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Research Article

Ginkgo biloba extract EGb® 761 increases endothelial nitric oxide production *in vitro* and *in vivo*

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Abstract. Beneficial effects of *Ginkgo biloba* on peripheral arterial occlusive disease have been repeatedly shown in clinical trials, especially after use of EGb® 761, a standardized special extract. Since the underlying mechanisms are widely unknown, we aimed to elucidate the molecular basis on which EGb® 761 protects against endothelial dysfunction *in vitro* and *in vivo*. Application of therapeutically feasible doses of EGb® 761 for 48 h caused endothelial nitric oxide (NO) production by increasing endothelial nitric oxide synthase (eNOS) promoter activity and eNOS expression *in vitro*. Phosphorylation of eNOS at a site typical for Akt (Ser 1177) was acutely enhanced by treatment with EGb® 761, as was Akt phosphorylation at Ser 478. Furthermore, the extract caused acute relaxation of isolated aortic rings and NO-dependent reduction of blood pressure *in vivo* in rats. These influences on eNOS represent a putative molecular basis for the protective cardiovascular properties of EGb® 761.

Keywords. Blood pressure, nitric oxide synthase, endothelium, vasodilation.

Introduction

Cardiovascular diseases are the major cause of death in the USA and Europe. Dysfunction of the vascular endothelium is a hallmark of cardiovascular diseases and is characterized by multiple factors including impaired vasodilation, tissue perfusion, hemostasis, and thrombosis. A variety of pathological processes, including hypertension and atherosclerosis, are associated with endothelial dysfunction involving a reduced nitric oxide (NO) bioavailability [1].

NO is crucial for maintaining vascular endothelial health and function and is produced by endothelial nitric oxide synthase (eNOS) [2]. It is a potent vasodilator by stimulating soluble guanylate cyclase and increasing cyclic guanosine monophosphate (cGMP) levels in smooth muscle cells. Additionally, NO also protects blood vessels against the onset of atherogenesis and thrombosis.

NO bioavailability depends on the balance of NO synthesis *via* eNOS and its degradation by oxidative stress [3]. Based on these facts, the enhancement of endothelial NO production in ageing and diseased endothelium by eNOS activation as well as expression is of great therapeutic interest.

Several medicinal plants offer potential for the prevention and treatment of cardiovascular diseases [4]. The best-selling herbal remedies in the USA and world-wide are formulations of *Ginkgo biloba*. Beneficial effects on senile dementia of primary degenerative, vascular, and mixed origin, as well as effects on

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peripheral arterial occlusive disease and various neurosensory disturbances have been repeatedly shown in clinical trials, especially based on the use of EGb® 761, a standardized special extract [5].

Although the efficacy of this standardized *Ginkgo biloba* extract has been well proven, the underlying molecular mechanisms and signalling pathways leading to Ginkgo's beneficial cardiovascular effects have as yet remained widely unknown. Thus, the aim of the study was to elucidate the molecular basis on which EGb® 761 might protect against endothelial dysfunction *in vitro* and *in vivo*. We hypothesized that EGb® 761 is able to influence the formation of NO in endothelial cells.

Methods

Cell culture. The human endothelial cell line EA.hy926 (kindly provided by Dr. C.-J. Edgell, University of North Carolina, Chapel Hill, NC) [6] was cultivated as described previously [7]. For experiments, cells were seeded in 6-well plates at a density of 0.4×10^6 cells/well. Assays were performed with confluent cells.

Reporter gene assay. EA.hy926 cells stably transfected with a plasmid containing 3600 bp of the human eNOS promoter driving a luciferase gene [8] (a kind gift of Dr. P. Wohlfart, Sanofi-Aventis, Germany) were stimulated for 24 h with EGb® 761 (kindly provided by Dr. Willmar Schwabe Pharmaceuticals, Karlsruhe, Germany) as indicated. Cells were washed and lysed and the assay performed according to the manufacturer's instructions (Luciferase assay system, Promega, Germany) with the use of a luminometer (AutoLumatPlus, Berthold Technologies, Germany).

Western blot analysis. *In vitro samples:* EA.hy926 cells were stimulated with EGb® 761 for 48 h, washed with ice-cold PBS, and lysed with RIPA buffer additionally containing 4% Complete® (protease inhibitor cocktail), 1 mM phenylmethylsulfonyl fluoride (PMSF, protease inhibitor), 1 mM NaF, and 1 mM activated Na_3VO_4. Supernatants were used for protein determination (BCA assay). Laemmli sample buffer was added, and the samples were heated at 95°C for 5 min. Samples (20 µg protein) were loaded on an SDS-polyacrylamide gel and transferred to PVDF-membranes by electroblotting. eNOS protein was detected using a monoclonal mouse anti-eNOS antibody (1:1000, BD Biosciences, Heidelberg, Germany). Antibodies against phospho-eNOS (Ser1177), Akt, and phospho-Akt (Ser473) were from Cell Signaling Technology (CST, Frankfurt, Germany). The protein levels were detected using an ECL™ detection system (Amersham Pharmacia Biotech, UK). *In vivo samples:* In anesthetized Sprague-Dawley rats, a bolus of EGb® 761 (5 mg/animal, n=2) or an equivalent volume of PBS (n=2) was injected *via* a catheter in the carotid artery. Five minutes after bolus administration, lung thoracic aortas were excised and immediately frozen in liquid nitrogen. The aortas were lysed by homogenization in a modified RIPA buffer and subjected to SDS-PAGE as described above.

[^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay. EA.hy926 cells were stimulated with EGb® 761 for 48 h. Cells were treated, and the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay was performed as described previously [7]. In brief, cells were washed and kept in a HEPES buffer for 45 min before addition of 0.32 µmol/L [^{14}C]L-arginine (313 mCi/mmol) and 1 µmol/L A23187. After incubation for 25 min at 37°C cells, were lysed with ice-cold ethanol (96%). Lysates were extracted with water. The water-supernatants were dried under vacuum. The extract was resolved in water/methanol (1:1) and spotted on a thin-layer chromatography plate. [^{14}C]L-arginine was separated from [^{14}C]L-citrulline using the solvent system water/chloroform/methanol/ammonium hydroxide (1/0.5/4.5/2 v/v/v/v). The chromatography plates were dried and analyzed with a phosphorimager (Fujifilm BAS-1500).

Confocal laser scanning microscopy. EA.hy926 cells were cultured on collagen-coated glass cover slips, treated as indicated, fixed with 3% formaldehyde (Sigma-Aldrich, Steinheim, Germany), permeabilized with 0.2% Triton X-100 (Roth, Karlsruhe, Germany), and incubated with the primary antibody [anti-phospho eNOS (Ser 1177), Cell Signaling] at a dilution of 1:100 for 1 h. As a secondary antibody, AlexaFluor633 goat anti-rabbit (Molecular Probes, Karlsruhe, Germany) was used at a dilution of 1:400 for 45 min. Images were obtained with a Zeiss LSM 510 META confocal microscope.

Rat thoracic aortic ring assay. Thoracic aortas from male Sprague-Dawley rats (Janvier, Le Genest, France) were immediately removed after decapitation and placed in Tyrode salt solution (mmol/L: NaCl 118.2; $NaHCO_3$ 24.8; KCl 4.6; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; glucose 10). After removal of fat and connective tissue, vessels were cut into 4 mm-long rings. The rings were mounted on stainless steel hooks in an organ chamber (Hugo Sachs, Hugstetten, Germany) and maintained at 37°C equilibrated with 95% O_2 and 5% CO_2. Isometric tension studies were performed using force transducers (Statham UC2, Hugo Sachs) connected to a four-channel recorder (Lineacorder, Graphtec). Integrity of vasomotion was tested by repetitive precontraction with phenylephrine (PE, 0.15 µg/mL). Endothelial function was evaluated by vascular relaxation to acetylcholine (ACh, 0.25 µg/mL) after PE-induced contraction.

Vasodilatory effects of EGb® 761 were studied by cumulative addition at concentrations from 6.4 up to 200 µg/mL (n=12) at the plateau of the PE-induced contraction. Finally, endothelial-dependent and -independent relaxation was tested by subsequent application of ACh (0.25 µg/mL) and papaverine (Pap; 37.6 µg/mL), respectively. As a solvent control, respective cumulative concentrations of DMSO were applied (n=4). Endothelial-independent relaxation was studied using endothelium-denuded aortic rings (n=8).

***In vivo* blood pressure measurement.** Male Spargue-Dawley rats (180–220 g) were purchased from Charles River Wiga GmbH (Sulzfeld, Germany). The animals had free access to chow (Sniff, Soest, Germany) and water up to the time of experiments. All animals received care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication 86–23, revised 1985). Studies were performed with the permission of the government authorities.

Animals were anaesthesized with a mixture of midazolam/fentanyl (2.0/0.005 mg/kg i.p.) and kept continuously anaesthesized by inhalation with isoflurane (1.3%). Blood pressure was continuously monitored by a catheter in the carotid artery. One group of animals served as solvent control. Another group received an i.v. injection of EGb® 761 (5 mg/animal), whereas a third group received an additional i.v. injection of L-NAME (4 mg/animal; Cayman Chemical Company, MI, USA) 30 min prior to the EGb® 761 application.

Data analysis. Data are expressed as mean ± SEM. Statistical analysis was performed using the SigmaStat software version 3.1 (Aspire Software International). Samples were analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's Method) and Student's t-test, as appropriate (indicated in the figure legend). $p \leq 0.05$ was the criterion for statistical significance.

Results

EGb® 761 up-regulates eNOS promoter activity, eNOS protein expression, and eNOS activity after long-term treatment.

After 24 h treatment, EGb® 761 significantly enhances eNOS promoter activity in a concentration-

dependent manner (10 to 500 µg/mL) up to 1.4-fold in EA.hy926 cells stably transfected with a 3.6-kb human eNOS promoter fragment (Fig. 1a). Accordingly, as analyzed by Western blot, EGb® 761 (500 µg/mL) clearly induced eNOS protein expression in EA.hy926 cells after a 48-h treatment (Fig. 1b). After the same period of time (48 h), when eNOS expression was already increased, NO production was measured indirectly using the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay. We detected an increase in L-citrulline production (Fig. 1c) of approximately 1.5-fold after incubation with EGb® 761 concentrations ranging from 10 to 100 µg/mL. To control for unspecific effects of the extract, we performed cell toxicity tests (ATP measurement and determination of cell number by crystal violet uptake) between 1 and 72 h of exposure with up to 500 µg/mL EGb® 761. No cytotoxic effects were detected (data not shown).

EGb® 761 promotes eNOS phosphorylation at Ser-1177.

In addition to the long-term influence of EGb® 761 on transcriptional regulation of eNOS, we evaluated whether eNOS is acutely activated in EA.hy926 cells by performing a time course for eNOS phosphorylation at Ser-1177 (Fig. 2a), a phosphorylation site important for eNOS activation [9]. Indeed, EGb® 761 (100 µg/mL) induced eNOS phosphorylation that reached a maximum at 60 min.

Furthermore, we investigated whether EGb® 761 exerts an effect on the intracellular distribution of phosphorylated eNOS by means of confocal microscopy. Indeed, phosphorylated eNOS was rapidly translocated to the plasma membrane upon treatment with 100 µg/mL EGb® 761 for 15 min (Fig. 2b).

Activation of eNOS *via* the PI(3)K/Akt pathway.

To test for involvement of Akt in phosphorylation of eNOS at Ser-1177, we examined whether EGb® 761 activates Akt by phosphorylation (at Ser-473). This phosphorylation of Akt was indeed found to be increased after EGb® 761 exposure and occurred in a time-dependent fashion (Fig. 3a). The maximum activation was achieved within 60 min after treatment with EGb® 761 (100 µg/mL), closely approximating the time course for eNOS Ser-1177 phosphorylation (Fig. 2a).

To investigate whether PI(3)K is involved in the EGb® 761-mediated phosphorylation of Akt, we incubated EA.hy926 cells with the PI(3)K inhibitor wortmannin before exposure to EGb® 761 (100 µg/mL). Wortmannin completely abolished EGb® 761-induced phosphorylation of not only Akt (data not shown) but also of eNOS (Fig. 3b). These results demonstrate that the EGb® 761-induced phosphorylation of eNOS at Ser-1177 is most probably Akt-dependent. An alternative explanation would be phosphorylation of eNOS by a different kinase downstream of PI(3)K.

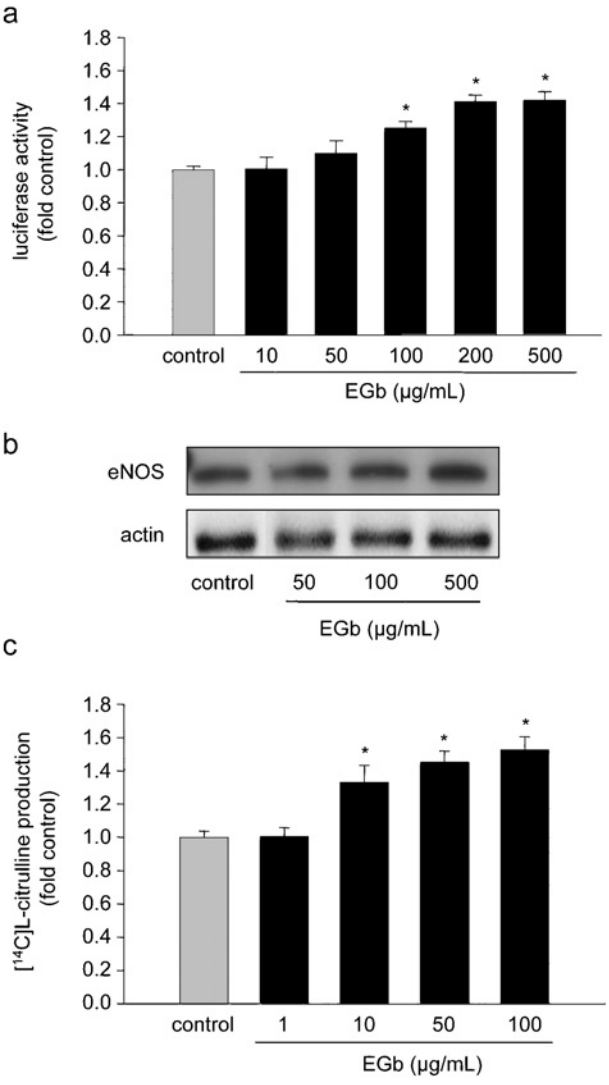

Figure 1. EGb® 761 increases eNOS promoter activity (*a*), eNOS protein levels (*b*), and eNOS activity/L-citrulline production (*c*) in endothelial cells. (*a*) Stably transfected EA.hy926 cells containing a 3.6-kB eNOS promoter driving a luciferase reporter gene were either kept untreated (control) or stimulated with increasing concentrations (10 to 500 µg/mL) EGb® 761 for 24 h. Cells were lysed and analyzed for luciferase activity. (*b*) EA.hy926 cells were either left untreated (control) or were treated with EGb® 761 (50 to 500 µg/mL) for 48 h. Levels of eNOS (upper panel) and actin (lower panel) protein were determined by Western blot analysis. One representative blot out of three independent experiments is shown. (*c*) eNOS activity was determined using the [^{14}C]L-arginine/[^{14}C]L-citrulline conversion assay. Cells were either left untreated (control) or treated with increasing concentrations (1 to 100 µg/mL) EGb® 761 for 48 h. Data are presented as mean ± SEM of three independent experiments (*$p<0.05$ *vs.* control, Kruskal-Wallis One Way Analysis of Variance on Ranks, Dunn's Method).

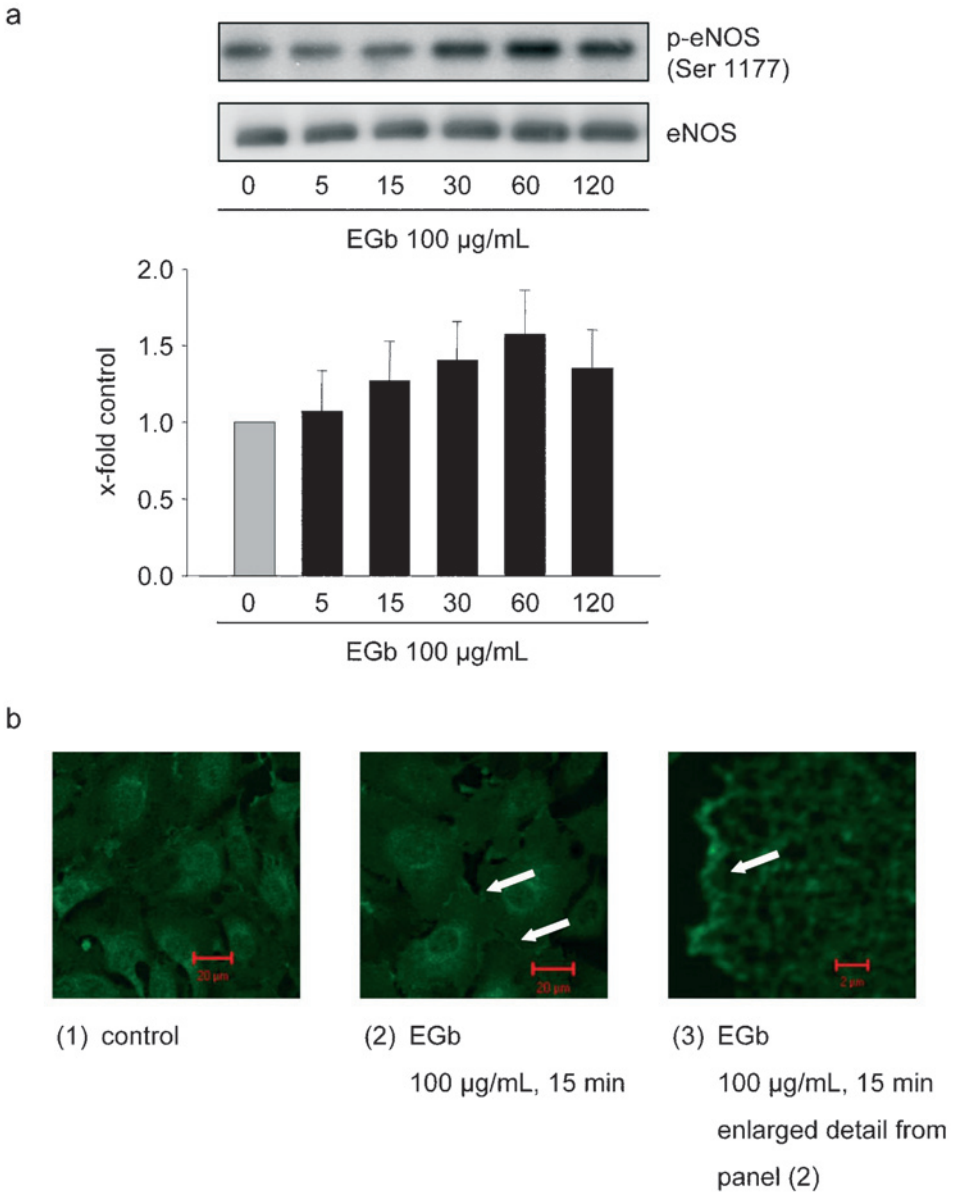

Figure 2. EGb® 761 increases eNOS phosphorylation and localization at the plasma membrane. (*a*) EA.hy926 cells were either left untreated (control) or were treated with EGb® 761 (100 µg/mL) for indicated times. Levels of phospho-eNOS (Ser-1177, upper panel) and total eNOS (lower panel) protein were determined by Western blot analysis. One representative blot out of three independent experiments is shown. (*b*) Representative photomicrographs are shown after immunofluorescent staining of EA.hy926 cells. Fluorescence indicates phosphorylated eNOS protein. In contrast to untreated cells (1), cells receiving 100 µg/mL EGb® 761 for 15 min (2 and 3) showed translocation of phosphorylated eNOS to the plasma membrane (arrows).

Vasorelaxant effect of EGb® 761 on rat thoracic aortic rings.

EGb® 761 (6.4 to 200 µg/mL) elicited dose-dependent relaxation of rat thoracic aortic rings with intact endothelium, with a maximal relaxation of 50% reached at a concentration of 200 µg/mL (Fig. 4a, b). Respective cumulative concentrations of the solvent used (DMSO) had no effects on aortic ring relaxation (Fig. 4c). Importantly, EGb® 761 failed to produce relaxation in aortas without functional endothelium (Fig. 4d), pointing to involvement of NO in the endothelium-dependent vasorelaxation induced by EGb® 761.

EGb® 761 acutely reduces systolic blood pressure in rats *via* NO release and augments eNOS phosphorylation in thoracic aortas.

To assess the effect of EGb® 761 on blood pressure, invasive blood pressure measurements were performed. EGb® 761 (5 mg/animal) significantly decreased systolic blood pressure: after 5 min the systolic blood pressure decreased from 120 mmHg to

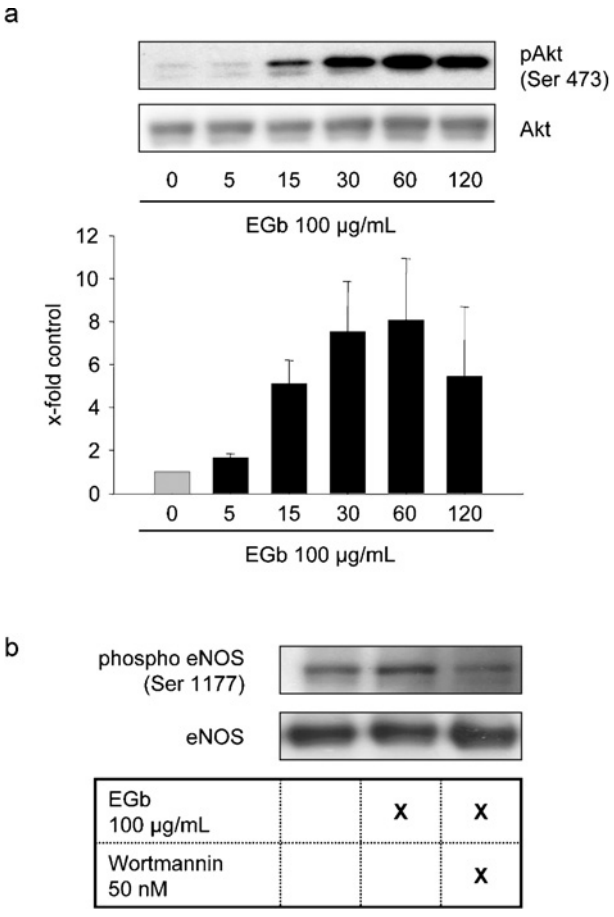

Figure 3. EGb®-induced phosphorylation of eNOS at Ser-1177 is mediated by the PI(3)K/Akt pathway. (*a*) EA.hy926 cells were either left untreated (control) or were treated with EGb® 761 (100 µg/mL) for indicated times. Levels of phospho-Akt (Ser-473, upper panel) and total Akt (lower panel) protein were determined by Western blot analysis. One representative blot out of three independent experiments is shown. (*b*) Endothelial cells were pretreated with wortmannin (50 nM) for 30 min before EGb® 761 (100 µg/mL, 60 min). Levels of phospho-eNOS protein were determined by Western blot analysis. One representative blot out of three independent experiments is shown.

65 mmHg (Fig. 5a). In order to causally link the blood pressure alterations to enhanced NO release, the animals were pretreated with the eNOS inhibitor L-NAME (4 mg/animal) 30 min before treatment with EGb® 761. Pretreatment of the animals with L-NAME prevented the reduction of blood pressure by EGb® 761 (Fig. 5a). In addition to the influence of EGb® 761 on blood pressure, we observed a significant increase in eNOS phosphorylation at Ser-1177 in thoracic aortas obtained from animals shortly after EGb® 761 treatment (Fig. 5b).

Discussion

Traditional herbal remedies are increasingly considered as a safe and effective alternative to modern synthetic drugs, even in western industrialized countries. In particular, *Ginkgo biloba* extract has been widely used as a herbal remedy in European countries and the USA [4]. The epidemiological and economical importance of this medicinal herb is documented by the fact that it was the top-selling plant product in 1998 in the USA [10, 11], a market with sales of herbal medicines of $4 billion per year at that time and with an annual growth rate of about 25% [12]. The indication for the use of Ginkgo extract that is best documented by clinical trials is dementia of various origins [13]. A second field of application for *Ginkgo biloba* is represented by diseases that are connected to dysregulation of vascular tone and endothelial dysfunction, such as intermittent claudication [14], Raynaud's disease [15], or tinnitus [16].

Despite its growing popularity and the increasing number of studies communicating a beneficial role of *Ginkgo biloba* extract in the treatment of cardiovascular diseases [14–16], its clinical use is still underrepresented due to the lack of knowledge about its cellular and molecular mechanisms of action [17]. One potential mode of action of *Ginkgo biloba* extract causing improved vascular perfusion would be a beneficial effect on pathologically altered hemorheology due to PAF antagonism [18]. However, decreased blood pressure and increased perfusion after treatment with Ginkgo extracts have also been shown in healthy humans with presumably normal hemorheological status [19, 20], hinting towards a second hypothesis that has repeatedly been proposed: the modulation of NO generation by *Ginkgo biloba* extracts.

In principle, there are two ways to increase NO production: induction of eNOS expression and post-translational activation of eNOS. The former is a more sustained process that would afford protracted elevation of NO levels, while the latter would lead to more acute effects. Several compounds of biogenic origin act *via* only one of these ways, while others use both [21–23]. In the present study, we show that EGb® 761 can enhance production of NO by both, induction of eNOS expression and post-translational activation of eNOS *in vitro* as well as *in vivo* at concentrations (100 µg/mL) that are likely to be achieved in blood after daily intake of 120 to 240 mg, representing normal dosage for effective therapy [24].

On the one hand, we observed an increase in eNOS promoter activity and higher expression of eNOS protein after long-term stimulation (for 48 h) with EGb® 761 (100 µg/mL). Interestingly, Cheung et al.

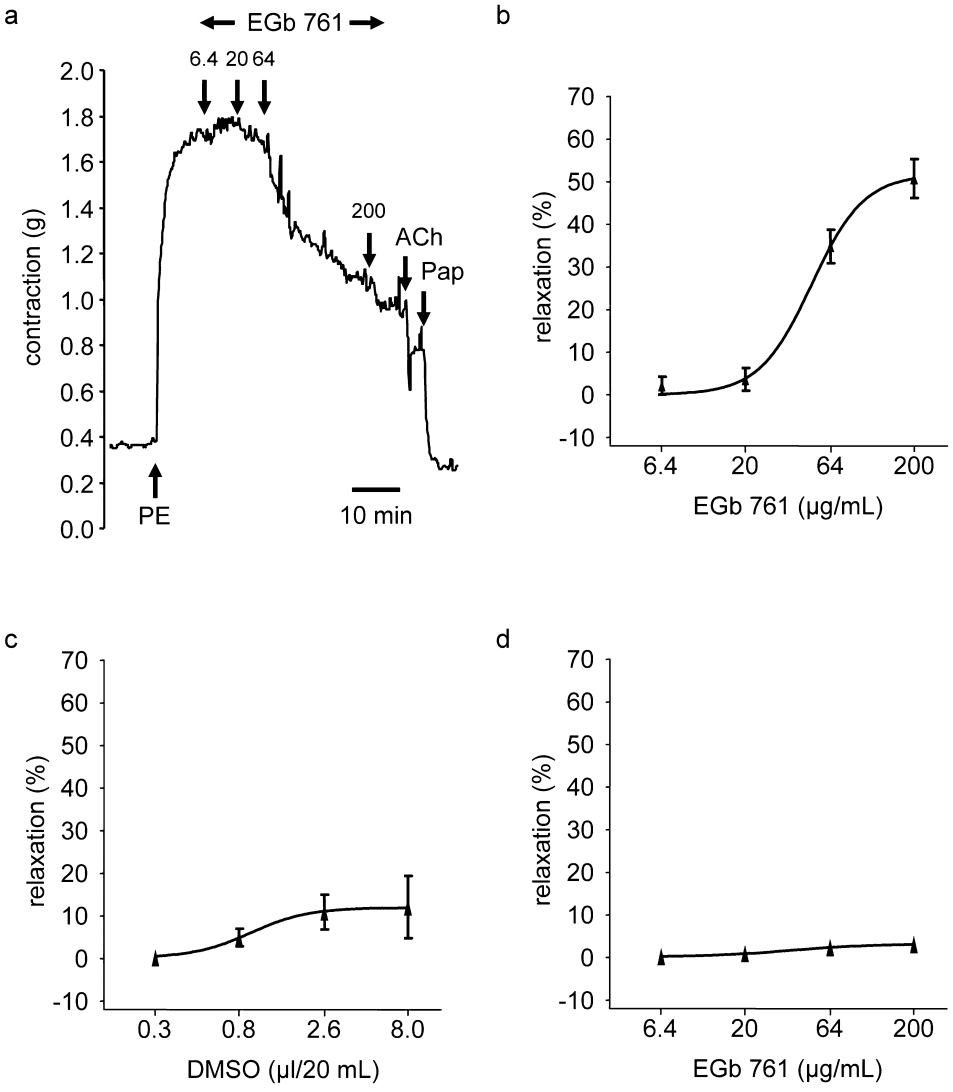

Figure 4. EGb® 761 concentration-dependently induces relaxation of pre-contracted thoracic aortic rings. (*a*) Relaxation of ring segments of rat thoracic aorta by cumulative addition of 6.4–200 µg/mL EGb® 761 (n=12) at the plateau of the PE-induced contraction (0.15 µg/mL), followed by application of ACh (0.25 µg/mL) and papaverine (Pap; 37.6 µg/mL) to control endothelial-dependent and -independent vasorelaxation. (*b*) Concentration-response curve of aortic ring relaxation following EGb® 761 treatment expressed as percentage of the phenylephrine-induced contraction. Values are presented as mean ± SEM (n=12). (*c*) Concentration-response curve of aortic ring relaxation following addition of DMSO. Data are expressed as percentage of the phenylephrine-induced contraction. Values are presented as mean ± SEM (n=4). (*d*) Concentration-response curve of endothelium-denuded aortic rings following EGb® 761 treatment. Data are expressed as percentage of the phenylephrine-induced contraction. Values are presented as mean ± SEM (n=8).

could not show an influence of Ginkgo extract on expression of eNOS [25, 26]. This study, however, utilized ECV 304 as an endothelial-like cell model, and the observation time was relatively short (only 2 or 4 h). Since ECV 304 have in the meantime been shown to be of epithelial origin, this discrepancy does not come as a surprise. An increase of eNOS mRNA after treatment with Ginkgo extract was previously shown in rat aortas [27]; however, data on eNOS protein levels were missing in this publication. One crucial problem with increasing eNOS expression seems to be that uncoupling of this enzyme may occur [28], leading to increased production of superoxide. However, this seems only to be the case after dramatic up-regulation, *e.g.* by overexpression (11-fold) [28], while moderate increases (<3-fold) as described for statins and other compounds [21] or, in our work, Ginkgo, seem to have no such adverse effects. On the other hand, in our study EGb® 761 caused a marked drop in blood pressure *in vivo* within minutes as well as significant relaxation of isolated aortic rings that cannot be due to altered eNOS expression. Similar observations were previously made with red wine polyphenols [29]; however, the underlying mechanisms are still unclear. The same applies to Ginkgo, where acute vasorelaxant effects have been observed

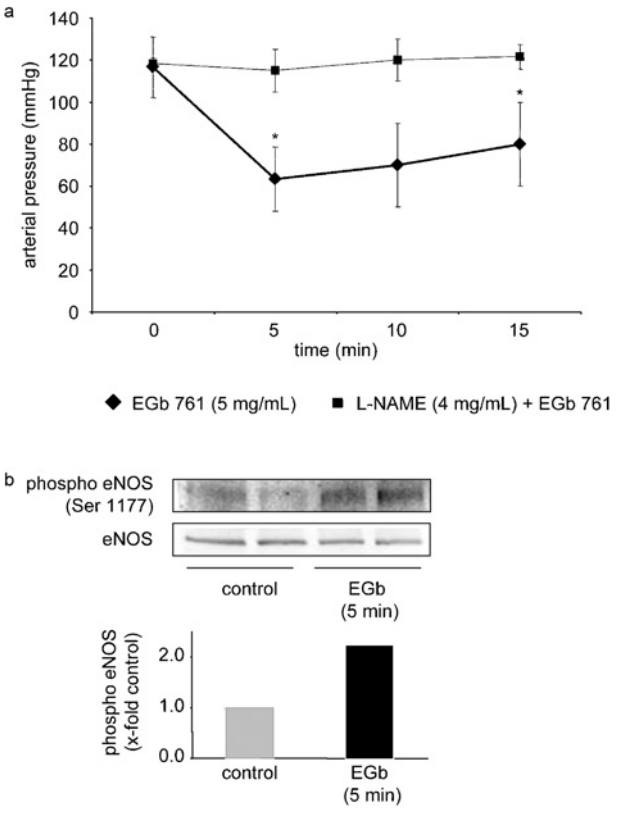

Figure 5. EGb® 761 rapidly decreases systolic blood pressure in Sprague-Dawley rats *via* NO release (*a*) and induces phosphorylation of eNOS at Ser-1177 in rat thoracic aortas (*b*). (*a*) Time course of systolic blood pressure after i.v. injection of EGb® 761 (5 mg/animal) or i.v. injection of L-NAME (4 mg/animal) 30 min prior to EGb® 761 application. Blood pressure was measured *via* catheterization of the carotid artery. Data are presented as mean ± SEM (n=3; *p<0.05 *vs.* L-NAME/EGb® 761-treated animals, Student's t-test). (*b*) Western blot analysis showing the level of phospho-eNOS protein (Ser-1177, upper panel) and total eNOS protein (lower panel) in thoracic aorta from untreated controls or after treatment with EGb® 761 (5 mg/animal for 5 min). The graph displays the signal intensities obtained by fluorimetric evaluation. Bars represent the mean of phospho-eNOS signals normalized by total eNOS signals (n=2).

[30]. An interesting hypothesis was recently suggested, namely that the vasorelaxant effect of Ginkgo could be due to inhibition of PDE-5 by biflavones [31]. However, in the special extract used by us, the content of biflavones is below 0.1%, which is approximately equivalent to 5 μM, a concentration that hardly caused PDE-5 inhibition in the work by Dell'Agli et al. As an alternative hypothesis, an increase in endothelial Ca^{2+} levels after Ginkgo treatment has been described [30]; however, no causal relation to vasodilation or eNOS activation was shown, and no further mechanistic insights were derived. In the present paper, we investigated the role of the PI(3)K/Akt pathway in this context, since it has been proposed as one of the candidates capable of activating eNOS directly by phosphorylation at Ser-1177 [32]. Indeed, we provide evidence for a rapid increase in phosphorylation of eNOS after treatment with EGb® 761, which was paralleled by phosphorylation of Akt at Ser-473. Since this phosphorylation site is a substrate for a kinase complex downstream of PI(3)K (PDK2 or mTOR/rictor), and these responses were abolished by the PI(3)K inhibitor wortmannin, we propose the following signalling cascade: Ginkgo extract causes an increase in endothelial Ca^{2+}, PI(3)K is activated, further downstream Akt is phosphorylated and, in turn, Akt phosphorylates eNOS. In addition to phosphorylation, the location of eNOS is important for its activity [33]. In good accordance, we found an increased level of phospho-eNOS located at the plasma membrane after stimulation with EGb® 761. Since translocation of eNOS to the plasma membrane does not depend on phosphorylation [33] and since we found an accumulation of phosphorylated eNOS but not total eNOS protein (data not shown) at the membrane, we conclude that this results from translocation of activated Akt to the plasma membrane [35] and from subsequent local phosphorylation of eNOS at the plasma membrane. This step increases the Ca^{2+} sensitivity of eNOS at a subcellular compartment, where the cytoplasmic concentration of Ca^{2+} is highest [34]. Interestingly, the higher phosphorylation status of eNOS following EGb® 761 treatment did not only occur *in vitro* but was also seen in rat aorta tissue after treatment *in vivo*. To the best of our knowledge, we report for the first time an influence of EGb® 761 on the PI(3)K-Akt-eNOS pathway and the phosphorylation of eNOS at the plasma membrane. Activation of the PI(3)K/Akt pathway is, in addition to its influence on eNOS, a key step for diverse biological effects mediating cell proliferation, growth, and survival [36]. Therefore, the cellular activities brought about by EGb® 761-induced Akt activation warrant further investigations. In conclusion, our study demonstrates that EGb® 761 stimulates up-regulation and activation of eNOS protein in endothelial cells, thus leading to enhanced production of NO. Our results suggest that EGb® 761 exhibits dual effects concerning both transcriptional and non-transcriptional activation of eNOS, including activation of the protein kinase Akt and subsequent eNOS phosphorylation. Keeping the character of our *in vitro* and *in vivo* systems and their limited transferability to the patient in mind, these basic findings may explain the cardiovascular protective effects of EGb® 761 in conditions associated with disturbed NO production. Our work adds *Ginkgo biloba* to the growing list of herbal remedies whose mode of action has been at least partially revealed on a molecular level [37, 38].

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1 Feletou M. and Vanhoutte P. M. (2006) Endothelial Dysfunction: a multifaceted disorder. Am. J. Physiol. Heart Circ. Physiol. 291, H985–1002.
2 Förstermann U. and Münzel T. (2006) Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 113, 1708–1714.
3 Förstermann U. (2006) Endothelial NO synthase as a source of NO and superoxide. Eur. J. Clin. Pharmacol. 62 Suppl 13, 5–12.
4 Ernst E. (2005) The efficacy of herbal medicine – an overview. Fundam. Clin. Pharmacol. 19, 405–409.
5 Defeudis F. V. (2003) A brief history of EGb 761 and its therapeutic uses. Pharmacopsychiatry 36 Suppl 1, S2-S7.
6 Edgell C. J., McDonald C. C. and Graham J. B. (1983) Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc. Natl. Acad. Sci. USA 80, 3734–3737.
7 Leikert J. F., Räthel T. R., Wohlfart P., Cheynier V., Vollmar A. M. and Dirsch V. M. (2002) Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells. Circulation 106, 1614–1617.
8 Li H., Oehrlein S. A., Wallerath T., Ihrig-Biedert I., Wohlfart P., Ulshofer T., Jessen T., Herget T., Förstermann U. and Kleinert H. (1998) Activation of protein kinase C alpha and/or epsilon enhances transcription of the human endothelial nitric oxide synthase gene. Mol. Pharmacol. 53, 630–637.
9 Sessa W. C. (2004) eNOS at a glance. J. Cell Sci. 117, 2427–2429.
10 Brevoort P. (1996) The US botanical market – an overview. HerbalGram 36, 49–57.
11 Brevoort P. (1998) The booming US botanical market – a new overview. HerbalGram 44, 33–46.
12 Muller J. L. and Clauson K. A. (1997) Pharmaceutical considerations of common herbal medicine. Am. J. Man. Care 3, 1753–1770.
13 Ernst E. and Pittler M. H. (1999) *Ginkgo biloba* for dementia: A systematic review of double-blind, placebo-controlled trials. Clin. Drug Invest. 17, 301–308.
14 Pittler M. H. and Ernst E. (2000) *Ginkgo biloba* extract for the treatment of intermittent claudication: a meta-analysis of randomized trials. Am. J. Med. 108, 276–281.
15 Muir A. H., Robb R., McLaren M., Daly F. and Belch J. J. (2002) The use of *Ginkgo biloba* in Raynaud's disease: a double-blind placebo-controlled trial. Vasc. Med. 7, 265–267.
16 Morgenstern C. and Biermann E. (2002) The efficacy of Ginkgo special extract EGb 761 in patients with tinnitus. Int. J. Clin. Pharmacol. Ther. 40, 188–197.
17 Zhou W., Chai H., Lin P. H., Lumsden A. B., Yao Q. and Chen C. (2004) Clinical use and molecular mechanisms of action of extract of *Ginkgo biloba* leaves in cardiovascular diseases. Cardiovasc. Drug Rev. 22, 309–319.
18 Koch E. (2005) Inhibition of platelet activating factor (PAF)-induced aggregation of human thrombocytes by ginkgolides: considerations on possible bleeding complications after oral intake of *Ginkgo biloba* extracts. Phytomedicine 12, 10–16.
19 Jezova D., Duncko R., Lassanova M., Kriska M. and Moncek F. (2002) Reduction of rise in blood pressure and cortisol release during stress by *Ginkgo biloba* extract (EGb 761) in healthy volunteers. J. Physiol. Pharmacol. 53, 337–348.
20 Mehlsen J., Drabaek H., Wiinberg N. and Winther K. (2002) Effects of a *Ginkgo biloba* extract on forearm haemodynamics in healthy volunteers. Clin. Physiol. Funct. Imaging 22, 375–378.
21 Li H., Xia N., Brausch I., Yao Y. and Forstermann U. (2004) Flavonoids from artichoke (*Cynara scolymus L.*) up-regulate endothelial-type nitric-oxide synthase gene expression in human endothelial cells. J. Pharmacol. Exp. Ther. 310, 926–932.
22 Li H., Hergert S. M., Schäfer S. C., Brausch I., Yao Y., Huang Q., Mang C., Lehr H. A. and Förstermann, U. (2005) Midostaurin upregulates eNOS gene expression and preserves eNOS function in the microcirculation of the mouse. Nitric Oxide 12, 231–236.
23 Wallerath T., Li H., Godtel-Ambrust U., Schwarz P. M. and Förstermann U. (2005) A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. Nitric Oxide 12, 97–104.
24 Biber A. (2003) Pharmacokinetics of *Ginkgo biloba* extracts. Pharmacopsychiatry 36 Suppl 1, S32-S37.
25 Cheung F., Siow Y. L., Chen W. Z. and O K. (1999) Inhibitory effect of *Ginkgo biloba* extract on the expression of inducible nitric oxide synthase in endothelial cells, Biochem. Pharmacol. 58, 1665–1673.
26 Cheung F., Siow Y. L. and O K. (2001) Inhibition by ginkgolides and bilobalide of the production of nitric oxide in macrophages (THP-1) but not in endothelial cells (HUVEC). Biochem. Pharmacol. 61, 503–510.
27 Sasaki Y., Noguchi T., Yamamoto E., Giddings J. C., Ikeda K., Yamori Y. and Yamamoto J. (2002) Effects of *Ginkgo biloba* extract (EGb 761) on cerebral thrombosis and blood pressure in stroke-prone spontaneously hypertensive rats. Clin. Exp. Pharmacol. Physiol. 29, 963–967.
28 Ozaki M., Kawashima S., Yamashita T., Hirase T., Namiki M., Inoue N., Hirata K., Yasui H., Sakurai H., Yoshida Y., Masada M. and Yokoyama M. (2002) Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. J. Clin. Invest. 110, 331–340.
29 Cishek M. B., Galloway M. T., Karim M., German J. B. and Kappagoda C. T. (1997) Effect of red wine on endothelium-dependent relaxation in rabbits. Clin. Sci. (Lond) 93, 507–511.
30 Kubota Y., Tanaka N., Umegaki K., Takenaka H., Mizuno H., Nakamura K., Shinozuka K. and Kunitomo M. (2001) *Ginkgo biloba* extract-induced relaxation of rat aorta is associated with increase in endothelial intracellular calcium level. Life Sci. 69, 2327–2336.
31 Dell'Agli M., Galli G. V. and Bosisio E. (2006) Inhibition of cGMP-phosphodiesterase-5 by biflavones of *ginkgo biloba.* Planta Med. 72, 468–470.
32 Fulton D., Gratton J. P. and Sessa W. C. (2001) Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? J. Pharmacol. Exp. Ther. 299, 818–824.
33 Garcia-Cardena G., Oh P., Liu J., Schnitzer J. E. and Sessa W. C. (1996) Targeting of nitric oxide synthase to endothelial cell caveolae *via* palmitoylation: implications for nitric oxide signaling. Proc. Natl. Acad. Sci. USA 93, 6448–6453.
34 Oess S., Icking A., Fulton D., Govers R. and Müller-Esterl W. (2006) Subcellular targeting and trafficking of nitric oxide synthases. Biochem. J. 396, 401–409.
35 Osaki M., Oshimura M. and Ito H. (2004) PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis 9, 667–676.
36 Woodgett J. R. (2005) Recent advances in the protein kinase B signaling pathway. Curr. Opin. Cell Biol. 17, 150–157.
37 Huang W., Zhang J. and Moore D. D. (2004) A traditional herbal medicine enhances bilirubin clearance by activating the nuclear receptor CAR. J. Clin. Invest. 113, 137–143.
38 Wallerath T., Deckert G., Ternes T., Anderson H., Li H., Witte K. and Förstermann U. (2002) Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. Circulation 106, 1652–1658.