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Anti-Inflammatory Mechanisms of the Tibetan Herbal Preparation Padma 28 in the Vessel Wall

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Key Words
Padma 28 · Atherosclerosis · Inflammation · C-reactive protein · E-Selectin · Heme oxygenase-1

Summary
Background: The Tibetan herbal preparation Padma 28 has been shown to act as an anti-atherosclerotic agent in advanced peripheral arterial occlusive disease. We tested the effect of aqueous Padma 28 extracts on both the C-reactive protein (CRP) induced expression of the pro-inflammatory cell adhesion molecule E-selectin and the anti-atherosclerotic protective enzyme heme oxygenase-1 (HO-1) in human aortic endothelial cells. **Methods and Results:** According to FACS analysis, quantitative RT-PCR and Western blot, CRP-induced E-selectin expression was completely prevented by aqueous Padma 28 extracts. Additionally, Padma 28 mediated an up to 60-fold upregulation of HO-1 mRNA as measured by quantitative RT-PCR. This upregulation could also be demonstrated on the protein level. **Conclusion:** Aqueous extracts of the Tibetan herbal preparation Padma 28 inhibit CRP-induced expression of the inflammatory cell adhesion molecule E-selectin and lead to upregulation of the vascular protective enzyme HO-1 in human aortic endothelial cells. These properties may be responsible for its anti-atherosclerotic effects in peripheral arterial occlusive disease.

Schlüsselwörter
Padma 28 · Arteriosklerose · Entzündung · C-reaktives Protein · E-Selektin · Hämoxygenase-1

Zusammenfassung
Hintergrund: Die tibetische Kräuterformel Padma 28 ist als wirksames Therapeutikum bei fortgeschrittener peripherer arterieller Verschlusskrankheit beschrieben worden. Wir untersuchten den Einfluss wässriger Padma-28-Extrakte auf die durch C-reaktives Protein (CRP) ausgelöste Expression des entzündungsassoziierten Zelladhäsionsmoleküls E-Selektin und des antiatherosklerotischen Schutzenzyms Hämoxygenase-1 (HO-1) in humanen aortalen Endothelzellen. **Methoden und Ergebnisse:** In FACS-Versuchen verhinderten wässrige Padma-28-Extrakte die CRP-induzierte Expression von E-Selektin an der Zelloberfläche. Dieser Effekt konnte auch in quantitativen RT-PCR-Versuchen auf mRNA-Ebene und im Western Blot gezeigt werden. Zusätzlich induzierte Padma 28 eine bis zu 60-fache Hochregulierung der HO-1 mRNA, ein Ergebnis, das auch auf der Proteinebene im Western Blot bestätigt werden konnte. **Schlussfolgerungen**: Wässrige Extrakte von Padma 28 führen zur Inhibition der CRP-induzierten E-Selektin-Expression und zur Hochregulierung des Schutzenzyms HO-1 in Endothelzellen. Diese Eigenschaften könnten zur antiatherosklerotischen Wirkung von Padma 28 bei peripherer arterieller Verschlusskrankheit beitragen.

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Introduction

Although atherosclerosis has long been considered a lipid storage disease, it is now widely accepted that it is a complex process that involves an ongoing inflammatory response [1]. Recent studies have established the fundamental role of inflammation for the onset, development and progression of the atherosclerotic lesion [2]. Elevated serum levels of C-reactive protein (CRP) [3, 4] have been considered to be sensitive, though unspecific markers of acute inflammation and are suggested as an independent risk factor for atherosclerosis and coronary heart disease [5–7]. CRP, at concentrations known to predict vascular disease, has multiple proinflammatory effects on cells of the vascular wall, favoring a proatherosclerotic phenotype [8–11]. CRP upregulates adhesion molecules on endothelial cells [8], including E-selectin that is involved in the earliest steps of atherogenesis [12]. The pro-atherogenic action of CRP extends beyond the endothelium to the vascular smooth muscle cells, where it upregulates angiotensin receptor 1, and stimulates smooth muscle cell migration and proliferation [13].

Cells of the vascular wall respond to injury by upregulating specific stress proteins, including heme oxygenase-1 (HO-1), a protective enzyme that has important anti-inflammatory, anti-apoptotic and anti-proliferative properties [14–17]. Some of these effects seem to be mediated by carbon monoxide generated by the enzyme [18, 19].

Padma 28 is a traditional Tibetan herbal medicine based on a *Gabur* (camphor) formula consisting of 20 different powdered herbal drugs, natural camphor and calcium sulphate [20]. In Tibetan medicine, *Gabur* formulas are used against different types of 'fevers' that could be equated with low-level acute and chronic inflammations. Consequently, Padma 28 has been shown to improve symptoms and to significantly increase maximal and pain-free walking distance in patients with peripheral arterial occlusive disease (PAOD) in randomized, double-blind studies [21–24].

We studied the effect of Padma 28 on the CRP-induced expression of E-selectin and the induction of HO-1 in human aortic endothelial cells.

Materials and Methods

Reagents: Endothelial cell (EC) media were from Clonetics® (Clonetics EGM-2 MV; Cambrex, East Rutherford, NJ, USA); Trypsin/EDTA and M-MLV reverse transcriptase were from Invitrogen™ (Carlsbad, CA, USA). Random hexamers and ECL reagents were purchased from Amersham Biosciences (Piscataway, NJ, USA). Capillaries and Fast Start DNA Master SYBR Green I kit were from Roche Diagnostics (Vienna, Austria). Collagenase H and hemin chloride were from Sigma (Vienna, Austria). Combi Reagent negative control was purchased from R and D Systems (Minneapolis, MN, USA) and RNeasy Mini Kit was from Qiagen (Hilden, Germany). Recombinant CRP was purchased from Calbiochem (Merck Biosciences, Nottingham, UK).

Cell culture: For the isolation of human aortic endothelial cells (HAEC), aortic tissue from organ donors was incubated in 20 mmol/l phosphate-buffered saline containing 50 mg of collagenase H per ml, for 30 min at 37 °C. Cells were sieved through a 100 µm mesh and plated on petri dishes in EGM-2 MV EC medium. After 12 h, the non-adherent cells were removed, and fresh medium was added to the adherent cells. The medium was changed every 3 days. After 5–10 days, the adherent cells were detached from the petri dish by incubation with 0.25% trypsin and 1 mmol/l EDTA and incubated with magnetic beads that were coated with anti-CD31 antibody (Dynal; Oslo, Norway), according to the manufacturer's instructions. Microbeads with bound cells were replated on petri dishes. The beading procedure was repeated after another 5–10 days of culture. Passage 3–8 HAECs were used for experiments.

Aqueous extracts of Padma 28: Powdered Padma® 28 (Padma AG; Schwerzenbach, Switzerland) (0.5 g) was mixed in a total volume of 10 ml in EGM-2 MV medium in a shaking incubator at 37 °C for 15 min. After vortexing for 2 min, the suspension was centrifuged at 4,000 rpm for 10 min. The supernatant was filtered through a 0.80 µm syringe driven filter unit and afterwards through a 0.22 µm micropore filter and immediately used for stimulation assays.

Stimulation of EC: Before stimulation, cells were exposed for 2 h to RPMI-1640 medium containing 100 U/ml penicillin, 100 µl/ml streptomycin, 5 µg/ml fungizone and 1% L-glutamine with 5% FCS. Stimulations were conducted with the same medium containing different mediators for 5 h for the surface antigen assay, the real-time RT-PCR, and for Western blot analysis.

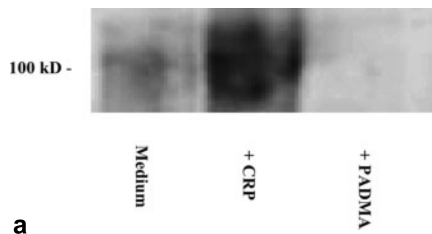
E-selectin expression on EC: To evaluate EC-surface expression of E-selectin after exposure to CRP (50 µg/ml) and Padma 28 (aqueous extract 1 : 4 and 1 : 8 in medium), cells were analyzed by flow cytometry. Confluent EC were detached by use of a collagenase-treatment (type Ia, 200 U/ml final concentration) for 10 min, washed twice in ice-cold PBS, and exposed to FITC labeled mAb directed against E-selectin (R&D Systems) for 30 min at 4 °C. Then, cells were washed twice in ice-cold PBS and analyzed. The reactivity of the antibodies with EC was determined by flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA). Irrelevant anti-IgG1+IgG2a (Combi Reagent, R&D) was used as negative control.

RNA-isolation and cDNA preparation: RNA was isolated from stimulated HAEC using Rneasy Mini Kit according to the protocols of the manufacturers. 1 µg of total RNA was reverse transcribed with M-MLV-RT using random hexamer primers.

Quantitative RT-PCR: Human E-selectin and HO-1 primers were designed using PRIMER 3 software from the Whitehead Institute for Biomedical Research [25]. The testing of primer specificity included melting curve analyses, agarose gel electrophoresis of the PCR products, and subsequent DNA sequencing. Quantitative RT-PCR was performed by Light Cycler technology (Roche Diagnostics) using SYBR Green I detection. In all assays, cDNA was amplified using a standardized program: (10 min denaturing step; 50 cycles of 15 min at 95 °C, 5 min at 63 °C and 25 min at 72 °C; melting curve analysis in 0.1 °C steps; final cooling step). Light Cycler capillaries were each loaded with 2 µl DNA Master Mix, 2.4 µl $MgCl_2$ (25 mmol/l), 12.8 µl H_2O, and 0.4 µl of each primer (10 µM). The final amount of cDNA corresponded to 40 ng of total RNA used for reverse transcription. Absolute quantification of target gene expression correlated to the house-keeping genes ABL and GAPDH, respectively, and was performed according to recommended standard procedures. The following primers were used:

HO-1 forward 5´-GTCTTCGCCCCTGTCTACTTCC-3´;
HO-1 reverse 5´-TTGGCCTCTTCTACTACCCTCTGC-3´;
E-selectin forward 5´-GGGACAACGAGAAGCCAACGTG-3´;
E-selectin reverse 5´-CGCATCTCACAGCTTCACATGTGG-3´;
ABL forward 5´-CAGCGGCCAGTAGCATCTGACTTTG-3´;
ABL reverse 5´-CCATTTTTGGTTTGGGCTTCACACCATTCC-3´;
GAPDH forward 5´-GAAGGTGAAGGTCGGAGTC-3´;
GAPDH reverse 5´-GAAGATGGTGATGGGATTTC-3´.

Western blot analysis: Confluent HAEC monolayers were exposed to CRP (50 µg/ml) with and without Padma 28 extract (1 : 4 in medium) for 5 h.



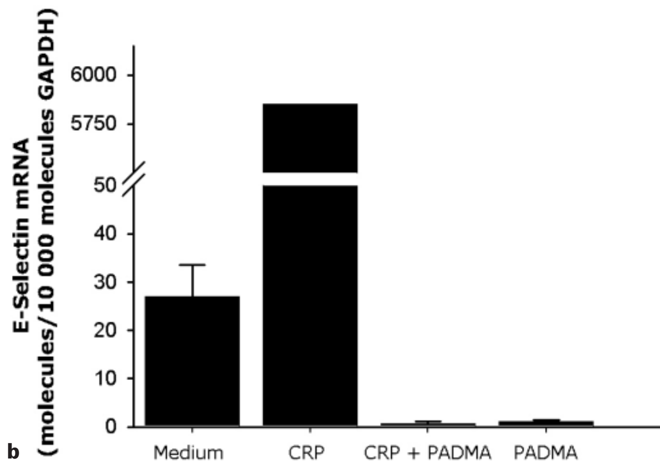
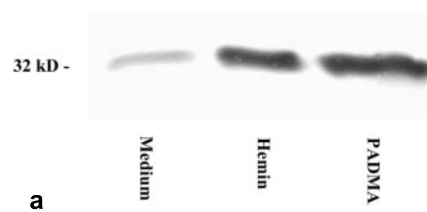


Fig. 1. Aqueous Padma 28 extracts inhibit CRP-induced E-selectin expression on HAEC: CRP induces E-selectin, while Padma 28 extracts inhibit CRP-induced E-selectin expression on HAEC as measured by **a** Western blot and **b** RT-PCR.



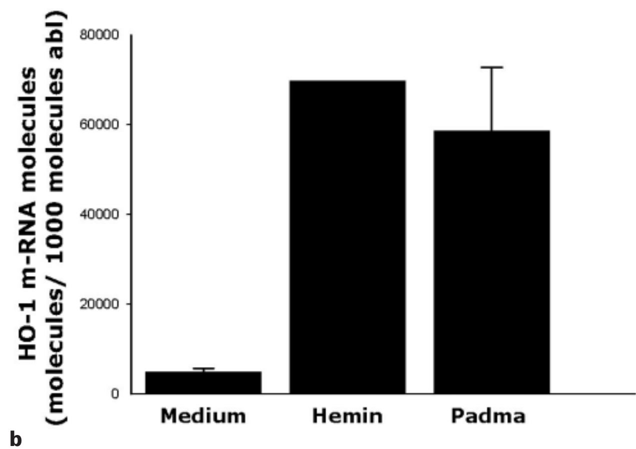


Fig. 2. Aquoeus Padma 28 extracts induce HO-1 expression in HAEC as assessed by **a** Western blot and **b** RT-PCR.

Thereafter, supernatant was removed and cells were lysed in 200 µl of Laemmli buffer. The proteins were separated by 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking membranes with blocking buffer (5% dry milk dissolved in PBS with 0.1% Tween 20), a sheep polyclonal antibody against E-selectin (AF724, 1 µg/ml, R&D Systems) or a rabbit polyclonal antibody against HO-1 (SPA-896, 1 : 5000; Stressgen, Victoria, BC, Canada) was applied for 30 min. Bound primary antibody was detected by the respective anti-IgG conjugated with peroxidase and subsequent chemoluminescent detection (ECL, Amersham).

Results

CRP enhanced the expression of the adhesion molecule E-selectin on endothelial cells, as demonstrated before [8]. A significant increase of E-selectin expression compared to unstimulated controls was achieved with a CRP concentration of 50 µg/ml and an incubation time of 5 hours as measured in the flow cytometric assay (data not shown) and in a Western blot analysis (fig. 1.a). In line with these results, E-selectin specific mRNA levels proved to be higher in CRP stimulated HAEC than in unstimulated controls, as measured by real time RT-PCR after an incubation time of 5 hours (fig. 1.b). The enhanced E-selectin expression by stimulation with CRP could be abrogated by the addition of aqueous Padma 28 extracts. Co-incubation of Padma 28 with CRP for 5 h completely inhibited E-selectin expression on HAEC in comparison to cells which were incubated with CRP only (flow cytometric assay, data not shown). The abrogation of CRP-induced E-selectin could also be shown by Western blot analysis (fig. 1.a). Consistently, E-selectin mRNA expression was inhibited by Padma 28 (fig. 1.b).

Incubation of HAEC with aqueous Padma 28 extracts markedly upregulated the expression of HO-1 mRNA levels as measured by RT-PCR (fig. 2.b) compared to untreated cells. Stimulation with hemin chloride (10 µmol/ml) served as positive control. Upregulation could also be shown by Western blot analysis (fig. 2.a).

Discussion

In this study we have shown that aqueous extracts of the Tibetan herbal preparation Padma 28 exert anti-atherosclerotic effects by downregulating CRP-induced E-selectin mRNA levels and E-selectin surface expression on human aortic endothelial cells and by upregulating HO-1 mRNA and protein levels. Padma 28 thereby counteracts the proatherosclerotic

action of CRP that has recently been discussed to be an active player in atherogenesis rather than merely a marker of inflammation.

CRP is an acute-phase protein whose serum levels increase rapidly up to 100-fold during acute inflammation. Even small increases can be used to predict progression of peripheral artery disease [26–29] or coronary events in apparently healthy subjects [30, 31] and patients with unstable angina [32–34]. CRP has been found to induce high levels of vascular adhesion molecules like E-selectin, ICAM-1 and VCAM-1 on endothelial cells [8]. This upregulation is an important factor in atherogenesis and adds to the inflammatory response in the vascular wall by recruiting monocytes and lymphocytes [35].

Our results confirm that Padma 28 interferes with one of the earliest steps in the development of the arteriosclerotic lesion, the rolling and tethering of white blood cells to the vascular endothelium that is mediated by E-selectin [36]. Since E-selectin mRNA levels were also diminished by Padma 28 extracts, we suggest that the anti-inflammatory influence on CRP-treated endothelial cells arises from a modulation of transcription rather than posttranslational modification. The downregulation of E-selectin may have clinical importance not only in arteriosclerotic disease, but also in other states of chronic inflammation where leukocyte entry into sites of inflammation is involved. This may explain the effectiveness of Padma 28 not only in PAOD [21–24, 37] but also in other inflammatory diseases like hepatitis, rheumatoid arthritis, multiple sclerosis and infections of the respiratory tract [38–41].

Our second finding that Padma 28 upregulates HO-1 mRNA and protein in arterial endothelial cells may have several clinical implications: heme oxygenases are the rate-limiting enzymes in heme degradation, the products of which have important biological functions [15, 16]. The inducible isoform HO-1 is increased as an adaptive response to several injurious stimuli including heme, hyperoxia, hypoxia, endotoxin and heavy metals [17]. In the vascular wall, HO-1 induction has anti-inflammatory and anti-apoptotic effects on endothelial cells and anti-proliferative effects on vascular smooth muscle cells [18, 19]. HO-1 induction by Padma 28 may therefore act as a protective mechanism targeting different pathogenetic pathways in the cascade leading to the arteriosclerotic lesion.

In conclusion, our findings indicate that the Tibetan herbal preparation Padma 28 inhibits CRP-induced expression of the inflammatory adhesion molecule E-selectin and leads to upregulation of the vascular protective enzyme HO-1 in human aortic endothelial cells. These properties may be responsible for its anti-atherosclerotic effects in peripheral arterial occlusive disease.

Limitations

Padma 28 is a complex multicompound medical product whose efficacy may be determined by the synergy of the 22 natural ingredients. We therefore did not determine the single components responsible for E-selectin suppression and HO-1 induction. Furthermore, aqueous extracts do not take into account lipid-soluble constituents that may also mediate the effects of Padma 28 in vivo.

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