Amygdalin Attenuates Atherosclerosis Progress Through Inhibiting of Toll-Like Receptors Expression and Activity

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Abstract: Toll-Like Receptors (TLRs) and TLR signaling cascade play a pivotal role in the innate immune responses, especially in initiation and progression of atherosclerosis. Thus in the present study, researchers examined the anti-atherosclerosis effects of amygdalin and explored whether the anti-atherosclerosis function was achieved via inhibiting TLRs expression and TLR signaling. ApoE knockout mice were fed on high fat diet and received daily injection of amygdalin. By comparing lipid profiles, atherosclerotic lesions and serum proinflammatory cytokine concentrations, mice untreated with amygdalin experienced more severe atherosclerosis compared with mice received amygdalin treatment. TLR2 and TLR4 mRNA and protein levels were found to be reduced after amygdalin treatment. Expression of key MyD88-dependent signaling components was inhibited by amygdalin. Amygdalin may play a protective role in the development of atherosclerosis via the inhibition of TLR2 and TLR4 expression.

Keywords: Amygdalin, Toll-Like Receptor (TLR), MyD88-dependent signaling, atherosclerosis, mice, protein

INTRODUCTION

Atherosclerosis is an inflammatory disease of the arterial wall. Hypercholesterolemia, smoking, male gender, hypertension, diabetes and age were traditionally considered as risk factors. However, recent research with accumulating evidence suggests that activation of inflammatory genes in the vessel wall with subsequent adhesion, chemo-atraction, subendothelial migration, retention and activation of inflammatory and immune cells such as monocytes and T cells are believed to play a critical role in the initiation, progression and destabilization of atherosclerosis (Binder et al., 2002; Hansson, 2005). The complex interplay between inflammatory cells, cytokines and degrading enzymes may lead to atherosclerotic lesion progression and unstable plaque formation or even plaque rupture eventually (Davies and Thomas, 1985). Toll-Like Receptors (TLRs) are important because of their diverse functions in the regulation and linking of immune and inflammatory processes (Miggin and O’Neill, 2006; Garcia and Marshall, 2005; Lim et al., 2009; Baatartsogt et al., 2011; Huang et al., 2011). TLRs, widely distributed on immune cells such as monocytes, macrophages, dendritic cells, neutrophils and B cells as well as mucosal epithelial and endothelial cells (Verstak et al., 2007) are a family of receptors that activate proinflammatory signaling pathways in response to microbial pathogens or pathogen-associated molecular patterns. Accumulating data from animal studies suggest the potential importance of Toll-Like Receptors (TLRs) and other key components of the innate immune system in atherosclerosis-based pathologies (De Kleijn and Pasterkamp, 2003). Intracellular propagation of TLRs on the cells by specific ligands leads to the increase in the expression of inflammatory mediators such as Interleukin-1 (IL-1), Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), Monocyte Chemoattractant Protein-1 (MCP-1), etc. via Nuclear Factor-κB (NF-κB) (Trinchieri and Sher, 2007) thereby influencing inflammatory responses (Zhang and Ghosh, 2001). A total deficiency of TLR4 is associated with reductions in lesion size, lipid concentration and macrophage infiltration in ApoE knockout mice fed a high cholesterol diet for 6 months (Michelsen et al., 2004). Collectively, TLRs recognize a variety of pathogen-associated microbial patterns. TLR-2 is essential for the recognition of bacterial lipoproteins whereas TLR-4 is activated by lipopolysaccharide. Because TLRs are expressed in a plethora of inflammatory diseases such as sepsis syndrome, asthma, rheumatoid arthritis, atherosclerosis, Systemic Lupus Erythematosus (SLE) and inflammatory bowel disease (O’Neill, 2003) and both TLR2 and TLR4 are present in human atheroma, diabetes and also have been shown in animal models to promote atherosclerosis (Edfeldt et al., 2002; Xu et al., 2001; Matijevic et al., 2011) here in we mainly studied on whether the anti-atherosclerotic effect of amygdalin relied on the inhibition of TLRs.

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Amygdalin (Vitamin B17 also called Laetrile) is extracted from Semen Persicae, the seed of Prunus persica L. Batsch. Besides its antitumor activity (Chang et al., 2006; Laster and Schabel, 1975; Milazzo et al., 2007), it has also been used for the treatment of asthma, bronchitis, emphysema, leprosy, diabetes and relieving the pain (Heikkila and Cabbat, 1980; Baroni et al., 2005; Hwang et al., 2008; Matin et al., 2001). Furthermore, Semen Persicae is a major component of Xuefu Zhuyu decoction which could relieve the symptom of atherosclerosis according to Traditional Chinese Medicine theory (TCM) and practice and amygdalin is the major compound extracted from Semen Persicae. The previous studies have indicated the therapeutic potential of amygdalin in ApoE knockout mice under hot or cold factors (Jiafang et al., 2011). Thus, the aim of this study was to examine whether amygdalin has antiatherosclerotic effect in vivo. Given the importance of TLR2 and TLR4 in atherosclerosis, we tested the effects of amygdalin on the activity of TLR2 and TLR4 in ApoE knockout mice.

**MATERIALS AND METHODS**

**Animals:** About 8 weeks old male ApoE deficient male mice on a C57BL/6 background (Plump et al., 1992) and their littermates were purchased from Jackson Laboratories and kept in 12 h light/dark cycle with free access to water and food. For C57BL/6 mice, a normal chow diet (containing 10 fat, 15 protein and 75% carbohydrate) was given. ApoE knockout mice fed a high fat diet (containing 40 fat, 14 protein and 46% carbohydrate) were randomly subdivided into two groups (amygdalin treated group and untreated group, 10-14 mice per group) for determining atherosclerosis development. Based on the results of the pilot study, we used a slightly higher dose of 10 mg kg\(^{-1}\) body weight delivered continuously for 1 month. All experiments were performed according to Sichuan University Medical Center Institutional Animal Care and Use Committee guidelines.

**Assessment of atherosclerosis in aortas and aortic sinus:** Aortas were excised and adherent (adventitial) fat was removed and were then fixed in HistoChoice (Amresco). Whole aortas were opened longitudinally from the aortic arch to the iliac bifurcation, mounted en face and stained for lipids with Oil red O. Hearts were embedded in OCT compound (Tissue Tek) and cross sections of the aortic sinus were stained with Oil red O. In a separate experiment, cross-sections (7 μm thick) of the aortic sinus were stained with hematoxylin-eosin. Detection of MCP-1 in aortic sections was accomplished by using antibody to mouse MCP-1 (10 ng mL\(^{-1}\), Abcam).

Digital micrographs were taken. Total area of atherosclerotic plaque in the aortic arch and the whole aorta was determined from on-screen images using Image Pro Plus 6.3 Software (Media Cybernetics). Image analysis was performed by a trained observer blinded to experimental groups.

**Lipid profiles:** Sera from mice were obtained at the time of killing, after an overnight fast. Total Cholesterol (TC) concentrations were determined in duplicate by using a colorimetric assay (infinity cholesterol reagent and Thermo Fisher). Triglyceride (TG) concentrations were determined by using the L-type triglyceride H assay according to the manufacturer’s instructions (Wako Chemicals). Low Density Lipoprotein-cholesterol (LDL-c) and High Density Lipoprotein-cholesterol (HDL-c) concentrations were determined by the CHOD-PAP Method (Shensuo, Shanghai).

**Serum levels of cytokines and chemokines:** Interleukin-12p40 (IL-12p40), MCP-1, TNF-α and IL-6 concentrations in the sera of mice were measured by high sensitive ELISA assays according to the manufacturer’s instructions (BD Biosciences for IL-12p40 and MCP-1 and R and D Systems for TNF-α and IL-6).

**Macrophage isolation:** Since, macrophage infiltration occurs in the early stage (3 months of age) of atherosclerosis development in vivo (Zhang et al., 1992) the animals were euthanized and peritoneal macrophages were isolated for TLR2 and TLR4 analysis after 1 month of drug delivery.

**Fluorescence Activated Cell aorter (FACS) analysis of TLR2 and TLR4:** Macrophages were treated with TLR2 agonist (S. aureus lipoteichoic acid, SLTA, 2 μg mL\(^{-1}\), Invitrogen) or TLR4 agonist (LPS, 100 ng mL\(^{-1}\), Sigma) in the presence or absence of amygdalin treatment. Following treatment, cells were washed twice with cold PBS and then incubated with anti-mouse TLR2 antibody (eBioscience) and anti-mouse TLR4 antibody (eBioscience) or isotype matched IgG controls (mouse IgG2a, eBioscience) in accordance with manufacturer’s instructions. Total 10,000 stained cells were analyzed with BD FACS Array Bicanalyzer (BD Biosciences) with CellQuest Software (BD).

**Reverse Transcription Polymerase Chain Reaction (RT-PCR):** Total RNA was prepared by RNeasy kit following the manufacture’s instructions (Qiagen). About 2 μg of RNA was used for cDNA synthesis. β-actin cDNA from each sample was used to normalize the samples for
differences in PCR efficiency. cDNA was amplified with Taq polymerase (Takara) and appropriate primers at 94°C for 40 sec, 60°C for 30 sec and 72°C for 40 sec for 30 cycles with an initial 5 min denaturation at 95°C and final 10 min extension at 72°C. The primers for TLR2 were 5'-GCAAACTTGGTTGATTGG-3' and 5'-TTGAAATGC TCCAGTCCTG-3'. The primers for TLR4 were 5'-GGAAG TTTCTCTGACTAACAAGTCTTAGA-3' and 5'-AAATGG TGAACACATTGAGTTCC-3'. The primers for β-actin were 5'-ATCCGTAAGAACTCTCTGCGC-3' and 5'-AACGCAGCTCAGTAACAGTC-3'. TLR2 and TLR4 yield a band between 450-700 bp and β-actin yield a band at 286 bp on 1.5% agarose gels. Band intensities were determined using Image Quant Software (GE).

**Immunoblotting:** Whole-cell lysates were prepared. Protein content was assessed by Bradford assay. Equal amounts of proteins were resolved on 4-12% NuPAGE Novex Bis-Tris Pre cast gels (Invitrogen) followed by electrophoresing onto polyvinyl difluoride Membrane (Millipore). Expression of TLR signaling proteins was assessed via anti-MyD88 (Abcam), anti-IL-1 receptor-associated kinase-1 (IRAK1, Cell Signal), anti-p65 (Abcam) and anti-β-actin (Abcam) antibodies and horseradish peroxidase-conjugated donkey anti-rabbit antibody (Jackson Immunolab) and visualized by enhanced chemiluminescence reagents (Amersham).

**Statistical analysis:** Data were expressed as mean±SEM. Statistical significance was determined by one-way ANOVA with Bonferroni correction and Student’s t-test. p<0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

**Amygdalin reduces serum cholesterol and triglyceride levels:** To study the role of amygdalin in the development of atherosclerosis, ApoE knockout mice and control littermates were chosen for this study. Mice were started on a high fat diet (at age 8 weeks) and then intraperitoneally injected amygdalin (10 mg kg⁻¹ body weight, dissolved in 0.9% NaCl) everyday for 1 month. High fat diet was maintained for 1 month until the time of sacrifice. To evaluate the effect of amygdalin on serum lipid concentrations, we measured total serum cholesterol concentrations. Significant decreases in serum triglyceride (Fig. 1a) and total cholesterol (Fig. 1b) concentrations were found after amygdalin treatment. Although, serum HDL-c concentrations were similar in amygdalin treated or untreated mice (Fig. 1c) but there were significant differences in LDL-c concentrations between amygdalin treated and untreated groups of mice (Fig. 1d).

**Fig. 1:** Lipid content in aortic sinus plaques is reduced after amygdalin treatment. Quantification of the; a) TC levels; b) TG levels; c) HDL-c levels and d) LDL-c in aortic plaques from ApoE knockout mice with and without amygdalin therapy (left and right, respectively). Shown are means and SD of the percentage of lipid content relative to total plaque areas (n = 10 per group). Relative lipid content in mice treated with amygdalin is significantly reduced compared with untreated mice. Data are presented as mean value±SEM; n = 10-12 for each group (**p<0.01)

**Amygdalin reduces the extent of aortic atherosclerosis in ApoE knockout mice:** Because ApoE knockout mice
fed high fat diet develop atherosclerotic lesions with morphological characteristics similar to those seen in humans (Breslow, 1996), herein in this study, ApoE knockout mice were given a diet that contained 40% fat, 14 protein and 46% carbohydrate. To evaluate the effect of amygdalin on the extent of aortic atherosclerosis, we measured the total lesion area by en face preparation of the aorta and Oil red O staining for lipids (Fig. 2a). Quantification of the lesion area of aortic sinus revealed a significant 66% reduction in lesion size in amygdalin-treated mice (Fig. 2b). Because of overcompensatory enlargement, a lumen area increase was observed after amygdalin treatment (p<0.05, Fig. 2c) and there was a significant reduction in plaque area (p<0.05, 48% average decrease, Fig. 2d) and plaque coverage percentage (p<0.05, 35% average decrease, Fig. 2e) after amygdalin treatment as well. So the athero-protective effect of amygdalin was proved by the decreased extent of atherosclerosis.

**Amygdalin results in diminished atherosclerotic plaques:** To further examine the effect of amygdalin on the composition of atherosclerotic plaques, histochemical and immunohistochemical analysis of aortic sinus lesions were performed. Amygdalin-treated mice exhibited a marked reduction in the thickness of the aortic sinus lesions (Fig. 3a and b), compared with ApoE deficient mice without amygdalin treatment. Representative pictures were taken under 40 and 100 x magnifications. Compared with amygdalin-treated mice, untreated mice also demonstrated more MCP-1 expression (Fig. 3c) in the endothelium and sub-endothelial lesions of aorta sinus plaque. Taken together, these data indicate that amygdalin treatment resulted in reduced plaque formation and less MCP-1 expression in the aortic sinus.

**Amygdalin treatment is associated with reduced levels of proinflammatory cytokines:** In order to investigate whether the anti-atherosclerotic effect of amygdalin...
Fig. 3: Amygdalin reduces expression of atherosclerosis markers in the aortic sinus. Representative staining of aortic sinus plaques from mice treated with amygdalin (left) and untreated with amygdalin (right). Sections were stained with H and E (a, 40x magnification; b, 100x magnification) and mouse MCP-1 (c, 100x magnification). The scale bar represents 100 μm.

depended on an anti-inflammatory mechanism, we therefore determined the circulating concentration of key proinflammatory cytokines and chemokines in mice with or without amygdalin treatment. ELISAs were performed to measure serum levels of TNF-α (Jovinge et al., 1996) IL-6 (Huber et al., 1999), IL-12p40 (Lee et al., 1999) and MCP-1 (Harrington, 2000). Amygdalin-treated mice had markedly reduced serum levels of MCP-1 (Fig. 4a), IL-12p40 (Fig. 4b), TNF-α (Fig. 4c) and IL-6 (Fig. 4d) compared with untreated mice.

Amygdalin treatment decreases TLR2 and TLR4 mRNA expression both in aorta and in peritoneal macrophages:

To confirm whether the anti-atherosclerotic function of amygdalin was achieved via TLR signaling pathway, RT-PCR was performed on aortic arch, the predominant site of atherosclerotic lesions. Constitutive TLR2 and TLR4 gene expression was detected in the aorta arch of untreated ApoE knockout mice but not in amygdalin treated mice (Fig. 5a and b). Judged from densitometric analysis of TLR2 mRNA and TLR4 mRNA in amygdalin treated or untreated mice, the ratio of TLR2/β-actin and TLR4/β-actin mRNA in aorta of treated mice were decreased significantly compared with that of amygdalin untreated mice (Fig. 5c and d). Because the earliest type of lesion is a pure inflammatory lesion, consisting only of monocyte-
Fig. 5: Evaluation of TLR2 and TLR4 expression in mice with or without amygdalin treatment. Representative RT-PCR of: a) TLR2 mRNA (left) and b) TLR4 mRNA from the aortic arch of mice treated or untreated with amygdalin, n = 4 in each group of mice. Lane 1, wild type C57/B6 mice; Lane 2, ApoE knockout mice without amygdalin treatment; Lane 3, ApoE knockout mice received amygdalin treatment for 1 month. Densitometric analysis of TLR2 mRNA and TLR4 mRNA in amygdalin treated or untreated mice (c and d). Each bar represents the average ± SEM of two band intensities (n = 4 separate experiments; *p<0.05, **p<0.001).

derived macrophages and T lymphocytes (Stary et al., 1994), researchers then examined TLR2 and TLR4 expression in peritoneal macrophages of ApoE knockout mice with or without amygdalin treatment. Significantly reduced TLR2 or TLR4 positive cells in macrophages of amygdalin-treated mice were observed (Fig. 6a and b).

Fig. 6: TLR2 and TLR4 expression is inhibited by amygdalin treatment. Macrophages were harvested and cultured with: a) 2 μg mL⁻¹ SLTA; b) 100 μg LPS, TLR2 and TLR4 surface expression was determined by FACS analysis using specific antibody and isotype control. Data are presented as mean values±SEM; n = 3 for each group. (*p<0.05, **p<0.01). c) Representative western blot of MyD88, phospho-IRAK1, IRAK1, p65 and β-actin protein in macrophages of amygdalin treated and untreated mice.
Amygdalin inhibits MyD88-dependent signaling: To further investigate whether TLR mediated MyD88-dependent signaling pathway (Michelsen et al., 2004) was activated in amygdyalin-treated mice, we determined protein levels of MyD88, phospho-IRAK1, IRAK1 and p65 by Western blotting. MyD88 and NF-κB are common downstream signaling components of both TLR2 and TLR4 pathways. In macrophages, amygdyalin significantly inhibited phosphorylation of IRAK-1 and decreased cytoplasmic MyD88 protein expression level and nuclear p65 protein expression level (Fig. 6c). However, the expression levels of TRIF were unchanged. Therefore, the inhibitory effects of amygdyalin on TLR2 and TLR4 could be judged from the deactivation of MyD88-dependent pathway.

Accumulating evidences in epidemiologic and experimental studies implicate a fundamental link of infection with the development of atherosclerosis and cardiovascular diseases. Notwithstanding, the precise mechanisms contributing to this link have remained elusive, identifying novel therapeutic drugs for the prevention and treatment of atherosclerosis still needs to be further explored. Herein, we demonstrated the antiatherosclerotic effect of amygdyalin using an ApoE deficient mouse model. To testify the athero-protective function of amygdyalin, plaque composition and lipid content were investigated in this study. Furthermore, the findings of reduced proinflammatory cytokine as IL-12p40, MCP-1, IL6 and TNF-α suggested that the athero-protective effect of amygdyalin might result from reduced inflammation. Together, these data suggested that amygdyalin could directly or indirectly inhibit development of atherosclerosis and promote a more stable plaque phenotype.

However, increasing the understanding of the antiatherosclerotic effect of amygdyalin will require the combination of atherosclerosis-based pathologies and related signaling networks. Several studies have shown that macrophages and chemokines, especially MCP-1, play a crucial role on the development of atherosclerosis (Chow and Taubman, 2004). Clinically, in a large cohort of patients with acute coronary syndromes, an elevated baseline level of MCP-1 was associated with multiple stages of atherosclerosis (De Lemos et al., 2003). Meanwhile, strong expression of MCP-1 was found in a small subset of cells in macrophage-rich regions of human and rabbit atherosclerotic lesions, suggesting an active role for this molecule in monocyte recruitment (Yu-Herttuala et al., 1991). Inactivation of MCP-1 or its receptor, CCR2 resulted in markedly reduced macrophage accumulation and attenuated the progression of dietary-induced atherosclerosis in mice (Gosling et al., 1999). It is important to note that these results together with the data presented in this study support a critical role of MCP-1 in the antiatherosclerotic function of amygdyalin.

Recently several investigators have shown that TLR signaling plays an important role in progression of atherosclerosis (De Kleijn and Pasterkamp, 2003; Michelsen et al., 2004). Some researchers found that TLR4 can directly interfere with cholesterol metabolism in macrophages (Castrillo et al., 2003), so we proposed that the reduction of cholesterol level in amygdyalin-treated mice could be attributed to the decreased expression of TLRs. It is important to note that in humans, the abundance of TLR2 and TLR4 has been testified by an immunohistochemical and mRNA transcript survey of aorta and the expression of TLR4 is found up-regulated in human atherosclerotic lesion (Edelst et al., 2002). So studies on TLR2 and TLR4 mRNA expression levels and related signaling pathway are of vital importance. In lesions, macrophages, dendritic cells, endothelial cells and vascular smooth muscle cells can all express TLR2 and TLR4 (Akashi et al., 2000). We examined TLR2 and TLR4 mRNA expression both in aorta arch and in macrophages with or without amygdyalin treatment. The findings are consistent with a number of studies exploring the TLRs expression in atherosclerosis and we found the elevated expression of TLRs could be partially inhibited by amygdyalin therapy. Thus, we suggest that the athero-protective mechanisms of amygdyalin are attributable to decreasing inflammation by inhibiting TLR2 and TLR4 expression.

TLR signaling cascade can be divided in Myeloid differentiation factor MyD88-dependent and MyD88-independent pathways. In the MyD88-dependent pathway, after the binding of TLR with MyD88 which induces the phosphorylation of IRAK1, NF-κB is subsequently released and thus activates transcription of different proinflammatory genes. MyD88 deficiency leads to reductions in plaque size, lipid content, expression of proinflammatory genes, cytokines and chemokines such as IL-12 and MCP-1 (Michelsen et al., 2004). In this study, we observed reduced MyD88-dependent activation with concomitant reduction in key inflammatory production as IL-12, MCP-1, IL-6 and TNF-α. Furthermore, evidence for the involvement of NF-κB in the development of atherosclerosis is substantial (Collins and Cybulsky, 2001) and in this study NF-κB p65 protein level was significantly decreased in amygdyalin-treated mice indicating the involvement of NF-κB p65-dependent activation. The results are consistent with the notion that MyD88-dependent pathway is activated in atherosclerosis and demonstrate that TLR and MyD88 signaling could be one important contributor to the anti-
atherosclerosis mechanisms of amygdalin. In MyD88-independent pathway, TLR signals through Toll/IL-1 Receptor (TIR) domain-containing adaptor (TRIF) protein that induces inflammatory cytokine production (Yamamoto et al., 2003). However, we found there were no differences in TRIF protein expression levels between amygdalin treated and untreated groups, suggesting that the anti-atherosclerosis function of amygdalin may not rely on MyD88-independent pathway.

CONCLUSION

In the present study, researchers show that amygdalin decreases TLR2 and TLR4 protein level and mRNA expression, reduces activation of MyD88-dependent pathway with concomitant reduction in key inflammatory production in vivo. Thus, we suggest that the anti-atherosclerosis mechanisms of amygdalin are ascribed to decreasing inflammation by inhibiting TLR2 expression and signaling cascade. This study shed light on the therapeutic function of amygdalin and its inhibition of TLR signaling in atherosclerosis treatment. However, further investigations is needed to clarify the precise mechanisms by which amygdalin inhibit TLR2 and TLR4 expression. Whether the expression of scavenger receptors, as macrophage scavenger receptor is involved in amygdalin induced athero-protective function will be an intriguing question to be addressed by future studies.

REFERENCES


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