Flavonoids from Artichoke (Cynara scolymus L.) Up-Regulate Endothelial-Type Nitric-Oxide Synthase Gene Expression in Human Endothelial Cells

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ABSTRACT

Nitric oxide (NO) produced by endothelial nitric-oxide synthase (eNOS) represents an antithrombotic and anti-atherosclerotic principle in the vasculature. Hence, an enhanced expression of eNOS in response to pharmacological interventions could provide protection against cardiovascular diseases. In EA.hy 926 cells, a cell line derived from human umbilical vein endothelial cells (HUVECs), an artichoke leaf extract (ALE) increased the activity of the human eNOS promoter (determined by luciferase reporter gene assay). An organic subfraction from ALE was more potent in this respect than the crude extract, whereas an aqueous subfraction of ALE was without effect. ALE and the organic subfraction thereof also increased eNOS mRNA expression (measured by an RNase protection assay) and eNOS protein expression (determined by Western blot) both in EA.hy 926 cells and in native HUVECs. NO production (measured by NO-ozone chemiluminescence) was increased by both extracts. In organ chamber experiments, ex vivo incubation (18 h) of rat aortic rings with the organic subfraction of ALE enhanced the NO-mediated vasodilator response to acetylcholine, indicating that the up-regulated eNOS remained functional. Caffeoylquinic acids and flavonoids are two major groups of constituents of ALE. Interestingly, the flavonoids luteolin and cynaroside increased eNOS promoter activity and eNOS mRNA expression, whereas the caffeoylquinic acids cyanin and chlorogenic acid were without effect. Thus, in addition to the lipid-lowering and antioxidant properties of artichoke, an increase in eNOS gene transcription may also contribute to its beneficial cardiovascular profile. Artichoke flavonoids are likely to represent the active ingredients mediating eNOS up-regulation.
Materials and Methods

Materials. Extracts from artichoke leaves were provided by Lichtwer Pharma AG (Berlin, Germany). Three types of extracts were used (Table 1). ALE was the dried supernatant of an aqueous extraction of artichoke leaves (commercially produced as L2220, Suprasens). An aqueous solution of ALE was further extracted with a mixture of ethyl acetate and n-butanol (2:1, v/v) resulting in an organic and an aqueous phase. The two phases were separated and the solvents were evaporated. This yielded two extracts, the organic subfraction (OSF) and the aqueous subfraction (ASF), respectively (Table 1).

Cynarin (1,3-dicaffeoylquinic acid) and cynaroside (luteolin-7-glucopyranoside) were obtained from AppliChem (Darmstadt, Germany); chlorogenic acid (5-O-caffeoylquinic acid) was obtained from Cayman Chemical (Ann Arbor, MI). Luteolin was obtained from Sigma (Taufkirchen, Germany). S-Nitroso-N-penicillamine (SNAP) and 5,6-dichloro-1-bromo-4-ribofuranosylbenzimidazole (DRB) were obtained from Merck (Darmstadt, Germany).

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion. HUVECs were cultured in endothelial cell growth medium (PromoCell, Heidelberg, Germany). HUVECs from passages 3 to 5 were used in the experiments. HUVEC-derived EA.hy 926 endothelial cells were grown under 10% CO₂ in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1× hypoxanthine, amethopterin/methotrexate, and thymine (Invitrogen, Karlsruhe, Germany) (Li and Förstermann, 2000b).

Analysis of eNOS Promoter Activity by Stable Transfection of EA.hy 926 Cells. A stable EA.hy 926 cell line was generated by transfection of EA.hy 926 cells with pGL3-eNOS-Hs-3500-neo, which contains a neomycin-resistance gene and a 3.5-kb promoter fragment of human eNOS driving the luciferase reporter gene (Li et al., 1998). Stable EA.hy 926 cells were cultured in medium containing 1 mg/ml compound G418. For analysis of eNOS promoter activity, the stably transfected cells were incubated with artichoke extracts for 18 h. Then, cell lysates were prepared and luciferase activities were determined as described (Li et al., 1998). The luciferase activity, normalized for protein concentration of cell lysates, was used as a determinant of eNOS promoter activity.

RNase Protection Assay for eNOS mRNA Analyses. Confluent HUVECs and EA.hy 926 cells were incubated with artichoke extracts for 18 h and total RNA was isolated. The expression of eNOS mRNA was analyzed by the RNase protection assay as described previously (Li et al., 1998; Li and Förstermann, 2000b).

Real-Time RT-PCR for eNOS mRNA Analyses. In some experiments, eNOS mRNA expression was analyzed with quantitative real-time RT-PCR using an iCycler iQ System (Bio-Rad, Munich, Germany). Confluent HUVECs and EA.hy 926 cells were incubated with cynarin, chlorogenic acid, luteolin, or cynaroside for 6 h and total RNA was isolated. Total RNA (0.5 µg) was used for real-time RT-PCR analysis with the QuantiTect Probe RT-PCR kit (Qiagen, Hilden, Germany). Sequences of used primers were GTGCGTGTCT-GCATGGACCT (forward) and CCACGATTTGGAACCTTGCT (reverse). The sequence of the dual-labeled TaqMan probe was AGTGGAATCAACGTTGCGCGTGC.

Western Blot for eNOS Protein Analyses. Confluent EA.hy 926 cells were incubated with artichoke extracts for 18 h and total protein was isolated. Western blotting was performed using 50 µg of protein and a monoclonal anti-eNOS antibody (BD Biosciences Pharmingen, San Diego, CA), as described previously (Li and Förstermann, 2000b). Immunocomplexes were developed using an enhanced horseradish peroxidase/luminol chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) according to the manufacturer’s instructions.

Determination of NO Synthesis. EA.hy 926 cells were treated with artichoke extracts for 18 h and then stimulated with 10 µM calcium ionophore A23187 for 1 h. The supernatants were collected and the oxidation products of NO, nitrite and nitrate, were assayed as a measure of NO synthesis. After reduction of nitrate with nitrate reductase, total nitrite was determined by NO-ozone chemiluminescence using a NOA 280 NO Analyzer (Sievers, Boulder, CO). Total protein content of the cells was determined (Bradford, 1976), and nitrite levels were normalized for protein (Li et al., 2002a, 2003).

Organ Chamber Experiment using Rat Aorta. Aortas were isolated from male Sprague-Dawley rats (250–300 g) and cut into rings of 3 mm width. The rings were washed twice with penicillin/streptomycin (100 U/ml, 100 µg/ml)-containing PBS and then maintained in cell culture incubators in Dulbecco’s modified Eagle’s medium (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1× hypoxanthine, amethopterin/methotrexate, and thymine (Invitrogen, Karlsruhe, Germany) (Li and Förstermann, 2000b).

Table 1

<table>
<thead>
<tr>
<th>Extract</th>
<th>Description of Extraction</th>
<th>Drug/Extract Ratio</th>
<th>Caffeoylquinic Acid Content</th>
<th>Flavonoid Content % (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALE</td>
<td>Aqueous extract of artichoke leaves</td>
<td>4–6:1</td>
<td>10.10</td>
<td>2.17</td>
</tr>
<tr>
<td>OSF</td>
<td>Organic subfraction of ALE</td>
<td>20–30:1</td>
<td>26.30</td>
<td>13.29</td>
</tr>
<tr>
<td>ASF</td>
<td>Aqueous subfraction of ALE</td>
<td>5–7:1</td>
<td>6.30</td>
<td>0.76</td>
</tr>
</tbody>
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ated (a contraction induced by 80 mM KCl was set at 100%). After washout, the rings were contracted again using 100 nM norepinephrine, and relaxation concentration-response curves were generated with acetylcholine in the absence or presence of the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 1 mM). Vasodilator response to the NO donor SNAP was achieved after precontraction with 100 nM norepinephrine in the presence of L-NAME.

**Statistics.** Statistical differences between mean values were determined by analysis of variance followed by Fisher’s protected least significant difference test for comparison of different means.

**Results**

**eNOS Promoter Activity in Human EA.hy 926 Endothelial cells.** An 18-h ALE treatment of EA.hy 926 cells stably transfected with a 3.5-kb human eNOS promoter fragment resulted in a concentration-dependent increase in eNOS promoter activity (Fig. 1). The maximal increase was seen with 100 μg/ml ALE. The cynara OSF was more potent than ALE. The ASF, however, was without any effect.

**eNOS mRNA Expression in Human Endothelial Cells.** Treatment of HUVECs and EA.hy 926 cells for 18 h with 100 μg/ml ALE or OSF resulted in a significant increase in eNOS mRNA expression, as analyzed with the RNase protection assay (Fig. 2).

**eNOS Protein Expression in EA.hy 926 Cells.** As analyzed with Western blot, both ALE and OSF increased eNOS protein in EA.hy 926 cells after an 18-h treatment (Fig. 3). Densitometric analyses of the three blots demonstrated an average increase to 185% of control after 100 μg/ml ALE and to 197% of control after 100 μg/ml OSF.

![Fig. 1. Effects of artichoke extracts on human eNOS promoter activity.](image)

Fig. 1. Effects of artichoke extracts on human eNOS promoter activity. Human EA.hy 926 endothelial cells were stably transfected with a 3.5-kb human eNOS promoter fragment driving a luciferase reporter gene. ALE is the crude aqueous extract from artichoke leaves; OSF and ASF are the organic and aqueous subfractions of ALE, respectively. The EA.hy 926 cells were treated with artichoke extracts for 18 h, and then luciferase activity was analyzed as a determinant of eNOS promoter activity. Symbols represent mean ± S.E.M. of three experiments (**, P < 0.01; *** P < 0.001 versus untreated cells).

![Fig. 2. Artichoke extracts increase eNOS mRNA expression in human endothelial cells.](image)

Fig. 2. Artichoke extracts increase eNOS mRNA expression in human endothelial cells. HUVECs and EA.hy 926 cells were treated for 18 h with ALE (100 μg/ml) or the OSF (100 μg/ml) thereof. Then, eNOS mRNA expression was analyzed with RNase protection assays. Panel A displays an original gel of an RNase protection assay (performed in triplicate). The gel shows the protected bands for eNOS (top) and for β-actin (bottom; used for normalization). Panels B and C illustrate the results of densitometric analyses of three different gels; columns represent mean ± S.E.M. of the three experiments (**, P < 0.01; *** P < 0.001 compared with control).

![Fig. 3. Artichoke extracts enhance eNOS protein expression.](image)

Fig. 3. Artichoke extracts enhance eNOS protein expression. EA.hy 926 cells were treated for 18 h with ALE (100 μg/ml) or the OSF (100 μg/ml) thereof, and eNOS protein expression was analyzed with Western blot using a monoclonal anti-eNOS antibody. The blot shown is representative of three independent experiments with similar results.
NO Production in EA.hy 926 Cells. Both ALE and OSF increased NO synthesis in EA.hy 926 cells after an 18-h treatment, as determined by the NO-ozone chemiluminescence assay (Fig. 4). OSF was more efficacious than ALE in stimulating endothelial NO production.

In contrast to the long-term effects on eNOS expression (and thus activity), ALE and OSF had no acute effect on eNOS activity. Incubation of EA.hy 926 cells for up to 30 min with ALE or OSF (up to 100 μg/ml), or with the flavonoid cynaroside (up to 30 μM), did not increase NO production.

Effects of Artichoke Flavonoids and Caffeoylquinic Acids. ALE is known to contain large amounts of polyphenolic compounds, with caffeoylquinic acids and flavonoids being major constituents. We therefore tested four commercially available compounds known to be present in ALE: two caffeoylquinic acids (cynarin and chlorogenic acid) and two flavonoids (luteolin and cynaroside). As shown in Fig. 5, luteolin and cynaroside, but not cynarin or chlorogenic acid, increased eNOS promoter activity in a concentration-dependent manner. In parallel, the two artichoke flavonoids also increased eNOS mRNA expression both in HUVECs and EA.hy 926 cells (Fig. 6). Cynarin and chlorogenic acid did not increase eNOS mRNA expression (three experiments).

eNOS mRNA Stability. To study eNOS mRNA stability, EA.hy 926 cells were treated either with 100 μg/ml OSF for 18 h or with 30 μM cynaroside for 8 h. After the pretreatment, transcription was stopped by adding 60 μM DBR, an inhibitor of RNA polymerase II transcription (Cai et al., 2001), to the culture medium. eNOS mRNA levels were determined with quantitative real-time RT-PCR at 0, 6, 12, and 24 h thereafter. As shown in Fig. 7, OSF and cynaroside had no significant effect on eNOS mRNA stability.

Vasomotor Responses of Rat Aorta ex Vivo. To investigate functional consequences of an eNOS up-regulation, we studied the effects of OSF and cynaroside on vasomotion. In organ chamber experiments, rat aortic rings pretreated with OSF for 18 h showed a decreased vasoconstriction in response to norepinephrine (Fig. 8A). The NOS inhibitor L-NAME (1 mM) did not change basal vascular tone. However, the norepinephrine-induced vasoconstriction was significantly enhanced by L-NAME, both in control and OSF-pretreated rings, although the OSF-exposed rings did not reach the same level of contraction as control vessels (Fig. 8A).

The vasodilator response to acetylcholine was significantly enhanced by OSF pretreatment (Fig. 8B). No significant relaxation was observed in the presence of the NOS inhibitor L-NAME (Fig. 8B). The vasodilator response to the NO donor SNAP was not changed by OSF (Fig. 8C).

Pretreatment of aortic rings with the flavonoid cynaroside had an effect on vasomotion similar to that of OSF. Cynaroside-pretreated rings showed decreased constriction response to norepinephrine (similar to Fig. 8A, three experiments). Also, the vasodilator response to acetylcholine was enhanced (similar to Fig. 8B, three experiments).

Additional experiments using aortic rings without OSF/cynaroside pretreatment showed that OSF and cynaroside has no acute effects on vasomotion. In the absence of acetylcholine, no relaxation to OSF (10–300 μg/ml) or cynaroside (1–100 μM) was observed within 10 min in rings precontracted with 100 nM norepinephrine (three experiments).

Discussion

In the present study, we investigated the effects of artichoke on the synthesis of endothelial NO. Long-term incubation with a crude extract from artichoke leaves or its organic subfraction increased eNOS promoter activity, eNOS mRNA expression, eNOS protein expression, and NO production in
cultured human vascular endothelial cells. Furthermore, long-term ex vivo incubation of aortic rings with the organic subfraction enhanced the endothelium-dependent, NO-mediated vasodilator response to acetylcholine.

NO synthesis can be modulated by eNOS activity and/or eNOS gene expression (Li et al., 2002b,c). Since short-term incubation with the artichoke extract had no effect on NO production in EA.hy 926 cells and did not change vascular tone of aortic rings, the effects of artichoke on endothelial NO synthesis seem to result mainly or exclusively from up-regulation of eNOS gene expression. Due to the antithrombotic, anti-atherosclerotic, and antihypertensive properties of endothelial NO, the eNOS enzyme could be an interesting target for the prevention or therapy of cardiovascular diseases. In vivo up-regulation of eNOS gene expression seems to be a reasonable and realistic strategy.

There is precedent for this strategy in the form of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins). Statins possess vasoprotective properties independent of their cholesterol-lowering effect, which include the up-regulation of eNOS expression in platelets and endothelial cells. This eNOS up-regulation was found to be associated with a decrease in platelet activation (Laufs et al., 2000b), an improvement of endothelial function in hypercholesterolemia (Wilson et al., 2001), an augmentation of cerebral blood flow (Endres et al., 1998; Laufs et al., 2000a), and a protection from stroke (Endres et al., 1998; Laufs et al., 2000a; Amin-Hanjani et al., 2001). Up-regulation of eNOS gene expression seems to be the predominant, if not the only, mechanism, because the protective effects are absent in eNOS-deficient mice.

However, one recent study has questioned the beneficial effects of eNOS up-regulation in vivo (Ozaki et al., 2002). In this study, a transgenic mouse strain (eNOS-Tg) was interbred with atherogenic apoE-deficient (apoE-KO) mice resulting in apoE-KO/eNOS-Tg mice. These mice expressed about 11-fold more eNOS than did the wild-type mice and, unexpectedly, developed larger atherosclerotic lesions than did apoE-KO mice (Ozaki et al., 2002). As discussed by the authors themselves (Ozaki et al., 2000), “uncoupling” of eNOS seems to occur under these circumstances. It is established that under certain pathological conditions, eNOS can generate superoxide rather than NO by dissociation of the ferrous-dioxygen complex (Vasquez-Vivar et al., 1998; Xia et al., 1998). Superoxide produced by the uncoupled eNOS may enhance the preexisting oxidative stress. The molecular mechanisms underlying eNOS uncoupling have not been completely understood. A relative lack of (6R)-5,6,7,8-tetrahydro-L-biopterin, an eNOS cofactor, seems to play a crucial role in many cases (Stuehr et al., 2001; Vasquez-Vivar et al., 2003).

In contrast to the extreme overexpression of eNOS mentioned above, the eNOS up-regulation by pharmacological compounds like statins is usually moderate (<3-fold). At these levels, the up-regulated eNOS seems to remain functional. In addition, a novel compound from Aventis (Strasbourg, France) (Cpd2431), which also moderately up-regulates eNOS expression, has been shown to reduce experimental atherosclerosis in apoE-KO mice (Wohlfart et
In conclusion, the present study demonstrates that artichoke leaf extracts increase eNOS gene expression and NO production in cultured human vascular endothelial cells. Artichoke leaf extract also enhances endothelium-dependent vasodilation in rat aorta. This novel action of the plant seems to be mediated, at least in part, by artichoke flavonoids. Based on the beneficial effects of endothelial NO, vasoprotective and anti-atherosclerotic effects are likely to ensue also in vivo.

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References
Li H, Oehrlein SA, Wallerath T, Ihrig-Biedler I, Wohlfart P, Ulschofer T, Jessen T, cranoside suggest that artichoke flavonoids represent one group of the compounds that are responsible for the eNOS up-regulation caused by cynara extracts.