Natural Product Extract of *Dicksonia sellowiana* Induces Endothelium-Dependent Relaxations by a Redox-Sensitive Src- and Akt-Dependent Activation of eNOS in Porcine Coronary Arteries

Yanna D. Rattmann^{a,b} Eric Anselm^a Jong-Hun Kim^a Stéphanie Dal-Ros^a
Cyril Auger^a Obdulio G. Miguel^c Adair R.S. Santos^d Thierry Chataigneau^a
Valérie B. Schini-Kerth^a

^aUMR CNRS 7213, Laboratoire de Biophotonique et Pharmacologie, Faculté de Pharmacie, Université de Strasbourg, Illkirch, France; ^bDepartment of Pharmacology, Centro Politécnico, and ^cDepartment of Pharmaceutical Sciences, Universidade Federal do Paraná, Curitiba, and ^dDepartment of Physiological Sciences, Universidade Federal de Santa Catarina, Florianópolis, Brazil

Key Words

Dicksonia sellowiana · Porcine coronary artery · Endothelium · PI3-kinase/Akt pathway · Nitric oxide

Abstract

Background/Aims: The consumption of polyphenol-rich food is associated with a decreased mortality from coronary diseases. This study examined whether a standardized hydroalcoholic extract of *Dicksonia sellowiana* (HEDS) triggered endothelium-dependent relaxations in porcine coronary artery rings and characterized the underlying mechanism. **Methods:** The phosphorylation level of Src, Akt and eNOS was assessed by Western blot analysis, the formation of reactive oxygen species by dihydroethidine staining and the level of eNOS Ser1177 phosphorylation by immunohistochemical staining in sections of coronary arteries. **Results:** HEDS-induced endothelium-dependent relaxations were strongly reduced by N^ω-nitro-L-arginine, an eNOS inhibitor, and by its combination with charybdotoxin plus apamin, inhibitors of endothelium-derived hyperpolarizing factor-mediated responses. These relaxations were markedly reduced by MnTMPyP (a membrane-permeant mimetic of superox-

ide dismutase), polyethylene glycol catalase (PEG-catalase; a membrane-permeant analog of catalase), and by wortmannin (an inhibitor of PI3-kinase). HEDS-induced sustained phosphorylation of Akt and eNOS in endothelial cells was abolished by MnTMPyP, PEG-catalase and wortmannin. Oral administration of HEDS induced a significant decrease of mean arterial pressure in spontaneously hypertensive rats. **Conclusion:** These findings indicate that HEDS caused endothelium-dependent relaxations of coronary artery rings through the redox-sensitive activation of the endothelial PI3-kinase/Akt pathway leading to the subsequent activation of eNOS by phosphorylation. HEDS also has antihypertensive properties.

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Introduction

Cardiovascular disease is the most prevalent disease worldwide and there is intense interest in pharmacological approaches to prevent and reduce the burden of this chronic disease. In this context, several epidemiological studies have indicated an inverse correlation between the

consumption of polyphenol-rich food such as fruits, vegetables and beverages (wine, grape juices and tea) and the risk of cardiovascular diseases [for reviews, see 1–4].

Some of the potential cardiovascular protective properties of polyphenols have been related to their antioxidant activity. In addition, these compounds have been suggested to decrease the risk of coronary artery disease by several additional mechanisms, i.e. attenuation of the oxidation of low-density lipoprotein [5, 6], inhibition of the inflammatory response mediated by nuclear factor- κ B [7], prevention of smooth muscle cell proliferation and migration [8–10], inhibition of platelet aggregation [11, 12] and leukocyte adhesion [13, 14]. Moreover, polyphenols can also exert a direct action on endothelial cells resulting in an enhanced formation of endothelium-derived factors playing a major role in the control of vascular homeostasis, such as nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) [1, 15–20].

Recently, we have shown that the hydroalcoholic extract of *Dicksonia sellowiana* (HEDS) Presl. Hook (Dicksoniaceae), a tree fern widely distributed in Central and South America [21, 22], decreases hypertension and induces endothelium-dependent relaxation mediated by activation of muscarinic receptors and stimulation of the NO pathway in rat aortic rings [23]. These effects were attributed to the presence of high amounts of polyphenolic compounds in the extract obtained from *D. sellowiana* leaves. This plant is popularly known as ‘xaxim’ or ‘samambaiaçu’ [24, 25] and its leaves are traditionally used to treat scabies, pruritus, parasitic diseases and asthma, mainly among the indigenous population [26]. In Brazil, *D. sellowiana* is now tested in volunteers for the development of phytomedicinal products against asthma, and it has also presented beneficial effects on several pathologies such as parasitic and skin diseases and also those related to the cardiovascular system such as hypertension.

The aim of the present study was to determine the vascular and endothelial effects of standardized HEDS leaves on isolated porcine coronary arteries, an important vascular bed, because coronary artery alterations often lead to cardiac diseases. Experiments were performed using isolated coronary artery rings suspended in organ chambers and cultured coronary artery endothelial cells in order to characterize the signaling pathway leading to eNOS activation in response to this extract. In addition, the ability of HEDS to decrease blood pressure in vivo was also investigated in spontaneously hypertensive (SHR) rats.

Methods

Plant Material and Preparation of HEDS

D. sellowiana leaves, harvested in the metropolitan region of Curitiba (Brazil), were identified in the Department of Botany of the Federal University of Paraná (Curitiba, PR, Brazil). Voucher specimens were deposited at the herbarium of the university under No. 46579.

The procedures used to prepare and analyze HEDS have been described previously [23]. Briefly, 10 g of powdered dry plant leaves was kept in a glass cartridge with a porous plate No. 1 and connected to a modified Soxhlet apparatus, where 200 ml of 85% aqueous ethanol was added for 3 h. The extract was concentrated to 100 ml and filtrated using a 0.45 μ m membrane filter. The calculated yield of the HEDS was 0.0341 g/ml. The standardized dry extract of *D. sellowiana* leaves was produced by the Humanus Botânica Indústria e Comércio LTDA (lot 0236/05) and supplied by the Natureza Pura Laboratório de Pesquisas Científicas LTDA. The phenolic content of HEDS was determined by the Folin-Ciocalteu method and corresponds to 494.2 mg/g gallic acid equivalent. High-performance liquid chromatography-photodiode array detection (HPLC-DAD) analysis showed that the extract contained 6.3 mg/g gallic acid, 8.7 mg/g protocatechuic acid, 17.1 mg/g chlorogenic acid, 28.0 mg/g coumaric acid, 12.9 mg/g ferulic acid, 8.45 mg/g sinapic acid and 18.3 mg/g cinnamic acid. Traces of other compounds were also detected but remained unidentified.

Reagents

All substances were of analytic grade. Apamin, charybdotoxin and iberiotoxin were from Latoxan. Tram-34, calmidazolium, atropine, superoxide dismutase (SOD), catalase, polyethylene glycol catalase (PEG-catalase), indomethacin, bradykinin, dihydroethidine (DHE), N ω -nitro-L-arginine (L-NA), KN-93 [2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine] and N-acetyl-L-cysteine (NAC) were obtained from Sigma. Wortmannin and the SOD mimetic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) were from Alexis Chemicals. U46619 (9,11-dideoxy-9 α -methanoepoxy prostaglandin F_{2 α}) was obtained from Cayman Chemical and compound C and PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine) were from Calbiochem. The tested substances were dissolved in Krebs bicarbonate or 10% (vol/vol) dimethyl sulfoxide. The solution of the HEDS was prepared in Krebs solution directly before use.

Preparation of Rings of Porcine Coronary Arteries

Hearts from pigs were collected from a local slaughterhouse and the left anterior descending coronary artery was dissected free of fat and connective tissue and cut into 4 mm rings. The rings were then mounted on two stainless steel hooks in 10 ml organ baths. One of these hooks was attached to a force transducer to measure changes in isometric tension and coupled to an amplifier and a computer for data collection. In experiments requiring endothelium-denuded rings, the endothelium was removed by rubbing the intimal surface with a pair of forceps. Tissues were maintained at 37°C in oxygenated (95% O₂/5% CO₂) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 1.25, NaHCO₃ 25, and D-glucose 11, pH 7.4) at a resting tension of 5 g. Rings were allowed to equilibrate

for at least 60 min, a period during which the tension was adjusted and the bathing solution was periodically changed.

Vascular Reactivity Studies

Following equilibration, the viability of each coronary ring was determined by repetitive contraction with KCl (80 mM). After a 30 min washout period, rings were contracted with the thromboxane mimetic U46619 (1–60 nM) to about 80% of the maximal contraction and then relaxed with bradykinin (0.3 μ M) to check the presence of a functional endothelium. After the removal of these drugs by repeated washings, the rings were contracted under the same conditions as described above before the addition of HEDS (from 0.1 to 100 μ g/ml). In some experiments, the rings were exposed to an inhibitor for 30 min before the addition of U46619.

In another set of experiments, cumulative contractile responses induced by U46619 (1 nM to 1 μ M) were obtained in the presence of HEDS (100 μ g/ml) under different time conditions: (a) HEDS incubated during 5, 30, 60 min or 3 h before the addition of U46619 in the presence of the extract; (b) HEDS incubated for only 5 min before washing and a subsequent 25, 55 min or 2 h 55 min waiting period before the contraction was induced by U46619 in the absence of the extract, and (c) both previous conditions in the presence of L-NA (100 μ M), an NO synthase inhibitor.

Determination of Vascular Reactive Oxygen Species Formation

The redox-sensitive fluorescent dye DHE was used to evaluate the in situ formation of reactive oxygen species (ROS) by the use of a method described by Miller et al. [27]. DHE permeates freely into cells and in the presence of ROS is oxidized to ethidium which is trapped by intercalating with the DNA. Porcine coronary rings (5 to 6 mm length) from the control group or treated with either HEDS (100 μ g/ml), HEDS plus SOD (500 U/ml), catalase (500 U/ml), MnTMPyP (100 μ M), PEG-catalase (500 U/ml) or NAC (10 mM) were embedded in OCT compound (Tissue-Tek; Qiagen, Hilden, Germany), and frozen in a liquid nitrogen bath before cryostat sections. These unfixed frozen coronary artery rings were cut into 25- μ m-thick sections and placed on poly-L-lysine-coated microscope slides. DHE (2.5 μ M) was applied to all tissue sections on the slide, which was then incubated in a light-protected humidified chamber at 37°C for 30 min before being sealed with a coverslip. Images were obtained with a Bio-Rad MRC-1024 laser-scanning confocal microscope equipped with a krypton/argon laser. Laser settings were identical for the acquisition of images from control and HEDS-treated groups. Ethidium was excited at 488 nm and fluorescence was detected with a 585 nm longpass filter.

Immunohistochemical Determination of Phosphorylated eNOS

Porcine coronary arteries were removed from the porcine hearts, embedded in OCT compound and frozen in liquid nitrogen. The frozen coronary arteries were cryosectioned at 14 μ m. Sections were air-dried for 1 h and stored at –80°C until use. The sections were placed in 4% paraformaldehyde for fixation, and thereafter treated with 10% serum in PBS containing 0.1% BSA and 0.05% Tween-20 for 45 min to block nonspecific binding. After rinsing, the sections were then incubated overnight at 4°C with control nonimmune serum or an antibody directed against

phosphorylated eNOS. The antibody used was a purified rabbit polyclonal against phosphorylated Ser1177 eNOS (sc-21871, 1:100 dilution; Santa Cruz Biotechnology). Sections were washed with PBS, incubated with the secondary antibody (Alexa Fluor 633-labeled anti-rabbit IgG, 1:200 dilution; Cell Signaling Technology), diluted in the same buffer for 2 h at room temperature in the dark and washed before being mounted and coverslipped. The sections were examined under a confocal microscope. For analysis of immunostaining intensity in coronary artery sections, we measured the fluorescence values of 6 arteries per group (on 3 randomly selected areas for each section). The fluorescence quantifications were assessed using the software ImageJ (NIH public domain software).

Culture of Porcine Coronary Artery Endothelial Cells

Segments of porcine coronary arteries were flushed with PBS without calcium to remove the remaining blood. Thereafter, endothelial cells were isolated by collagenase treatment (type I, Worthington, 1 mg/ml for 15 min at 37°C), and cultured in culture dishes containing MCDB 131 medium (Invitrogen) supplemented with 15% fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 μ g/ml) and L-glutamine (2 mM) (all from Cambrex) and grown for 48–72 h. All experiments were performed with confluent cultures of cells used at first passage. Cells were exposed to a serum-free culture medium in the presence of 0.1% bovine serum albumin (QBiogene) for 6 h prior to treatment.

Western Blot Analysis

The level of phosphorylation of Src, Akt and endothelial eNOS was determined in cultured endothelial cells using Western blot analysis. After treatment, the endothelial cells were washed twice with PBS and then lysed in an extraction buffer of the following composition in mM: Tris/HCl 20 (pH 7.5; QBiogene), NaCl 150, Na₃VO₄ 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogen). Total proteins (20 μ g) were separated on 8% SDS-polyacrylamide (Sigma) gels at 70 V for 2.5 h. Separated proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (Amersham) at 100 V for 120 min. Membranes were blocked with a blocking buffer containing 3% bovine serum albumin for p-Akt and p-eNOS, and I-block for p-Src, Tris-buffered saline solution (Biorad) and 0.1% Tween 20 (Sigma) for 1 h. For detection of phosphorylated proteins, membranes were incubated with the respective primary antibody (p-Src Tyr418, Biosource; p-Akt Ser473 and p-eNOS Ser1177, Cell Signaling Technology; dilution 1:1,000) overnight at 4°C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit IgG, dilution 1:5,000; Cell Signaling Technology) at room temperature for 60 min. Prestained markers (Invitrogen) were used for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham). Ponceau staining was performed to verify the quality of the transfer and equal amounts of proteins in each lane. Densitometric analyses were performed with the software ImageJ.

Antihypertensive Effects of HEDS

The putative dose-dependent antihypertensive effect of HEDS was examined in SHR rats. The extract was administered by gavage at 30–300 mg/kg. After a 1-hour treatment period, the rats

Fig. 1. Characterization of HEDS-induced relaxations in isolated porcine coronary artery rings. Intact and endothelium-denuded rings were contracted with U46619 before the addition of cumulative concentrations of HEDS. **A** Endothelium-dependent relaxations induced by HEDS. **B** Effect of L-NA (100 μM), apamin (APA, 100 nM) plus charybdotoxin (CTX, 100 nM), and the combination of L-NA and apamin plus charybdotoxin on HEDS-induced relaxations. **C** Effect of indomethacin (10 μM) on HEDS-induced relaxations in coronary artery rings with endothelium. **D** Relaxant effect of HEDS in the presence of atropine (1 μM) in intact arteries. Results are shown as means $\pm$ SEM of 6 different experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for inhibitory effect versus control.

were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg), given by intramuscular route. A polyethylene tube (PE 50) filled with heparinized saline was catheterized into the left carotid artery. The catheter was connected via a pressure transducer to a MacLab® recording system for online measurement of blood pressure. During the measurement period the rats were placed on a warm bed maintained at 36°C.

Statistical Analysis

The results are expressed as means $\pm$ SEM of 3–8 experiments. Statistical significance was determined through a one-way analysis of variance (ANOVA) followed by Bonferroni's test or with Student's *t* test for paired data as required. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, Calif., USA). Values of $p < 0.05$ were considered statistically significant.

Results

HEDS Induces Endothelium-Dependent Relaxations in Coronary Artery Rings

The addition of cumulative concentrations of HEDS on isolated porcine coronary artery rings contracted with U46619 induced concentration-dependent relaxations in endothelium-intact but not in endothelium-denuded preparations (fig. 1A). The endothelium-dependent relaxations started at concentrations greater than 1 μg/ml and reached a near maximal value at 100 μg/ml ($E_{\max} = 95.2 \pm 3.2\%$). Relaxations to HEDS were significantly reduced by L-NA (100 μM), an inhibitor of eNOS (approx. 54% of inhibition of $E_{\max}$), minimally affected (around 18%) by the combination of charybdotoxin (100 nM) plus apamin

Fig. 2. Role of calmodulin and CaMKII on endothelium-dependent relaxations induced by HEDS in intact coronary artery rings. Rings were incubated with either calmidazolium (10 μ M) (**A**) or KN-93 (10 μ M) (**B**) for 30 min before the contraction with U46619 and subsequent addition of HEDS. Results are shown as means $\pm$ SEM of 6 different experiments. *** $p < 0.001$ for inhibitory effect versus control.

(100 nM), two inhibitors of EDHF-mediated responses, and almost abolished (94% of inhibition) by the combination of L-NA with charybdotoxin plus apamin (fig. 1B). Indomethacin (10 μ M) induced a leftward shift of the concentration-response curve for HEDS-induced relaxations without affecting the maximal relaxation (fig. 1C). Tram-34 plus apamin-resistant relaxations to HEDS were unaffected by iberiotoxin, a selective inhibitor of BK_{Ca} channels ($n = 8$, data not shown), indicating that these channels are unlikely to be involved. Atropine (1 μ M), a selective muscarinic receptor antagonist, induced a significant potentiation of the relaxations elicited by HEDS (fig. 1D) without significantly affecting the maximal relaxation. Altogether, these findings indicate that HEDS causes endothelium-dependent relaxations, mainly dependent on an L-NA-sensitive component and also, to some extent, on an EDHF component and rule out the involvement of muscarinic receptors. In addition, prostanoids seem to oppose the relaxant effect induced by HEDS.

Role of Calmodulin and Calmodulin-Dependent Kinase II in HEDS-Induced Relaxations

In endothelial cells, eNOS has been shown to be a constitutive and Ca²⁺/CaM-dependent enzyme. An increase in the intracellular-free Ca²⁺ concentration, as observed following receptor-dependent and receptor-independent stimulation, enhances endothelial NO formation and elicits vasodilatation. eNOS can be phosphorylated and activated by a calmodulin-dependent enzyme such as calmodulin-dependent kinase II (CaMKII) [28]. Incubation of

rings with calmidazolium (10 μ M), a calmodulin inhibitor, caused a partial reduction of the relaxations induced by HEDS (approximately 40% of inhibition of E_{max}) (fig. 2A), whereas KN-93 (10 μ M), a selective CaMKII inhibitor, was without effect (fig. 2B). These results indicate that HEDS-induced relaxations partially involve eNOS activation by a calmodulin-dependent but not a CaMKII-dependent mechanism. Treatment of coronary artery rings with compound C (50 μ M), an inhibitor of AMP-activated protein kinase, did not significantly affect endothelium-dependent relaxations to HEDS at low concentrations and even potentiated them at 1 and 3 μ g/ml indicating that AMP-activated protein kinase does not play a role in HEDS-induced eNOS activation ($n = 8$, data not shown).

Role of ROS, Src Kinase and the PI3-Kinase/Akt Pathway in HEDS-Induced Relaxations

Recent findings indicate that polyphenolic compounds can induce NO-mediated relaxations in porcine coronary arteries by a mechanism involving the endothelial production of ROS and activation of the PI3-kinase/Akt pathway [15, 20] leading to eNOS phosphorylation. The consequent activation of eNOS through this pathway has been found to occur even in a Ca²⁺-free medium [29]. Therefore, experiments were performed using SOD (500 U/ml), MnTMPyP (100 μ M), a membrane-permeant SOD mimetic, catalase (500 U/ml) and PEG-catalase (500 U/ml), a membrane-permeant catalase mimetic, to determine whether a ROS signaling pathway is involved in HEDS-induced relaxations. The relaxations induced by

Fig. 3. Role of the redox-sensitive Src kinase and the PI3-kinase/Akt pathway in HEDS-induced endothelium-dependent relaxations. Coronary artery rings with endothelium were incubated with native SOD (500 U/ml) or MnTMPyP (100 μ M), a cell permeable SOD mimetic (**A**), native catalase (CAT, 500 U/ml) or PEG-catalase (PEG-CAT, 500 U/ml), a membrane-permeant analog of catalase (**B**), the Src kinase inhibitor PP2 (10 μ M) (**C**) and the PI3-kinase inhibitor wortmannin (30 nM) (**D**) for 30 min before contraction to U46619 and subsequent relaxation to HEDS. Results are shown as means $\pm$ SEM of 6 different experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for inhibitory effect versus control.

HEDS (0.1–100 μ g/ml) were markedly reduced by the membrane-permeant SOD mimetic MnTMPyP (approx. 89% of inhibition of $E_{\max}$), and partially inhibited by the cell-permeable PEG-catalase, whereas native SOD and native catalase did not have such an effect (fig. 3A, B, respectively).

Moreover, it has been shown that Src, a redox-sensitive kinase, acts as an upstream activator of the PI3-kinase/Akt pathway leading to the polyphenol-induced eNOS activation [30, 31]. Experiments were performed, therefore, to determine the role of the Src kinase/PI3-kinase/Akt pathway in relaxations to HEDS. Inhibition of Src kinase with PP2 (10 μ M) partially but significantly reduced relaxations to HEDS (fig. 3C) and inhibition of the PI3-kinase by wortmannin (30 nM) almost abolished relaxations to HEDS (80% of inhibition) (fig. 3D).

HEDS Stimulates the Formation of ROS and eNOS Ser1177 Phosphorylation in Sections of Porcine Coronary Arteries

As indicated previously, polyphenols cause a pro-oxidant response in endothelial cells which in turn causes activation of the PI3-kinase/Akt pathway leading to NO formation [1]. To evaluate the ROS production induced by HEDS, sections of porcine coronary arteries were exposed to DHE and the ability of HEDS to stimulate the formation of superoxide anions was evaluated in situ in the presence of different treatments. In comparison to control (fig. 4A, a), HEDS (100 μ g/ml) consistently increased the fluorescence signal predominantly at the endothelial layer (fig. 4A, b). Treatment of artery sections with native SOD (500 U/ml) or catalase (500 U/ml) did not affect the fluorescence signal (fig. 4A, c, d). How-

Fig. 4. HEDS induces the formation of ROS in sections of porcine coronary arteries and stimulates Ser1177 eNOS phosphorylation. **A** Sections of arteries loaded with 10 µM DHE were either untreated (**a**) or exposed to HEDS (**b**) in the presence of SOD (500 U/ml) (**c**), catalase (CAT, 500 U/ml) (**d**), MnTMPyP (100 µM) (**e**), PEG-catalase (PEG-CAT, 500 U/ml) (**f**) or NAC (10 mM) (**g**) for 30 min. DHE fluorescence was monitored over 10 min using a confocal microscope. **B** Corresponding cumulative data. **C** Immunohistochemical staining in coronary artery sections using antibody against phosphorylated Ser1177 eNOS. Representative original microphotographs showing an increased eNOS phosphorylation signal after treatment with HEDS (100 µg/ml, 10 min) (**b**) compared to control (**a**). **D** Corresponding cumulative data. Results are shown as means $\pm$ SEM of 4 or 6 different experiments. ** $p < 0.01$ and *** $p < 0.001$ versus control; ### $p < 0.001$ versus HEDS; °° $p < 0.01$ versus SOD; +++ $p < 0.001$ versus CAT.

ever, the membrane-permeant substances MnTMPyP (100 µM) and PEG-catalase (500 U/ml) and the antioxidant N-acetylcysteine (NAC, 10 mM) abolished the HEDS-induced endothelial ROS production (fig. 4A, e–g).

In endothelial cells, eNOS activation can occur by Akt-dependent phosphorylation of eNOS at Ser1177 [29]. Therefore, to assess the effects of HEDS on eNOS activation by Ser1177 phosphorylation, immunohistochemical

experiments were performed in coronary artery sections using an antibody directed against phosphorylated eNOS Ser1177. HEDS (100 µg/ml, 10 min) induced a strong increase in phosphorylation of eNOS Ser1177 (fig. 4C, b) compared to basal conditions (fig. 4C, a). Densitometric quantification showed a significant increase in staining intensity for phosphorylated eNOS following HEDS stimulation up to $287 \pm 32\%$ (fig. 4D).

Fig. 5. HEDS causes a concentration-dependent phosphorylation of Akt at Ser473 (**B**) and eNOS at Ser1177 (**C**) in cultured porcine coronary artery endothelial cells. Results are shown as means $\pm$ SEM of 3–4 different immunoblots (**A**). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for inhibitory effect versus control.

HEDS Causes the Redox-Sensitive Activation of Src with Subsequent PI3-Kinase/Akt-Dependent Phosphorylation of eNOS

To better characterize the signaling pathway involved in eNOS activation in response to HEDS, levels of phosphorylated Src, Akt and eNOS were assessed in endothelial cells by Western blot analysis.

Fig. 6. HEDS causes a time-dependent phosphorylation of Akt at Ser473 (**B**) and eNOS at Ser1177 (**C**) in endothelial cells. Results are shown as means $\pm$ SEM of 3–4 different immunoblots (**A**). * $p < 0.05$ for inhibitory effect versus control.

HEDS (1–100 $\mu\text{g/ml}$, 10 min) evoked the concentration-dependent phosphorylation of Akt at Ser473 and eNOS at Ser1177 in endothelial cells (fig. 5A–C). Exposure of endothelial cells to HEDS (100 $\mu\text{g/ml}$) caused the appearance within 1–5 min of a strong phosphorylation signal of Akt and eNOS. The increased phosphorylation signals persisted for up to 60 min (fig. 6A–C).

Fig. 7. Role of calmodulin and CaMKII in HEDS-induced phosphorylation of Src, Akt and eNOS in endothelial cells. Cells were incubated with either calmidazolium (CALMID, 10 μ M) or KN-93 (10 μ M) for 30 min before the addition of HEDS (100 μ g/ml). The levels of p-Src (**B**), p-Akt (**C**) and p-eNOS (**D**) were determined by Western blot analysis. **A** Representative immunoblots. Results are shown as means $\pm$ SEM of 3–4 different experiments. * $p < 0.05$ and ** $p < 0.01$ versus control.

In addition, HEDS (100 μ g/ml) enhanced the phosphorylation level of Src at Tyr418 (fig. 7A, B, 8A, B, 9A, B). Treatment of the cells with calmidazolium (10 μ M) or KN-93 (10 μ M) did not significantly affect the phosphorylation of Src, Akt and eNOS (fig. 7B–D, respectively). In contrast, HEDS-induced phosphorylation of Akt and eNOS was abolished by MnTMPyP (100 μ M) and PEG-catalase (500 U/ml) (fig. 8C, D, respectively) and that of Src by MnTMPyP but not by PEG-catalase (fig. 8A, B). Also, the PI3-kinase inhibitor wortmannin prevented phosphorylation of Akt and eNOS (fig. 9A, C, D) but not that of Src (fig. 9A, B). The treatment with PP2 (10 μ M) reduced the phosphorylation level of Src (fig. 9A, B), almost abolished that of p-Akt (fig. 9A, C) and caused a small but significant reduction of p-eNOS (fig. 9A, D). These data indicate that ROS act as upstream mediators of Src kinase which induces activation of the PI3-kinase/Akt pathway leading to eNOS phosphorylation in response to HEDS.

In endothelial cells treated for only 5 min with HEDS (100 μ g/ml), the increased phosphorylation level of Akt

and eNOS was long-lasting. It reached a peak value within 25–55 min after washing and then returned gradually to baseline at 2 h 55 min (fig. 10A–C).

HEDS Inhibits the Contractile Responses Induced by U46619 in Coronary Arteries

Exposure of coronary artery rings to HEDS (100 μ g/ml) during 5, 30, 60 min or 3 h prior to the addition of cumulative concentrations of U46619 (1 nM to 1 μ M) significantly reduced contractions in rings with endothelium (fig. 11). Incubation of rings with HEDS for only 5 min before washing was also associated with a reduced contraction to U46619 up to 55 min after washout (fig. 11C). The inhibitory effect of HEDS on contractile responses was prevented by L-NA (100 μ M, 30 min), indicating the involvement of the activation of eNOS (fig. 11A–D).

HEDS has Antihypertensive Effects on SHR Rats

Oral administration of HEDS induced a significant and dose-dependent decrease in mean arterial blood pres-

Fig. 8. Role of ROS in HEDS-induced phosphorylation of Src, Akt and eNOS in endothelial cells. Cells were incubated with either SOD (500 U/ml), MnTMPyP (100 µM), PEG-catalase (PEG-CAT, 500 U/ml), or catalase (CAT, 500 U/ml) for 30 min before the addition of HEDS (100 µg/ml). The levels of p-Src, p-Akt and p-eNOS were determined by Western blot analysis. **A** Representative immunoblots. **B–D** Corresponding cumulative data. Results are shown as means $\pm$ SEM of 3–4 different experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control; # $p < 0.05$ and ### $p < 0.001$ versus HEDS.

sure of SHR rats (-11.0 ± 1.3 , -17.6 ± 1.4 and -23.5 ± 1.5 mm Hg at 30, 100 and 300 mg/kg, respectively, $n = 5$) in comparison to vehicle (water, -3.7 ± 1.0 mm Hg).

Discussion

In the present study, standardized HEDS leaves extract was found to be a potent stimulus for endothelium-dependent relaxation in isolated porcine coronary arteries and eNOS activation in cultured porcine coronary endothelial cells. These responses are partially calmodulin-dependent and involve a ROS-dependent activation of Src kinase and subsequent PI3-kinase/Akt-dependent

phosphorylation of eNOS at Ser1177 leading to smooth muscle relaxation.

HEDS caused potent endothelium-dependent relaxations in coronary artery rings which were reduced by L-NA and minimally affected by the combination of charybdotoxin plus apamin. However, the combination of L-NA, charybdotoxin and apamin abolished HEDS-induced relaxations. These findings indicate a major role for an L-NA-sensitive component of relaxation and also to some extent for an EDHF-mediated component. They are, at least in part, in agreement with a previous study performed using rat aorta rings [23].

In porcine coronary artery rings, HEDS-induced relaxations were not due to the formation of relaxing pros-

Fig. 9. Role of the PI3-kinase/Akt pathway and the Src kinase in HEDS-induced phosphorylation of Akt and eNOS in endothelial cells. Cells were incubated with either wortmannin (WORT, 30 nM) or PP2 (10 µM) for 30 min before the addition of HEDS. The level of p-Src, p-Akt and p-eNOS was determined by Western blot analysis. **A** Representative immunoblots. **B–D** Corresponding cumulative data. Results are shown as means $\pm$ SEM of 3–4 different experiments. ** $p < 0.01$ and *** $p < 0.001$ versus control; # $p < 0.05$ and ### $p < 0.001$ versus HEDS.

tanoids or the activation of endothelial muscarinic receptors. Indeed, relaxations were slightly potentiated in the presence of indomethacin, a cyclooxygenase inhibitor, and also atropine, a muscarinic receptor antagonist. In contrast, NO-mediated relaxations induced by several substances or plant extracts in rat aortic rings have been shown to involve activation of muscarinic receptors [32–34]. In addition, acetylcholine is not able to relax porcine coronary artery rings [35] and can even induce a contractile response [36–39]. However, in agreement with the present findings, it has already been shown that endothelium-dependent modulation of vascular tone with acetylcholine or lionfish venom are potentiated by atropine in porcine coronary artery rings [40, 41]. A potentiating effect of indomethacin on HEDS-induced relaxations is observed. These findings suggest that arachidonic acid metabolites counteract the relaxation to HEDS [43].

Endothelial NO release can be activated by receptor-dependent and receptor-independent agonists as a consequence of an increase in the intracellular concentration of free Ca^{2+} and the association of a Ca^{2+} /calmodulin (CaM) complex with eNOS [44]. The subsequent NO formation and vasodilatation can be inhibited by CaM inhibitors [45]. In addition, calmodulin-dependent kinase, CaMKII, has been involved in the rapid phosphorylation of eNOS at Ser1177 and vasorelaxation after bradykinin stimulation, an effect inhibited by KN-93 [28, 46]. In the present study, the incubation with a calmodulin inhibitor, calmidazolium, caused approximately a 40% inhibition of HEDS-induced relaxation in coronary artery rings, whereas KN-93 was inactive. Also, none of the inhibitors affected the HEDS-induced phosphorylation of eNOS in cultured endothelial cells.

Fig. 10. Long-lasting effect of HEDS in coronary endothelial cells. The phosphorylation level of Akt (**B**) and eNOS (**C**) in endothelial cells was assessed at different times after incubation of the cells with HEDS (100 μ M) for only 5 min followed by washing. **A** Representative immunoblots. Results are shown as means $\pm$ SEM of 3–4 different experiments. * $p < 0.05$ and ** $p < 0.01$ versus control; # $p < 0.05$ versus HEDS 25 min.

Altogether, these findings indicate that the HEDS-induced relaxant effect is partially dependent on Ca^{2+} /CaM but not on CaMKII.

Previous studies have indicated that polyphenols are able to activate eNOS through two distinct mechanisms:

an increase in intracellular Ca^{2+} and a phosphorylation of eNOS by the PI3-kinase/Akt pathway [1], a phenomenon which is strictly dependent upon the intracellular formation of superoxide anions in endothelial cells [19, 20, 47]. Therefore, experiments were performed to determine the role of ROS in HEDS-induced relaxations. In the presence of membrane-permeant analogs of either SOD or catalase (MnTMPyP and PEG-catalase, respectively), there was a strong reduction in the relaxations to HEDS, whereas native SOD and native catalase were inactive, indicating a key role of intracellular superoxide anions (O_2^-) and to some extent of intracellular hydrogen peroxide (H_2O_2). In addition, MnTMPyP and PEG-catalase also prevented HEDS-induced phosphorylation of eNOS at Ser1177. Moreover, direct evidence that HEDS is able to cause a NAC-, MnTMPyP- and PEG-catalase-sensitive pro-oxidant response was obtained with the redox-sensitive fluorescent probe DHE in coronary artery sections. Although the specific endothelial source of ROS remains to be determined, potential sources include NADPH oxidase, xanthine oxidase, cytochrome P450 monooxygenases and the polyphenolic compounds themselves [48–51]. A similar pathway involving an increased endothelial formation of superoxide anions has also been observed in eNOS activation in response to several other sources of polyphenols such as red wine and grape juices [15, 47, 52].

In endothelial cells, the formation of superoxide anion is able to activate the Src-PI3-kinase/Akt pathway leading to eNOS phosphorylation at Ser1177 in response to polyphenols [1]. This pathway can also be activated in response to several physiological stimuli including estrogens, shear stress, vascular endothelial growth factor, H_2O_2 and high-density lipoprotein [53–58]. HEDS caused activation of the PI3-kinase/Akt pathway in a redox-sensitive manner as indicated by the concentration- and time-dependent phosphorylation of Akt at Tyr418 and eNOS at Ser1177, and their inhibition by intracellular scavengers of ROS (MnTMPyP and PEG-catalase) and by wortmannin, an inhibitor of PI3-kinase, in endothelial cells and also in isolated coronary arteries. The present findings also indicate that Src kinase, a redox-sensitive protein kinase, acts upstream of the PI3-kinase/Akt pathway since the Src kinase inhibitor PP2 partially prevented HEDS-induced phosphorylation of Src, Akt and eNOS in endothelial cells and HEDS-induced endothelium-dependent relaxations in isolated coronary arteries. In addition, HEDS-induced phosphorylation of Src was prevented selectively by the intracellular scavenger of superoxide anions MnTMPyP but was not affected by

Fig. 11. Effect of HEDS on contractile responses in coronary artery rings with endothelium. The graph shows contractile curves to U46619 assessed either in the presence or absence of HEDS (100 µg/ml), with or without L-NA (100 µM, 30 min), and at different times: 5 min (**A**), 30 min (**B**), 60 min (**C**) and 3 h (**D**). As indicated, some intact rings were exposed to HEDS 5 min before washout followed by a resting period before U46619-induced contractions. Results are shown as means $\pm$ SEM of 6 different experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for inhibitory effect versus control.

PEG-catalase, whereas both types of scavengers prevented phosphorylation of Akt and eNOS. This suggests that O_2^- seems to play a more important role in the activation of Src. Additionally, O_2^- can be partially converted to H_2O_2 and together they contribute to a potent activation of the PI3-kinase/Akt pathway [58].

Although polyphenols caused a modest pro-oxidant signal in endothelial cells, they did not induce the formation of ROS in the vascular smooth muscle of coronary arteries. In vascular smooth cells, it was reported that polyphenols prevented the growth factor-induced NADPH oxidase-dependent formation of ROS [59]. Moreover, they are able to scavenge superoxide anions, peroxy

radicals, hydroxyl radicals and peroxynitrite [60–62], to prevent the expression of pro-oxidant enzymes, such as NADPH oxidase and xanthine oxidase and to increase that of antioxidant enzymes such as catalase [63, 64].

HEDS induced long-lasting activation of eNOS even after short-term exposure to the extract. Within 1–5 min, HEDS caused the phosphorylation of eNOS at Ser1177 in endothelial cells. Moreover, the changes in the phosphorylation level of eNOS at Ser1177 were maintained for at least 3 h in the presence of HEDS and 55 min after a 5-min exposure period to HEDS. This effect was followed by a slow decline of eNOS phosphorylation level to near baseline levels. In addition, HEDS caused a similar time-

dependent inhibition of U46619-induced contractions in coronary artery rings.

The ability of HEDS to induce a decrease in blood pressure in SHR rats is in agreement with previous observations [23] and indicates that, like other polyphenol-rich extracts [1], HEDS could be of interest for the treatment of hypertensive states.

In summary, the present study indicates that HEDS causes an acute and potent activation of the NO pathway in endothelial cells from coronary arteries and that this

effect involves both the calmodulin-dependent (but not CaMKII-dependent) and ROS/PI3 kinase/Akt-dependent activation of eNOS. These mechanisms could help explain the antihypertensive effects of HEDS in SHR rats.

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