (Mock), the model group showed a significant increase in TC, TG and LDL and a notable decrease in HDL, suggesting the hyperlipidemia in model group. In Rosuvastatin group (positive control), the levels of TC, TG and LDL were decreased, and the levels of HDL were increased in a time-dependent manner. In the groups treated with PrA, the serum levels of the four indices were changed in a time and dose dependent manner (n=12). Effects of high dose PrA and rosuvastatin on these serum biochemical indices were almost equal. It suggested that PrA might alleviate

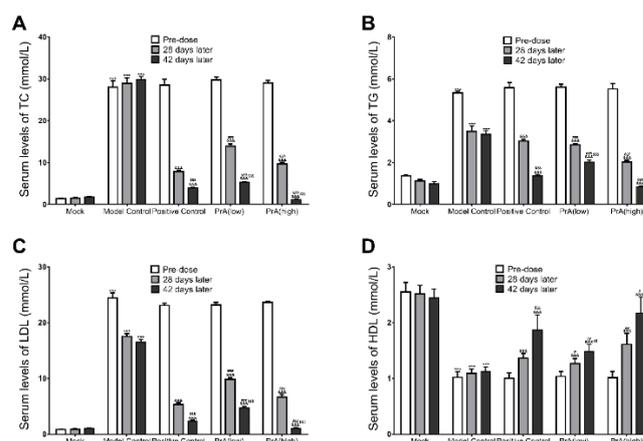


Figure 2. Protosappanin A markedly decreased serum levels of cholesterol (A), triglyceride (B) and low-density lipoprotein (C), and increased serum levels of high-density lipoprotein (D) in rabbits with hyperlipidemia in a time- and dose-dependent manner. n=12, mean \pm SD. *** $P < 0.001$ vs. Mock control in corresponding period; &&& $P < 0.001$ vs. Model control in corresponding period; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. positive control (Rosuvastatin group) in corresponding period; \$\$ $P < 0.05$, \$\$\$ $P < 0.001$ vs. 28 days later in the same medication group

AS by inhibiting hyperlipidemia like the positive control rosuvastatin (29).

PrA decreased the serum levels of inflammatory cytokines

In order to determine the influence of PrA on inflammatory cytokines, the serum levels of MMP-9, IL-6 and TNF- α were detected. Results showed that, compared with the healthy control (Mock), levels of all the three inflammatory cytokines were increased in the model group. Treatment with rosuvastatin and PrA for 42 days significantly decreased the levels of detected cytokines. The effects of PrA were in a dose dependent manner (n=12, $P < 0.001$). It suggested that PrA might alleviate AS through the way of anti-inflammation by reducing the serum levels of MMP-9, IL-6 and TNF- α (Figure 3).

PrA inhibited the nuclear NF- κ B p65 protein expression

To explore the possible molecular mechanisms how PrA alleviated AS, the nuclear NF- κ B p65 protein expression was examined. Results showed that compared with healthy control (Mock), NF- κ B was

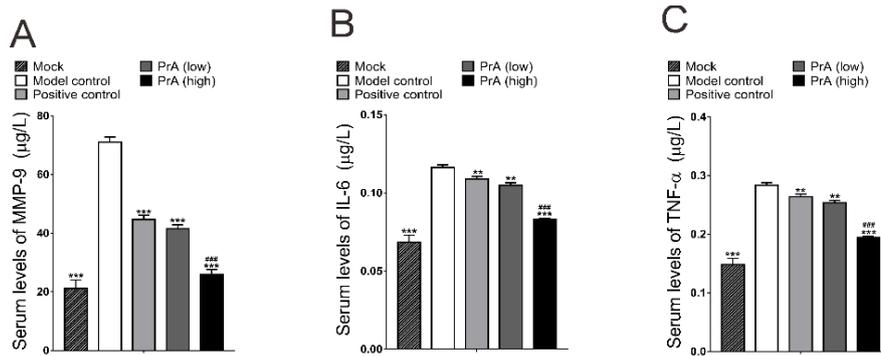


Figure 3. The serum levels of inflammatory cytokines were decreased by protosappanin A. Compared with the model control, levels of MMP-9 (A), IL-6 (B) and TNF-α (C) were decreased in rosuvastatin treatment groups (positive control), and were also decreased in PrA treatment groups in a dose dependent manner. n=12, mean±SD. **P<0.01, ***P<0.001 vs. model control; ###P<0.001 vs. positive control (Rosuvastatin group)

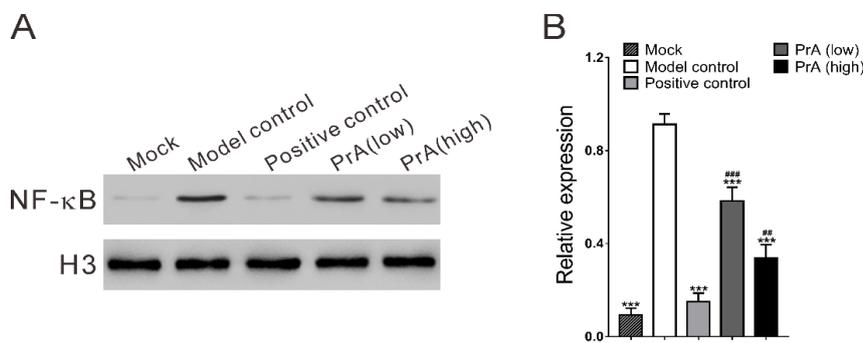


Figure 4. Effects of protosappanin A on the nuclear NF-κB p65 protein expression. A. The nuclear protein levels of NF-κB were down-regulated by PrA compared with the model and positive control. B. Quantitation of Western blot was performed by Image J software and shown. n=3, mean ± SD. ***P<0.001 vs. model control; #P<0.05, ###P<0.001 vs. positive control (Rosuvastatin group)

increased in the model group, and 42 days of treatment with PrA dose-dependently decreased NF-κB (Figure 4). The positive control rosuvastatin showed a more obvious effect than PrA. It suggested that PrA might alleviate AS by regulating NF-κB signaling pathway.

PrA suppressed the expression of IFN-γ and IP10

To get a further insight into the possible molecular

mechanisms of PrA on AS, the expression levels of IFN-γ and IP10 were detected as subsequent downstream genes of NF-κB. Results showed that the expression levels of IFN-γ and IP10 were down-regulated by PrA with gene expression levels relative to the reference GAPDH (Figure 5A). Serum production of IFN-γ and IP10 was significantly decreased in PrA treatment group as indicated by ELISA assay (Figure 5B).

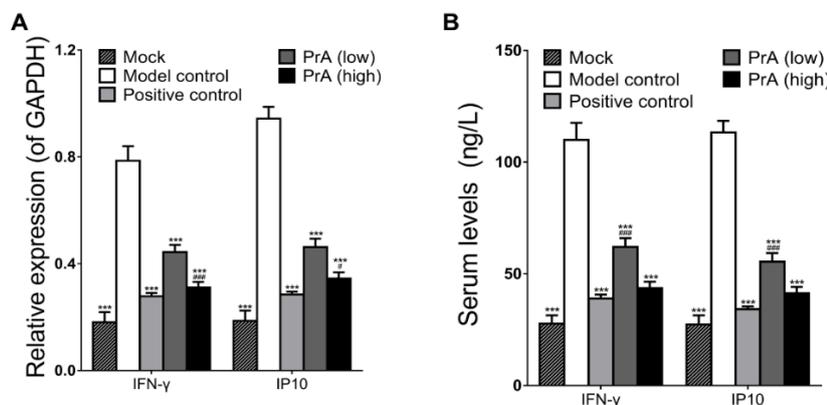


Figure 5. Protosappanin A suppressed the mRNA and serum levels of interferon-γ (IFN-γ) and interferon-gamma-inducible protein 10 (IP10). A. The mRNA levels of IFN-γ and IP10 were significantly decreased by PrA, compared with the model control. B. Serum production of IFN-γ and IP10 were inhibited in PrA treatment group. n=12, mean ± SD. ***P<0.001 vs. model control; #P<0.05, ###P<0.001 vs. positive control (Rosuvastatin group)

Discussion

Establishing the atherosclerosis (AS) model is the foundation of the study on measuring the effects of therapy. In this study, the AS model were successfully established in rabbits by high-fat-diet feeding according to the previous reports (10). Protosappanin A (PrA) is an active ingredient in the ethanol extract of *Caesalpinia sappan* L (5). The effects of PrA on AS have been not reported. In this current study, we showed that PrA inhibited high-fat-diet-induced AS. Hypertriglyceridemia were closely related with the pathologies of AS. Increase in blood lipids induces endothelial damage and lipid deposition in vascular wall, leading to the formation of plaques and AS (30). Therefore, we elucidated the effects of PrA on hyperlipidemia by measuring the serum levels of TC, TG, LDL and HDL. As expected, rabbits in model group were suffering from hyperlipidemia as compared to healthy control. Notably in treatment groups, the serum levels of TC, TG and LDL was significantly decreased in a time- and dose-dependent manner, while HDL levels were increased. Our data suggested that PrA might protect against AS through reducing hyperlipidemia (31).

In addition, studies have shown that AS is associated with the inflammation responses (32). For patients with hyperlipidemia, with the increase of blood lipid levels, the serum levels of inflammatory cytokines, such as TNF- α and IL-6 will be correspondingly increased (33-35). TNF- α and IL-6 play a critical role in fat metabolism, involving in the inflammatory response. Excess of TNF- α and IL-6 can cause direct damage to vascular endothelial cells (36, 37). Moreover, MMP-9 plays an important role in the formation and rupture of the unstable AS plaques by promoting the degradation of extracellular matrix in fiber membrane of AS plaques (19-21). Here, in PrA treatment groups, the serum levels of MMP-9, TNF- α and IL-6 was significantly decreased, indicating that PrA might protect against AS by decreasing inflammation.

As Wu *et al.* reported, PrA can induce immunosuppression via NF- κ B signaling pathway (6), we further detected nuclear NF- κ B p65 protein expression. In our study, NF- κ B was greatly reduced by PrA treatment. These results were in line with the study of Wu *et al.* (6). IFN- γ and IP10, downstream genes of NF- κ B signaling (24), may promote the development of AS (18, 25). Here, we indicated that mRNA expression and serum concentrations of IFN- γ and IP10 were reduced in PrA-treated rabbits. The results supported our presumption that NF- κ B signaling pathway might involve in the immunosuppressive action of PrA, although it still needs to be explored further.

Conclusion

We established a rabbit model with AS. PrA alleviated AS, and markedly inhibited the serum levels of

TC, TG and LDL in a time and dose-dependent manner with HDL being increased. PrA decreased the serum production of the inflammatory cytokines, MMP-9, IL-6 and TNF- α , and might exerted its functions through NF- κ B signaling pathway. And it can be speculated that PrA might be a new drug during the treatment of AS.

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Conflict of interest

The authors declare that no conflict of interest exists.

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