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2,3-*cis*-Procyanidins Elicit Endothelium-Dependent Relaxation in Porcine Coronary Arteries via Activation of the PI3/Akt Kinase Signaling Pathway

Aurica M. Kaufeld, Heinz H. Pertz, and Herbert Kolodziej*

Institute of Pharmacy, Pharmaceutical Biology, Freie Universität Berlin, Königin-Luise-Strasse 2, 14195 Berlin, Germany

ABSTRACT: Polyphenols including procyanidins have been reported to reduce the risk of cardiovascular diseases. However, polyphenolic extracts represent complex mixtures, and detailed information on their chemical composition is commonly lacking. The aim of this study was to investigate the potential of a highly purified and chemically defined 2,3-*cis*-procyanidin sample (di- to hexameric [4β,8]-linked oligomers) from *Nelia meyeri* to relax coronary arteries and to get insight into the underlying mechanisms. The procyanidins produced a concentration-dependent relaxation in endothelium-intact vascular rings by activation of the NO and endothelium-derived hyperpolarizing factor (EDHF)-signaling pathway via PI3/Akt kinase in a redox-sensitive manner, with O_2^- as key species predominantly produced by xanthine oxidase and NADPH oxidase. Our observations in tissue bath studies were confirmed by Western blotting; 2,3-*cis*-procyanidins induced phosphorylation of eNOS and Akt in a ROS-dependent manner. These findings provide a basis for comparing the relaxant response and mode of action with that of structurally related proanthocyanidins. Our results may contribute to a better understanding of the potential link between the beneficial effects of proanthocyanidins on vascular health and their broad distribution in many fruits, natural food sources, and foodstuffs.

KEYWORDS: *Nelia meyeri, 2,3-cis-procyanidins, NO, EDHF, PI3/Akt kinase, ROS, superoxide anion, relaxation, coronary heart disease*

INTRODUCTION

The endothelium produces a large variety of substances that cause dilatation or constriction of the vascular smooth muscle cell. Endothelial dysfunction is a key event in the development of cardiovascular diseases such as atherosclerosis, hypertension, and hyperlipidemia.[1,2] These pathological conditions include an impairment of endothelium-dependent vasorelaxation caused by a reduced release or enhanced inactivation of vasodilators such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF). In addition, endothelium-derived contracting factors (EDCF) such as prostaglandin endoperoxides may counteract endothelium-dependent vasorelaxation.[3−6]

The regulatory function of the endothelium is altered by cardiovascular risk factors with apparently detrimental functional consequences as well as long-term effects. For example, oxidative stress associated with an excessive vascular production of reactive oxygen species (ROS) plays an important role in the pathogenesis of vascular disorders.[7] Notably, superoxide anion (O_2^-) levels are markedly increased in such pathological conditions. Furthermore, an impairment of endothelium-dependent relaxation has been reported in hypercholesterolemic rabbits due to a lack of NO.[1] Epidemiological studies have indicated that regular consumption of polyphenol-rich food and beverages reduces the risk of cardiovascular diseases.[8,9] Numerous lines of evidence have demonstrated that polyphenols possess antioxidant and radical scavenging activities, thereby explaining, at least in part, the claimed beneficial effects of ingested polyphenolic-rich diets on cardiovascular diseases and inflammatory conditions.[10,11] Vascular protection may also be due to the direct effect of polyphenols on endothelial cells as exemplarily demonstrated for red wine, tea, strawberries, and raspberries.[12−15] Previous experimental studies have indicated activation of the phosphatidylinositol-3 (PI3) kinase/protein kinase B (Akt) signaling pathway by polyphenols, which subsequently induces the endothelial formation of NO.[16] In addition, the EDHF signaling pathway may be involved in endothelium-dependent relaxation via the PI3-kinase/Akt pathway.[14] Paradoxically, it has been reported that the generation of ROS is apparently crucial for procyanidin-dependent relaxation prior to activation of the PI3/Akt kinase.[17]

Within the structurally heterogeneous group of polyphenols, procyanidins and procyanidin-rich fractions have been reported to elicit endothelium-dependent relaxation in arteries of different species.[17−22] However, the hitherto tested procyanidin samples are chemically poorly defined. The information on structural features for activity is largely limited to the hydroxylation patterns and the degree of polymerization, while knowledge about the mode of bonding and the stereochemistry of flavanyl constituent units and interflavanyl linkages in oligomers is commonly missing or structures have been assumed. *Nelia meyeri* Schwant (Mesembryanthemaceae) has been shown to be a rich source of 2,3-*cis* oligomeric procyanidins (PC).[22] The availability of a highly purified and chemically well characterized fraction from this plant, comprising a mixture of di- to hexameric 2,3-*cis*-procyanidins

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with 4β,8-interflavanyl linkages (Figure 1), prompted the present work to study (i) the relaxant response to 2,3-*cis*

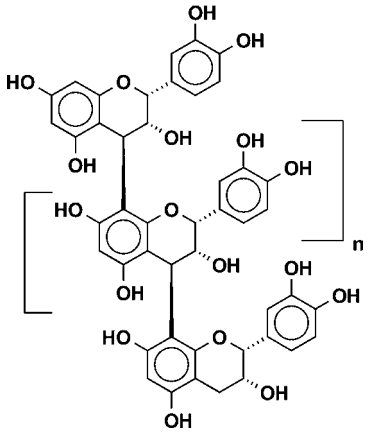

Figure 1. Representative structure of 2,3-*cis* oligomeric procyanidins from leaves of *Nelia meyeri* with [4β,8]-linked epicatechin constituent units.

oligomeric procyanidins in porcine coronary arteries and (ii) the molecular mechanism(s) of relaxation with special focus on the PI3/Akt pathway and the origin and role of ROS, with relevance to a variety of plant foodstuffs and beverages containing quantities of proanthocyanidins.

MATERIALS AND METHODS

Materials. 9,11-Dioxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) was a gift from Upjohn (Kalamazoo, MI). Allopurinol, *N*-acetylcysteine (NAC), apocynin, N^G-nitro-L-arginine methyl ester (L-NAME), indomethacin, 2′,7′-dichlorofluorescein, and wortmannin were obtained from Sigma-Aldrich (Taufkirchen, Germany). Apamin, carbenoxolone, charybdotoxin, (*Z*)-1,3-dihydro-3-(1*H*-imidazol-4-yl-methylene)-5-methoxy-2*H*-indol-2-one (SU5416), 7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)-nonyl]estra-1,3,5(10)-triene-3,17β-diol (ICI 182,780), 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), pertussis toxin, and rotenone were purchased from Tocris (Bristol, UK). Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) was ordered from Axxora GmbH (Lörrach, Germany).

Procyanidin Sample. Isolation, purification, and characterization of procyanidins from *N. meyeri* were reported previously.[22] Briefly, the ethyl acetate soluble portion (1.6% yield related to fresh weight), obtained from the parent methanol extract of leaves, was chromatographed on Sephadex LH-20 using ethanol as eluent. Further purification with acetone afforded oligomeric flavan-3-ols, identified as 2,3-*cis*-procyanidins by means of chemical methods, mass spectroscopy, NMR analysis, and circular dichroism. Repeated chromatography yielded a highly purified procyanidin mixture (0.53% yield related to fresh weight), comprised of dimeric to hexameric 2,3-*cis* procyanidins in the proportions dimers/trimers/tetramers/pentamers and hexamers 1:1.6:2.3:7.5. The sample was dried *in vacuo* and stored at room temperature in a tightly closed container.

For organ chamber studies, the procyanidin sample was dissolved in ethanol/Krebs−Henseleit solution (KHS; V/V 1:1) that did not contain Mg^{2+} and Ca^{2+} (composition of Krebs−Henseleit solution, see below). For Western blotting, the sample material was dissolved in ethanol (stock solution 50 mg/mL). The stock solution was diluted with Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline (PBS). The final ethanol concentration in Western blotting experiments was lower than 0.5%.

Tissue Preparation. Porcine hearts were obtained from the local slaughterhouse (Lehr- und Versuchsanstalt für Tierzucht und Tierhaltung; Teltow-Ruhlsdorf, Germany). During transport to the laboratory, the hearts were placed in ice cold preoxygenated KHS (95% O_2 and 5% CO_2; pH 7.4) of the following composition (in mM): NaCl (118), KCl (4.7), $CaCl_2$ (1.6), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $NaHCO_3$ (25), and glucose (11.5). Coronary arteries (left anterior descending and left circumflex) were removed from the hearts and cleared of fat and adhering tissue. The vessels were stored overnight at 4 °C in prior gassed KHS. Preliminary experiments have shown that tissue storage overnight does not impair the contractility of the smooth muscle. On the following day, the vessels were cut into rings (3−4 mm long and 2−3 mm i.d.). In experiments with endothelium-denuded rings, the endothelium was removed by gently rubbing the intimal surface with a pair of tweezers. The arterial rings were mounted between two L-shaped stainless steel hooks (300 μm diameter) in a 20-mL organ chamber filled with oxygenated KHS at 37 °C. Preparations were connected to an isometric force transducer (FMI TIM-1020; FMI Föhr Medical Instruments, Seeheim-Jugenheim, Germany), coupled to a TSE 4711 transducer (TSE Systems, Bad Homburg, Germany), and a Siemens C1016 compensograph (Siemens AG, Erlangen, Germany) for the continuous recording of changes in tension.

Relaxant Responses to 2,3-*cis*-Procyanidins. Following an equilibration period of 90 min with a resting tension of 20 mN, the tissue rings were precontracted once with 30 mM of KCl and three times with 30 nM of U46619 (thromboxane A2 mimetic). After the first and the second U46619-induced contractions had stabilized, the tissues were allowed to relax following application of bradykinin (100 nM). This procedure verified the integrity of the endothelium. When the third precontraction induced by U46619 had reached a plateau, the procyanidin sample was added cumulatively to the tissues in half-logarithmic increments (1−32 μg/mL). Inhibitors were added 30 min before the third U46619-induced contraction. All experiments were conducted in the continuous presence of indomethacin (5 μM) to inhibit the activity of cyclooxygenase.

Cell Culture. Human umbilical vein endothelial cells (HUVECs; Promocell, Heidelberg, Germany) were cultured in tissue dishes containing special HUVEC growth medium (Promocell), supplemented with 2% fetal calf serum (FCS). Passages 0 to 5 were used for the experiments. All cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. Before addition of the samples, cells were treated with serum-free culture medium.

Electrophoresis and Immunoblotting. To prepare cell lysates for Western blotting, the cells were incubated with the 2,3-*cis*-procyanidin sample (50, 100, and 200 μg/mL). Inhibitors were added to the culture flasks 30 min before the addition of the procyanidin sample. The cells were washed twice with Mg^{2+}- and Ca^{2+}-free PBS and then treated with lysis buffer (25 mM Tris × HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor (Roche Applied Science GmbH, Mannheim, Germany) and phosphatase inhibitor (Pierce, Bonn, Germany). All working steps were made on ice. After centrifugation at 16 000*g* for 15 min, the supernatants were collected, and the total protein concentration was determined with the bicinchoninic acid (BCA) protein assay reagent (Pierce) using bovine serum albumin (BSA) as a standard. Aliquots were stored at −70 °C until use.

Equal amounts of total cell lysate proteins were separated by gel electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gels (nUView precast gels, Peqlab, Erlangen, Germany). Proteins were subsequently transferred to polyvinylidene difluoride membranes (Whatman, Carl Roth, Karlsruhe, Germany) at 0.2 A for 45 min. The membranes were incubated in blocking buffer, which contained 5% skim milk powder (SKP; Sigma-Aldrich) dissolved in TBS-T (tris-buffered saline with 0.1% Tween 20), at room temperature for 1 h. Membranes were incubated with primary antibody (p-eNOS Ser1177, 48 μg total protein (TP); p-Akt Ser473, 20 μg TP, 1:1000; Cell Signaling Technology, Danvers, MA) dissolved in BSA-blocking buffer overnight at 4 °C. After four washing steps with TBS-T (each 10 min), membranes were incubated with secondary antibody (goat anti-rabbit antibody; Invitrogen, Darmstadt, Germany) at a dilution of 1:3000 with SKP blocking buffer for 90 min. Immunodetection was performed using an enhanced chemiluminescence detection kit (Amersham ECL

Western Blotting Detection System; GE Healthcare, Freiburg, Germany). A loading control (Akt-kinase and eNOS at a dilution of 1:1000; Cell Signaling Technology) was used for standardization. Quantification of bands was obtained by digital image analysis using NIH Image (U.S. National Institutes of Health; http://rsb.info.nih.gov/nih-image/). The mean value of control was set at 100%, and all data were expressed as percentages relative to the control.

Intracellular ROS Assay. After trypsinization, HUVECs were diluted in medium and added to each well of a black 96-well tissue plate with clear bottom (Costar, Corning, NY). After reaching confluence, cells were washed 2 times with PBS (Ca^{2+}- and Mg^{2+}-free) and incubated with *Nelia* procyanidins in medium without FCS for 30 min. After a washing step, cells were treated with 5 μM of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 20 min. DCF fluorescence intensity was read using a plate reader (Tecan, Infinite M200 pro, Männedorf, Germany; excitation wavelength 485 nm and emission wavelength 525 nm). The difference of fluorescence between treated and untreated cells was calculated.

Statistical Analysis. Data are presented as mean values ± standard error of the mean (SEM) for n animals (one vascular ring per animal for each treatment) or n individual experiments. Student's t test was used to assess differences between two mean values. Multiple comparisons between treatment groups were performed using the analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison test. P values <0.05 were considered to be significant.

RESULTS AND DISCUSSION

2,3-*cis*-Procyanidins Induce an Endothelium-Dependent Relaxation in Isolated Coronary Arteries via the NO Signaling Pathway. The 2,3-*cis*-procyanidin sample induced a concentration-dependent relaxation in endothelium-intact porcine coronary arterial rings (Figure 2A). To underline the importance of a functional endothelium, experiments with endothelium-denuded arteries were performed. As expected, endothelium-denuded arterial rings showed no relaxation after stimulation with *Nelia* procyanidins, clearly demonstrating that the relaxant response to *Nelia* procyanidins is endothelium-dependent (Figure 2A). This finding is consistent with the pharmacological effects of other polyphenols in coronary arteries.[11,23] To further substantiate the endothelium dependency of the vasorelaxant response to the 2,3-*cis*-procyanidins, relaxation was studied in the presence of the NO synthase (NOS) inhibitor L-NAME (200 μM). Under these experimental conditions, the maximal effect of the concentration–response curve to the procyanidin sample was significantly depressed but not abolished suggesting that NO is not the sole endothelial vasodilator substance for procyanidins (Figure 2A). On the other hand, NO has multiple targets in the cell, especially guanylyl cyclase. Therefore, procyanidin-treated arteries were incubated in the presence of ODQ (30 μM), an inhibitor of the NO-sensitive soluble guanylyl cyclase (sGC). Addition of ODQ caused an inhibition of the procyanidin-induced relaxation (Figure 2B). Thus, the endothelial NO synthase (eNOS) pathway plays an essential role in the relaxant effect of *Nelia* 2,3-*cis*-procyanidins. Confirmatory evidence of the activation of eNOS by the procyanidin sample was obtained from Western blot experiments (see below).

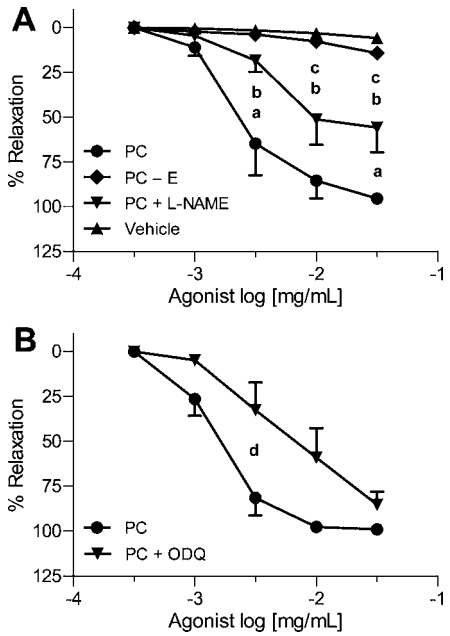

Figure 2. Endothelium-dependent relaxation to PC in porcine coronary arteries. (A) Effects of PC on endothelium-intact rings, in the presence of L-NAME (NOS inhibitor; 200 μM), and on endothelium-denuded rings (-E). In addition, the effect of vehicle ethanol/KHS (V/V 1:1; Mg^{2+} and Ca^{2+} free KHS) is shown. (B) Effects of PC in the absence and presence of ODQ (sGC inhibitor; 30 μM). Values are the mean ± SEM (n = 4–7); a indicates P < 0.05 PC plus L-NAME vs PC; b indicates P < 0.05 PC minus endothelium (-E) vs PC; c indicates P < 0.05 PC minus endothelium (-E) vs PC plus L-NAME; d indicates P < 0.01 PC plus ODQ vs PC.

Contribution of EDHF in the Relaxant response to 2,3-*cis*-Procyanidins. Under physiological conditions, endothelium-dependent relaxation is mediated by both endothelium-derived relaxing factor (EDRF syn. NO) and EDHF. It should be noted that EDHF is a minor contributing factor to inhibition of the vascular tone by hyperpolarizing the vascular smooth muscle cells in most types of large arteries.[24] The relaxant effect has been suggested to be mediated by Ca^{2+}-activated K^+ channels in the endothelial and smooth muscle plasmalemma.[25] As a consequence, EDHF-mediated responses induce a relaxation of blood vessels, often combined with NO-dependent relaxation.[26] In order to establish the role of EDHF in our experiments, apamin (APA; 100 μM), which is an inhibitor of small conductance Ca^{2+}-sensitive K^+ channels (SK_{Ca}), and charybdotoxin (CTX; 100 μM), an inhibitor of large conductance Ca^{2+}-sensitive K^+ channels (BK_{Ca}), were simultaneously added to the bathing medium to identify K^+ channels involved in the EDHF pathway.[27] Conspicuously, the procyanidin-induced relaxation remained unaffected (Figure 3A), demonstrating that Ca^{2+}-activated K^+ channels played only a minor role here. This should be seen in the light that EDHF release in arteries depends on their internal diameter.[24] However, by adding L-NAME to the combination of inhibitors, the inhibitory effect was more pronounced than in the presence of L-NAME alone as evident from the markedly abolished relaxation (Figure 2A and 3A). This observation suggested that the inhibition of the NO-signaling pathway may unmask the EDHF effect in the relaxant response to 2,3-*cis*-procyanidins.

Role of PI3/Akt Kinase in the Relaxant Response to 2,3-*cis*-Procyanidins. PI3/Akt kinase is involved in multiple cellular processes such as the regulation of the vascular tone, apoptosis, cell proliferation, and transcription[28,29] and can be activated by a wide variety of stimuli such as shear stress[29,30] or insulin.[31] Recent findings have indicated that polyphenols are able to activate the PI3/Akt kinase pathway and in turn induce a NO- and EDHF-mediated relaxation in rabbit aorta and porcine coronary arteries.[13,32] In line with a potential role of PI3/Akt kinase in the relaxation to polyphenols, addition of

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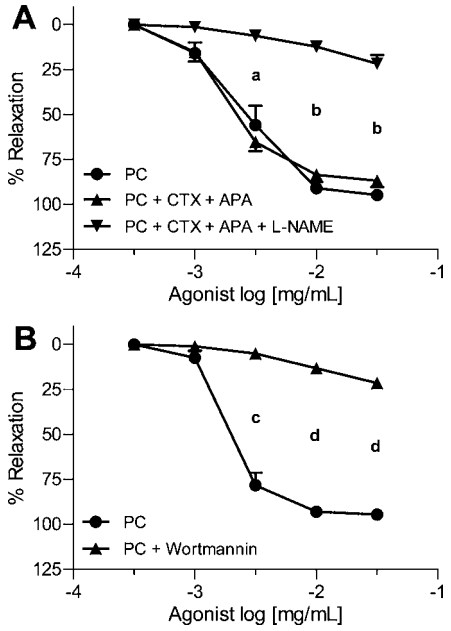

Figure 3. Relaxant response to PC in the absence and presence of (A) charybdotoxin (CTX) plus apamin (APA) (Ca^{2+}-activated K^+ channel inhibitors; each 100 nM), CTX plus APA plus L-NAME (200 μM), and (B) wortmannin (PI3/Akt kinase inhibitor; 30 nM) in porcine coronary arteries with intact endothelium. Values are the mean ± SEM (n = 6): a indicates $P < 0.001$; b indicates $P < 0.0001$ PC plus CTX plus APA plus L-NAME vs PC; c indicates $P < 0.001$; d indicates $P < 0.0001$ PC plus wortmannin vs PC.

wortmannin (inhibitor of PI3/Akt kinase; 30 nM) abolished the procyanidin-induced effects (Figure 3B). Thus, the 2,3-*cis*-procyanidins caused a PI3/Akt-dependent relaxation in porcine coronary arteries. Independent support for this was provided by Western blotting through the activation of Akt by phosphorylation of Ser473 upon stimulation with the procyanidin sample (see below).

Role of ROS in 2,3-*cis*-Procyanidin-Induced Relaxation. Several endothelial cell enzymes such as eNOS, xanthine oxidase, NADPH oxidase, and cyclooxygenases are capable of generating a broad spectrum of ROS including O_2^-.[1,33] Previous studies suggested that an enhanced O_2^- production by polyphenols triggered the NO- and EDHF-induced relaxation in the vascular system by phosphorylation of Akt kinase.[14,34] Following such reports on a key role of an intracellular redox-sensitive event with production of ROS early in the endothelium-dependent relaxation, experiments were performed to examine the role and origin of ROS in relaxation to the 2,3-*cis*-procyanidins. In the presence of MnTMPyP, a cell-permeable mimic of superoxide dismutase (100 μM) that catalyzes the dismutation of O_2^- into hydrogen peroxide and oxygen, the relaxant response to the procyanidin sample was abolished (Figure 4A) due to decreased O_2^- levels. Since the source of the endogenous ROS in the relaxant response to procyanidins is still unknown, the focus was on the origin of O_2^-. Therefore, experiments were performed in the presence of allopurinol (nonselective inhibitor of xanthine oxidase; 10 μM) and apocynin (inhibitor of the NADPH oxidase; 100 μM). The xanthine oxidase generates O_2^- by conversion of hypoxanthine to uric acid and the NADPH oxidase by an electron transfer of NADPH to $NADP^+$. The combination of both drugs induced a significant inhibition of the relaxant response to the 2,3-*cis*-

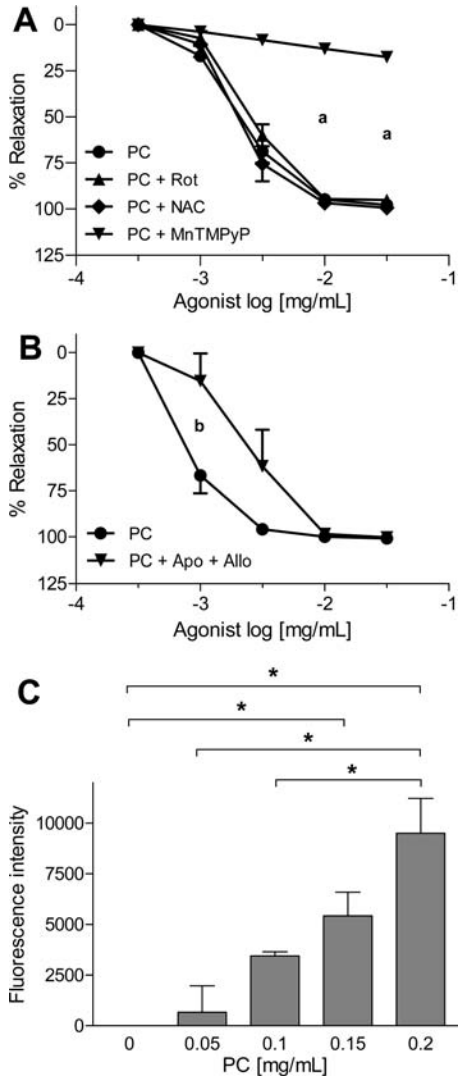

Figure 4. Role of ROS in the relaxant response to PC in porcine coronary arteries with intact endothelium. (A) Effects of PC in the absence and presence of MnTMPyP (cell permeable mimic of superoxide dismutase; 100 μM), rotenone (mitochondrial electron transport chain inhibitor; 10 μM), and NAC (nonselective ROS scavenger; 10 mM). (B) Effects of PC in the absence and presence of inhibitors of xanthine oxidase (allopurinol; 10 μM) and NADPH-oxidase (apocynin; 100 μM). (C) Effects of PC on intracellular ROS production in HUVECs using the fluorescent probe DCFH-DA. Values are the mean ± SEM (n = 4–6): a indicates $P < 0.0001$ PC plus MnTMPyP vs PC; b indicates $P < 0.01$ PC plus APO plus Allo vs PC; *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ compared with controls (0).

procyanidin sample (Figure 4B), providing a clue for a potential source of O_2^- in the present study. This finding clearly indicated for the first time that O_2^- played a major role in endothelium-dependent relaxation to 2,3-*cis*-procyanidins. Interestingly, rotenone (inhibitor of mitochondrial complex I; 10 μM)[35] as well as NAC (nonselective ROS scavenger; 10 mM), which may both reduce ROS production, had no effect on relaxation (Figure 4A). The ineffectiveness of NAC to inhibit procyanidin-induced relaxation cannot satisfactorily be explained. Further studies are needed to clarify this phenomenon. To get more information about ROS production, a DCFH-DA assay was used to quantify intracellular ROS species. Selection of test concentrations (50–200 μg/mL)

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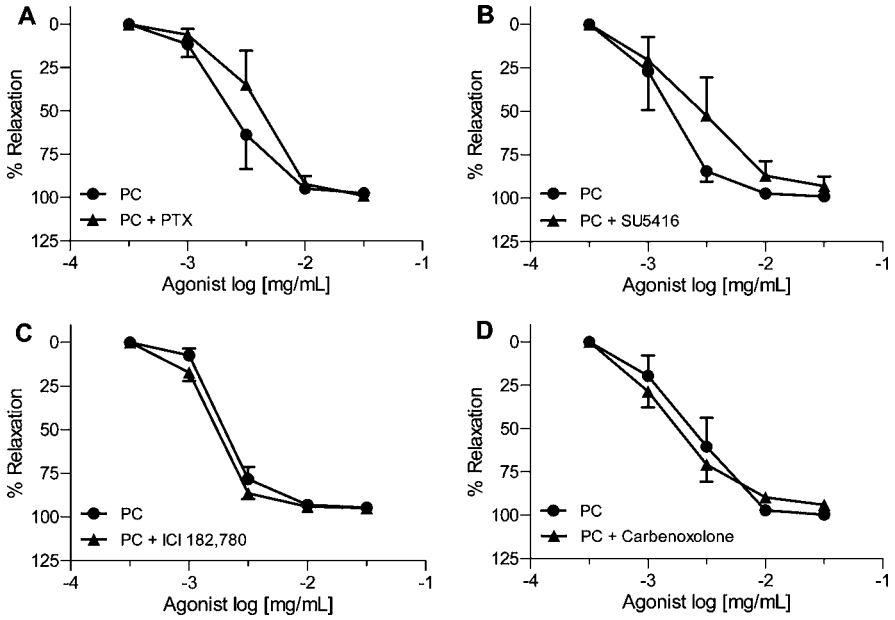

Figure 5. Effects of PC in porcine coronary arteries in the absence and presence of (A) pertussis toxin (PTX; inhibitor of Gi proteins; 100 ng/mL; 3 h), (B) SU5416 (VEGFR inhibitor; 50 μM; 30 min), (C) ICI 182,780 (estrogen receptor antagonist; 10 μM; 30 min), and (D) carbenoxolone (nonspecific inhibitor of GAP junctions; 100 μM). Effects of procyanidins on endothelium-intact rings in the absence of inhibitors served as control. Values are the mean ± SEM (n = 6).

higher than those used in our tissue bath studies (1−32 μg/mL) was made on the basis of a previous report using even a higher concentration (300 μg/mL) for comparing the effects.[32] The nonfluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) penetrates the cell membrane readily and is deacetylated by intracellular esterases to 2′,7′-dichlorofluorescein (DCFH), which is rapidly oxidized to highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) by ROS.[36] In our experiments, 2,3-*cis*-procyanidins concentration-dependently increased ROS production, indicating that the procyanidin sample caused a pro-oxidant response in endothelial cells (HUVECs), consistent with similar responses observed to grape and hawthorn samples.[10,32] The concentration of 0.2 mg/mL of the procyanidin sample showed the highest fluorescence intensity (Figure 4C). A large body of evidence indicates that ROS may trigger the activation of redox-sensitive kinases,[37] giving credence to the procyanidin-induced activation of the redox-sensitive PI3 kinase/Akt signaling pathway in a similar manner. It is also worth mentioning that polyphenol-induced pro-oxidant responses have been reported to be limited to the endothelium, while ROS production was inhibited in vascular smooth muscle and in platelets.[32] These protective effects may be attributable to their scavenging capabilities or control of pro-oxidant and antioxidant enzymes. The dual action of procyanidins and related polyphenols on vascular cells may plausibly explain their vasoprotective effects. Clearly, more studies are needed to get an overall picture of the dual role of procyanidins in biological responses.

Role of Cell Surface and Intracellular Receptors in the Relaxation Response to 2,3-*cis*-Procyanidins. It has been demonstrated that soy isoflavones activate PI3/Akt kinase and eNOS signaling pathway in an inhibitory G-protein (Gi)-dependent manner.[38] To find out whether Gi proteins were involved in relaxation in response to the 2,3-*cis*-procyanidin sample, coronary arterial rings were incubated with pertussis toxin (PTX; 100 ng/mL). As shown in Figure 5A, PTX had no effect on relaxation, thus excluding the involvement of Gi in our set of experiments.

Polyphenols such as green tea catechins are known to inhibit vascular endothelial growth factor receptors (VEGFR) with regard to tumor angiogenesis.[39] It might be assumed that PI3/Akt kinase is phosphorylated by activation of VEGFR. However, inhibition of VEGFR by semaxanib (SU5416; 50 μM) failed to affect relaxation to the procyanidin sample (Figure 5B).

Previous studies have demonstrated that resveratrol and black tea polyphenols induce endothelium-dependent relaxation via estrogen receptors (ERs).[15,40] To investigate the possible involvement of ERs in the relaxation in response to 2,3-*cis*-procyanidins, experiments were carried out in the presence of the ER antagonist ICI 182,780 (10 μM). Concentration−response curves to the procyanidin sample were not affected by ICI 182,780 (Figure 5C). Thus, Gi protein coupled receptors, VEGFR receptors, and estrogen receptors apparently play no decisive role in endothelium-dependent relaxation to 2,3-*cis*-procyanidins.

Role of Gap Junctions in the Relaxant Response to 2,3-*cis*-Procyanidins. Gap junctions may be involved in the vasorelaxant response to polyphenols,[41] as demonstrated for the EDHF response in rat mesenteric arteries.[42] Therefore, organ chamber experiments were performed in the presence of the unspecific gap junction inhibitor carbenoxolone (100 μM). In porcine coronary arteries, the effect on gap junction regarding relaxation to the procyanidin sample remained unaffected (Figure 5D). It has been postulated that hyperpolarizing current can spread from the endothelial to the smooth muscle cell through gap junctions.[25] The ineffectiveness of carbenoxolone in our experiments may be explained by the fact that EDHF plays only a minor role in relaxation of large arteries such as the coronary artery in contrast to NO.[24]

2,3-*cis*-Procyanidins Elicit Phosphorylation of Akt Kinase and eNOS in HUVECs. To further substantiate the

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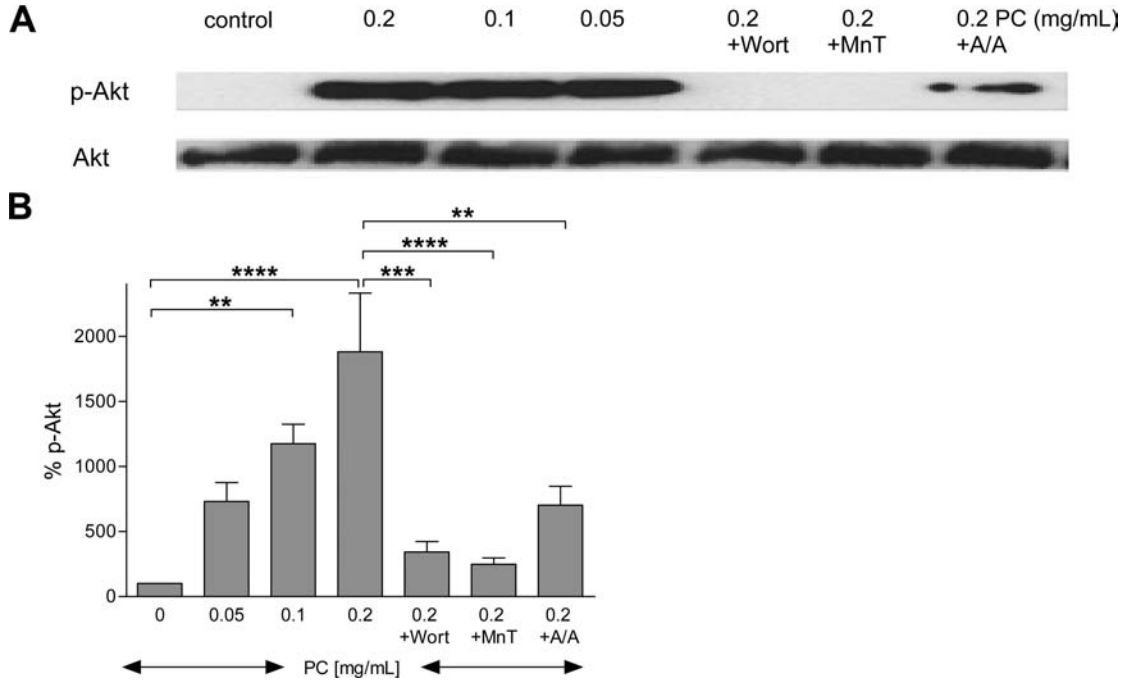

Figure 6. Western blotting analyses in HUVECs. (A) Concentration-dependent effects of PC on the phosphorylation level of Akt at Ser473 in HUVECs. Role of wortmannin (Wort), MnTMPyP (MnT), and allopurinol/apocynin (A/A) in the PC-induced phosphorylation of Akt in HUVECs. All inhibitors were exposed 30 min before the addition of the procyanidin sample. The levels of Akt served as the loading control. (B) p-Akt levels from four different experiments. The mean value of the controls (0) was set at 100 (control value), and all data were expressed as percentages relative to control value. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ compared with controls (0).

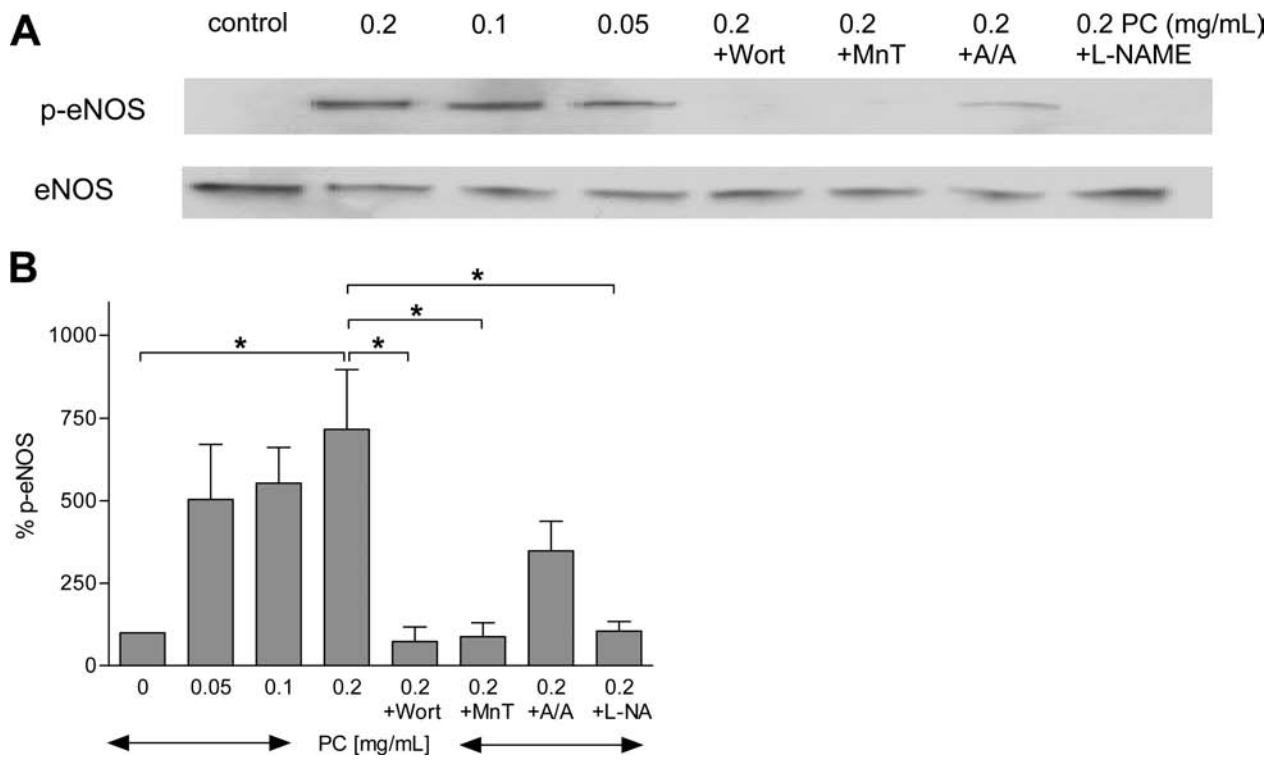

Figure 7. Western blotting analyses in HUVECs. (A) Concentration-dependent effects of PC on the phosphorylation level of eNOS at Ser1177 in HUVECs. Role of wortmannin (Wort), MnTMPyP (MnT), allopurinol/apocynin (A/A), and L-NAME (L-NA) in the PC-induced phosphorylation of eNOS in HUVECs. All inhibitors were exposed 30 min before adding PC. The levels of eNOS served as the loading control. (B) p-eNOS levels from four different experiments. The mean value of the controls (0) was set at 100 (control value), and all data were expressed as percentages relative to control value. $^{*}P < 0.05$ compared with controls (0).

demonstrated observations of the organ chamber studies, Western blotting analyses were employed for phosphorylation of eNOS and Akt using HUVECs. Treatment of cells with the procyanidin sample at different concentrations (0.2, 0.1, and

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0.05 mg/mL; 30 min; for selection of concentration range, see above) produced phosphorylated PI3/Akt kinase (p-Akt) (Ser473) (Figure 6) and phosphorylated eNOS (p-eNOS) (Ser1177) (Figure 7). For a better understanding of the signaling pathways of relaxation mediated by the 2,3-*cis*-procyanidins, HUVECs were individually pretreated with MnTMPyP, wortmannin, and a combination of allopurinol/apocynin (allo/apo) and L-NAME 30 min before the addition of the procyanidins. The effects of the Western blotting experiments were consistent with those observed in our organ chamber studies: (i) inhibition of O_2^- production by MnTMPyP abolished the phosphorylation of Akt and eNOS, (ii) the combination of allopurinol plus apocynin induced a significant reduction of the phosphorylation of Akt, (iii) wortmannin abolished Akt and eNOS phosphorylation, and (iv) L-NAME inhibited eNOS phosphorylation (Figure 6 and 7). Thus, the 2,3-*cis*-procyanidins were able to induce phosphorylation of Akt and eNOS. Furthermore, endogenous ROS apparently acts upstream of the PI3/Akt and eNOS pathway in response to 2,3-*cis*-procyanidins.

In conclusion, the present findings indicated that 2,3-*cis*-procyanidins of *N. meyeri*, and seemingly from other plant sources, are potential endothelium-dependent vasodilators of isolated porcine coronary arteries. The relaxant response is predominantly mediated by an activation of PI3/Akt kinase via the NO and, to a lesser extent, via the EDHF pathway. Furthermore, the DCFH-DA assay confirmed that ROS production is increased in a concentration-dependent manner and represents an early crucial step in the response to procyanidins. In this context, it appears appropriate to note that O_2^- was found to be a key species in the apparently ROS-triggered activation of the PI3/Akt signaling pathway. However, further studies are needed to elucidate the role of individual oxygen species. This study provides a basis for testing homogeneous 2,3-*trans*-procyanidins and analogous samples with flavanyl constituents units of "mixed" stereochemistry and other structural modifications to compare their relaxant potentials and mode of actions. Most effective procyanidin-containing diets and pharmaceuticals may best benefit the risk of cardiovascular diseases, once these data are available. It should be noted that the composition of the procyanidin sample comprised of di- to hexameric oligomers is considered to exemplify a key portion of procyanidins with reasonable bioavailability in the human diet.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +49 30 83853731. Fax: + 49 30 83853729. E-mail: kolpharm@zedat.fu-berlin.de.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

APA, apamin; BCA, bicinchoninic acid; BK_{Ca}, large conductance Ca^{2+}-sensitive K^+ channels; BSA, bovine serum albumin; CTX, charybdotoxin; DCF, 2′,7′-dichlorodihydrofluorescein; DCFH, 2′,7′-dichlorofluorescein; DCFH-DA, dichlorofluorescein diacetate; EDCF, endothelium-derived contracting factor; EDHF, endothelium-derived hyperpolarizing factor; ER, estrogen receptor; FCS, fetal calf serum; sGC, guanylyl cyclase; HUVEC, human umbilical vein endothelial cell; ICI 182,780, 7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)-nonyl]estra-1,3,5(10)-triene-3,17β-diol; KHS, Krebs−Henseleit solution; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)-porphyrin; NAC, *N*-acetylcysteine; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, NO synthase; NOX, NADPH oxidase; O_2^-, superoxide anion; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PBS, phosphate-buffered saline; PC, 2,3-*cis* procyandins from *Nelia meyeri*; PI3/Akt kinase, phosphatidylinositol-3 (PI3) kinase/protein kinase B (Akt); PTX, pertussis toxin; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; Ser, serine; SK_{Ca}, small conductance Ca^{2+}-sensitive K^+ channels; SKP, skim milk powder; SU5416, (*Z*)-1,3-dihydro-3-(1*H*-imidazol-4-ylmethylene)-5-methoxy-2*H*-indol-2-one; TBS-T, tris buffered saline with 0.1% Tween 20; TP, total protein; U46619, 9,11-dioxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$; VEGFR, vascular endothelial growth factor receptors; XOD, xanthine oxidase

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