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A Chemically Defined 2,3-*trans* Procyanidin Fraction from Willow Bark Causes Redox-Sensitive Endothelium-Dependent Relaxation in Porcine Coronary Arteries

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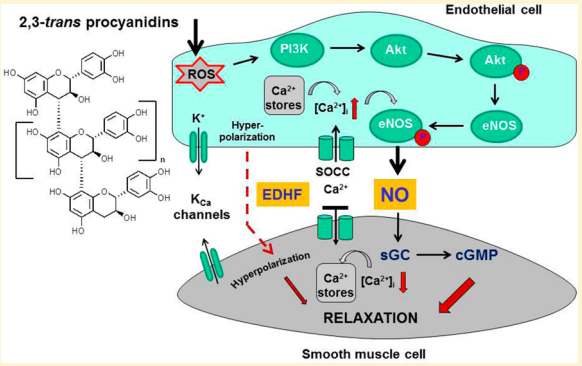
ABSTRACT: Extracts of the bark of willow species (*Salix* spp.) are popular herbal remedies to relieve fever and inflammation. The effects are attributed to salicin and structurally related phenolic metabolites, while polyphenols including procyanidins are suggested to contribute to the overall effect of willow bark. This study aimed at investigating the relaxant response to a highly purified and chemically defined 2,3-*trans* procyanidin fraction in porcine coronary arteries. The procyanidin sample produced a concentration-dependent relaxation in U46619-precontracted tissues. Relaxation was predominantly mediated through the redox-sensitive activation of the endothelial phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway, leading to the subsequent activation of endothelial nitric oxide synthase (eNOS) by phosphorylation, as evidenced by Western blotting using human umbilical vein endothelial cells (HUVECs). That the relaxant response to *Salix* procyanidins was reactive oxygen species (ROS)-dependent with O_2^- as the key species followed from densitometric analysis using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA assay) and employment of various ROS inhibitors, respectively. The data also suggested the modification of intracellular Ca^{2+} levels and K_{Ca} channel functions. In addition, our organ bath studies showed that *Salix* procyanidins reversed the abrogation of the relaxant response to bradykinin by oxidized low-density lipoproteins (oxLDL) in coronary arteries, suggesting a vasoprotective effect of willow bark against detrimental oxLDL in pathological conditions. Taken together, our findings suggest for the first time that 2,3-*trans* procyanidins may contribute not only to the beneficial effects of willow bark but also to health-promoting benefits of diverse natural products of plant origin.



Extracts of the bark of willow species (*Salix* spp.) are highly valued in a number of countries for their curative properties and have a long tradition of use in the treatment of rheumatic diseases, fever, and headache.[1] From clinical studies and traditional medical practices, derivatives of salicylic alcohol are of particular interest, representing the underlying biologically active principle.[2] However, on the basis of pharmacokinetic studies, it was concluded that salicin and its derivatives are unlikely to be solely responsible for the overall efficacy of medicinally used willow bark extracts.[3] Willow bark is known to contain a complex mixture of polyphenols[4,5] suggested to contribute to the medicinal effects. Earlier work on polyphenolic extractives provided proof of the occurrence of a series of 2,3-*trans* procyanidins,[6] accompanied by a unique biflavonoid, [2′,2′]-catechin-taxifolin.[7]

Our interest in willow bark is explained by beneficial effects of procyanidins on the function of the endothelium, reducing the risk of cardiovascular diseases.[8,9] Very recently, we have demonstrated that 2,3-*cis* procyanidins produced a concentration-dependent relaxation in endothelium-intact vascular rings by activation of the nitric oxide (NO)- and endothelium-derived hyperpolarizing factor (EDHF)-signaling pathway via phosphatidylinositol-3-kinase (PI3K)/Akt in a redox-sensitive manner.[10] In continuation of this work and the conceivable therapeutic relevance of willow bark polyphenols, we embarked on a similar set of experiments using a highly purified and chemically defined 2,3-*trans* procyanidin sample from this plant source, comprising a mixture of predominantly [4α,8]-linked di- to hexameric flavan-3-ols to get insight into the relaxant potential and mode of action. It should be noted that the present work provides a detailed account on the vasorelaxant potential of 2,3-*trans* procyanidins for the first time. Previous studies tested commonly chemically poorly defined or even nonanalyzed polyphenolic extracts and do not allow conclusions regarding the contribution of individual phenolic groups to the observed effects. Furthermore, having in mind that several lines of evidence demonstrated that oxidized low-density lipoproteins (oxLDLs) are implicated in the initiation and development of cardiovascular diseases including atherosclerosis[11,12] and that procyanidins have recently been shown to be potent inhibitors of oxLDL receptor-1 (LOX-1),[13] we examined the potential of the 2,3-*trans* procyanidin sample to inhibit the detrimental effect of oxLDL on porcine coronary arteries.

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RESULTS AND DISCUSSION

Endothelium-Dependent Relaxation by *Salix* 2,3-*trans* Procyanidins in Porcine Coronary Arteries. In endothelium-intact porcine coronary artery rings precontracted with the thromboxane A2 mimetic U46619, *Salix* 2,3-*trans* procyanidins (Figure 1) produced a concentration-dependent vasorelaxation

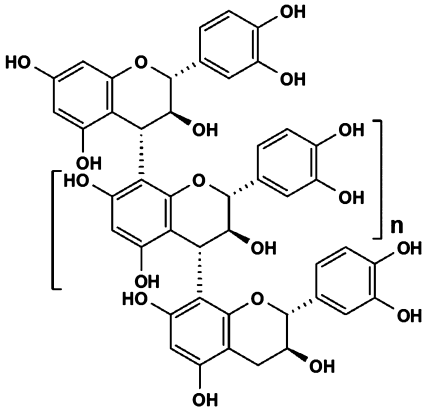

Figure 1. Representative structure of 2,3-*trans* oligomeric procyanidins from willow bark.

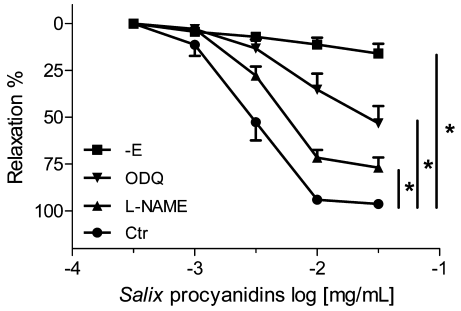

Figure 2. Relaxant response to *Salix* procyanidins in porcine coronary arterial rings. Experiments were performed in endothelium-denuded rings (-E) and endothelium-intact rings in the presence of ODQ (3 μM) or L-NAME (200 μM) and in the absence of inhibitors as a control (Ctr). Values are the mean ± SEM (n = 4–6). *p < 0.05 as compared to the respective control value.

(Figure 2), consistent with previous findings for a number of polyphenols.[14,15] Denudation of the endothelium abolished the relaxant response to the 2,3-*trans* procyanidins. Nitric oxide is a powerful vasodilator, produced by endothelial nitric oxide synthase (eNOS). In vascular smooth muscle cells, soluble guanylyl cyclase (sGC) is the primary target of NO and cGMP is the main effector of its action. Activation of cGMP-dependent protein kinases induces vasorelaxation by reduction of intracellular Ca^{2+} levels in smooth muscle cells. To evaluate the involvement of the endothelium-dependent NO–cGMP signaling pathway in the procyanidin-induced vasorelaxation, the effects of L-NAME (nonselective eNOS-inhibitor; 200 μM) and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; selective inhibitor of sGC, 3 μM) were assessed. Relaxation was significantly attenuated but not abolished by L-NAME or ODQ (Figure 2). These findings suggested that the relaxation was predominantly mediated via NO production, but also that other mechanisms may contribute to vasorelaxation.

Role of PI3K/Akt in the Endothelium-Dependent Relaxant Response. To evaluate the involvement of PI3K/Akt, an upstream effector of the NO signaling pathway,[16,17] the selective inhibitor wortmannin (30 nM) was employed. Under these experimental conditions, the endothelium-dependent relaxation in response to *Salix* 2,3-*trans* procyanidins was abrogated (Figure 3), indicating that the PI3K/Akt pathway

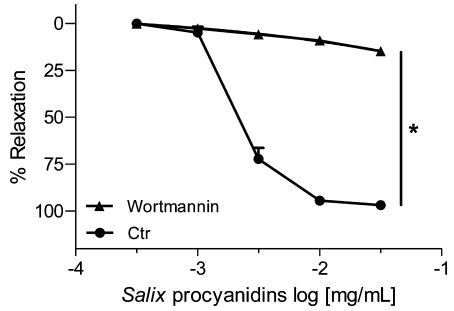

Figure 3. Role of the PI3K/Akt signaling pathway in the relaxant response to *Salix* procyanidins in porcine coronary arterial rings. Experiments were performed in the presence of wortmannin (30 nM) and in its absence (Ctr). Values are the mean ± SEM (n = 5). *p < 0.05 as compared to the respective control value.

played an essential role. Indeed, phosphorylation of Akt activates eNOS,[17] leading to the production of NO. Confirmatory evidence of Akt-eNOS signaling was obtained by Western blotting (formation of phosphorylated Akt and eNOS; see below).

Role of ROS in the Vasorelaxant Action of 2,3-*trans* Procyanidins. Previous studies have demonstrated that polyphenol-induced ROS production is an early and critical event in the PI3K/Akt signaling pathway.[10,18,19] To get information about the role of ROS in the current study, experiments were performed to identify the type and origin of ROS. As expected, endothelium-dependent relaxation in response to 2,3-*trans* procyanidins was markedly depressed by the cell-permeable mimic of superoxide dismutase Mn(III)-tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) (100 μM) and slightly but significantly reduced by the mitochondrial electron transport chain inhibitor rotenone (10 μM) (Figure 4A).[20] These results suggested not only a crucial role of superoxide anion (O_2^-) in the early event of the PI3K/Akt signaling pathway but also mitochondria as a source of ROS. However, differences in the origin of O_2^- were noted with respect to the procyanidin samples in that rotenone did not show any effect on the 2,3-*cis* procyanidin-induced relaxation in our previous work.[10] Other potential sources of O_2^- such as NADPH oxidases and xanthine oxidase are obviously not involved in the response to 2,3-*trans* procyanidins, as evidenced by the failure of apocynin and allopurinol to inhibit relaxation (Figure 4A).

In the present study, quantification of intracellularly generated ROS was measured with a DCFH-DA assay using human umbilical vein endothelial cells (HUVECs). The nonfluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) penetrates the cell membrane and is deacetylated by intracellular esterases to 2′,7′-dichlorofluorescein (DCFH), which is rapidly oxidized to highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) by ROS. As shown in Figure 4B, the 2,3-*trans* procyanidins increased ROS production in a concentration-dependent manner, consistent

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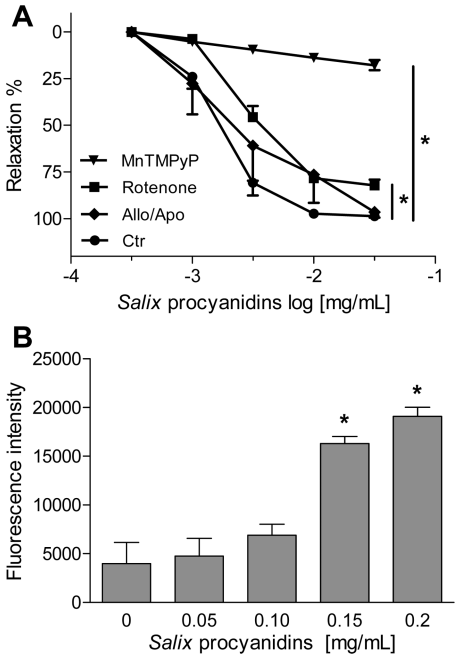

Figure 4. Role of ROS in the relaxant response to *Salix* procyanidins in porcine coronary arteries. Effects are shown in the presence of MnTMPyP (100 μM), rotenone (10 μM), and a combination of allopurinol (Allo, 10 μM)/apocynin (Apo, 100 μM) as well as in the absence of inhibitors as a control (Ctr) (A). Effects of different concentrations of *Salix* procyanidins on intracellular ROS production in HUVECs using the fluorescent probe DCFH-DA (B). Values are the mean ± SEM (n = 4–6). *p < 0.05 as compared to the respective control value.

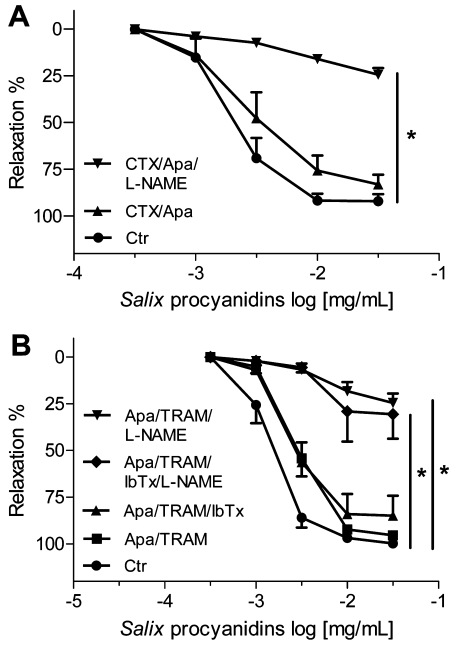

Figure 5. Role of Ca^{2+}-activated K^+ channels in the relaxant response to *Salix* procyanidins in porcine coronary arteries. Experiments were performed in the presence of combinations of inhibitors and in their absence as a control (Ctr). For clarity, the effects are shown in graphs (A) and (B). Concentration of inhibitors was as follows: charybdotoxin (CTX, 100 nM), apamin (Apa, 100 nM), L-NAME (200 μM), TRAM 34 (1 μM), iberiotoxin (IbTx, 100 nM). Values are the mean ± SEM (n = 4–6). *p < 0.05 as compared to the respective control value.

with recent studies.[10,21] This finding further suggested that a redox-sensitive event was involved in polyphenol-induced vasorelaxation via the PI3K/Akt pathway. The two-edged role of ROS is not yet defined but has been associated with different regulatory mechanisms in endothelial and smooth muscle cells.[22]

Role of EDHF in the Endothelium-Dependent Relaxant Response to 2,3-*trans* Procyanidins. Numerous lines of evidence have shown that endothelium-derived hyperpolarizing factors are commonly involved in blood vessel relaxation.[23,24] EDHF-mediated responses are caused by very different factors including the activation of endothelial K_{Ca} channels, IK_{Ca} (intermediate conductance Ca^{2+}-sensitive K^+ channels), and SK_{Ca} (small conductance Ca^{2+}-sensitive K^+ channels), which cause opening of K^+ channels of vascular smooth muscle cells. The resulting reduction in intracellular Ca^{2+} levels induces relaxation.[23,25] To assess the possible roles of K_{Ca} channels in the 2,3-*trans* procyanidin-induced vasorelaxation, the effects of various K_{Ca} channel blockers were evaluated (Figure 5A). Charybdotoxin (CTX, 100 nM), a nonselective blocker of K_{Ca}, and apamin (100 nM), a selective SK_{Ca} blocker, failed to affect the procyanidin-induced relaxation. This implied that the EDHF pathway was apparently less important in our experiments. Supporting evidence is provided by reports indicating that EDHF usually plays only a minor role in large vessels such as the coronary artery.[26] For example, EDHF did not significantly contribute to the relaxant response to acetylcholine in rat aorta and pulmonary arteries.[23,27] However, when CTX and apamin were administered in combination with L-NAME, vasorelaxation was abrogated (Figure 5A), differing from only inhibitory effects observed in experiments with L-NAME alone (Figure 2). A plausible explanation may be that the EDHF-mediated response was boosted upon inhibition of eNOS and became obvious. Activation of endothelial K_{Ca} channels elicited Ca^{2+} entry, thereby providing for effective eNOS activity.

As mentioned above, CTX is a nonselective K_{Ca} inhibitor with blocking properties at both IK_{Ca} and BK_{Ca} (large conductance K^+ channels).[28,29] To further define differences in the contribution of the two K_{Ca} channel subtypes to the EDHF response, the effect of TRAM 34, a selective IK_{Ca} blocker (1 μM), was studied. TRAM 34 alone did not inhibit the 2,3-*trans* procyanidin-induced relaxation (data not shown). Similarly, combinations of TRAM 34/apamin and TRAM 34/apamin/iberiotoxin (selective blocker of BK_{Ca}) also failed to affect the relaxation to 2,3-*trans* procyanidins (Figure 5B). Consistent with the above findings, addition of L-NAME to these combinations again abolished the relaxant response (Figure 5B), suggesting that IK_{Ca} and SK_{Ca} channels located on the endothelial cell were apparently stimulated to compensate a loss of "classical" NO production.

Effects of Modulators of Intracellular Ca^{2+} Homeostasis on Vasorelaxation. Changes in the intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels regulate a broad spectrum of cellular processes. The increase in $[Ca^{2+}]_i$ by release from intracellular stores and influx from external sources into the endothelial cell may enhance eNOS activity.[30−32] Increased $[Ca^{2+}]_i$ by mobilization of Ca^{2+} from intracellular stores leads to the opening of K_{Ca} channels, subsequent K^+ efflux and hyperpolarization of endothelial cells, providing a driving force for Ca^{2+} entry. Notably, depletion of intracellular Ca^{2+} stores activates Ca^{2+} influx by opening of store-operated calcium channels (SOCCs)

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in the plasma membrane. In order to verify the role of $[Ca^{2+}]_i$, modulators of Ca^{2+} environment and $[Ca^{2+}]_i$ were selected, including ryanodine (10 μM; inhibitor of Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores), thapsigargin (100 nM; inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase, SERCA), and 2-aminoethyl diphenylborinate (2-APB, 100 μM) (inhibitor of SOCCs) (Figure 6A,B). Although 2-APB was

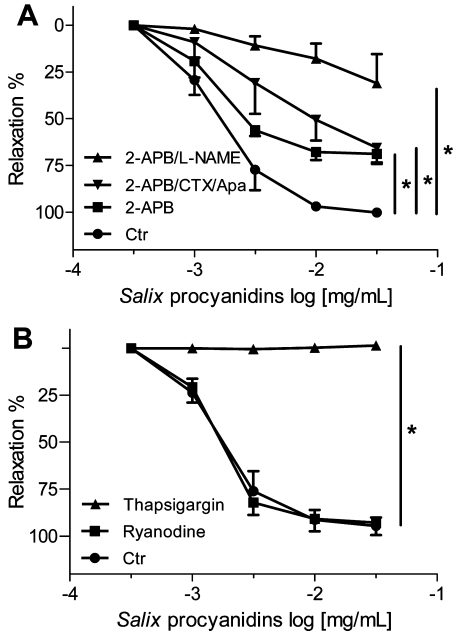

Figure 6. Role of Ca^{2+} in the relaxant response to *Salix* procyanidins in porcine coronary arteries. Experiments were performed in the presence of inhibitors alone or different inhibitor combinations or in their absence as a control (Ctr). For clarity, the effects are shown in graphs (A) and (B). Concentration of inhibitors was as follows: 2-APB (100 μM), L-NAME (200 μM), charybdotoxin (CTX; 100 nM), apamin (Apa; 100 nM), thapsigargin (100 nM), ryanodine (10 μM). Values are the mean ± SEM (n = 4–8). *$p < 0.05$ as compared to the respective control value.

originally characterized as a cell-permeable IP_3 receptor antagonist,[33] subsequent work revealed lack of specificity.[34] However, 2-APB produced a direct inhibitory effect on SOCCs. Exposure of porcine coronary artery rings to 2-APB significantly depressed the maximal effect of the concentration–response curve to 2,3-*trans* procyanidins, suggesting inhibition of SOCCs (Figure 6A). It should be noted that 2-APB/CTX/apamin showed similar inhibitory effects, but 2-APB in combination with L-NAME abolished the relaxant response, again demonstrating the pivotal role of NO signaling associated with $[Ca^{2+}]_i$. Thapsigargin abolished the vasorelaxant response to 2,3-*trans* procyanidins (Figure 6B). As shown in Figure 2, the relaxation to 2,3-*trans* procyanidins is mediated by NO and cGMP. A plausible explanation for the effect of thapsigargin is that the nonrefilling of intracellular Ca^{2+} stores in the smooth muscle cell in the presence of this compound leads to depletion of these stores and in turn to influx of Ca^{2+} via SOCCs associated with an increase in vascular tone.[35] In contrast to the effect of thapsigargin, relaxation remained unaffected in the presence of ryanodine, indicating that ryanodine-sensitive Ca^{2+} storage played no substantial role in the relaxant response to *Salix* procyanidins in porcine coronary arteries.

Procyanidin-Induced Phosphorylation of Akt Kinase and eNOS in HUVECs. To substantiate the findings of organ bath experiments, Western blot analyses were performed to show phosphorylation of Akt (p-Akt; Ser473) and eNOS (p-eNOS; Ser1177) using HUVECs. Endothelial cells were exposed to different sample concentrations (0.2, 0.1, 0.05, and 0.025 mg/mL) for 30 min, and expressions of p-Akt (Figure 7A) and p-eNOS were measured (Figure 7C). In addition, HUVECs were individually pretreated with the inhibitors MnTMPyP, wortmannin, rotenone, and L-NAME as internal controls for the signaling pathways involved. In the presence of these inhibitors, the levels of p-Akt and p-eNOS were markedly reduced, consistent with our organ chamber studies (see above). As shown in Figure 7B and D, densitometric analyses verified that the 2,3-*trans* procyanidin-induced expression of p-Akt and p-eNOS was concentration-dependent.

Effects of 2,3-*trans* Procyanidins on Oxidized LDL-Induced Impaired Endothelium-Dependent Relaxation. Oxidized low-density lipoproteins play a key role in the pathogenesis of atherosclerosis.[36,37] Recently, procyanidins were reported to inhibit the binding of oxLDL to the lectin-like oxidized low-density lipoprotein receptor-1.[13] While LOX-1 inhibition represents a plausible explanation for vascular protective effects, evidence for the sustained function of the endothelium requires its verification. To evaluate the functional integrity, coronary artery rings were incubated with oxLDL or LDL (200 mg/mL each) for 4 h in the absence or presence of 2,3-*trans* procyanidins (10 μg/mL, 1 h before lipoprotein treatment) (Figure 8). In untreated arteries, bradykinin induced a concentration-dependent relaxation, providing evidence for an intact endothelium in the vessels. LDL alone or in combination with 2,3-*trans* procyanidins did not affect the relaxation to bradykinin (Figure 8). In contrast, rings incubated with oxLDL abolished the vasorelaxant effect of bradykinin. However, pretreatment with the procyanidin sample inhibited the detrimental effect of oxLDL. As shown, in the presence of 2,3-*trans* procyanidins relaxation was comparable with that of the bradykinin control. It should be noted that the abolished relaxation to bradykinin in the presence of oxLDL was surmounted by a subsequent bolus injection of sodium nitroprusside (SNP; 100 μM); the vascular rings showed complete relaxation to 100% (Figure 8, inset). The experiments showed not only that LDL and the procyanidin sample did not cause damage to the endothelium but also, and more importantly, that the 2,3-*trans* procyanidins may exhibit vasoprotective effects against oxLDL in pathological conditions such as atherosclerosis.

Impact of Vessel Diameters on Vasorelaxant Mechanisms. The coronary artery is a large conducting vessel and may not reflect responses that occur in smaller arteries. Indeed, some reports indicated different contributions of EDHF and NO in large and small conduit arteries, with predominantly EDHF-mediated contribution to vasorelaxation in smaller vessels.[26] To evaluate the impact of 2,3-*trans* procyanidins on the signaling pathways in vessels with small internal diameters, experiments were extended to coronary arteries ≤ 0.5 mm.[38] Compared to large arteries (2–3 mm i.d.), the relaxant responses were very similar in most experiments. Conspicuous differences included less sensitivity to the endothelium-dependent NO-mediated vasorelaxation, as evident from higher sample concentrations required to induce complete relaxation (Figure 9A,B). Interestingly, the addition of L-NAME caused a

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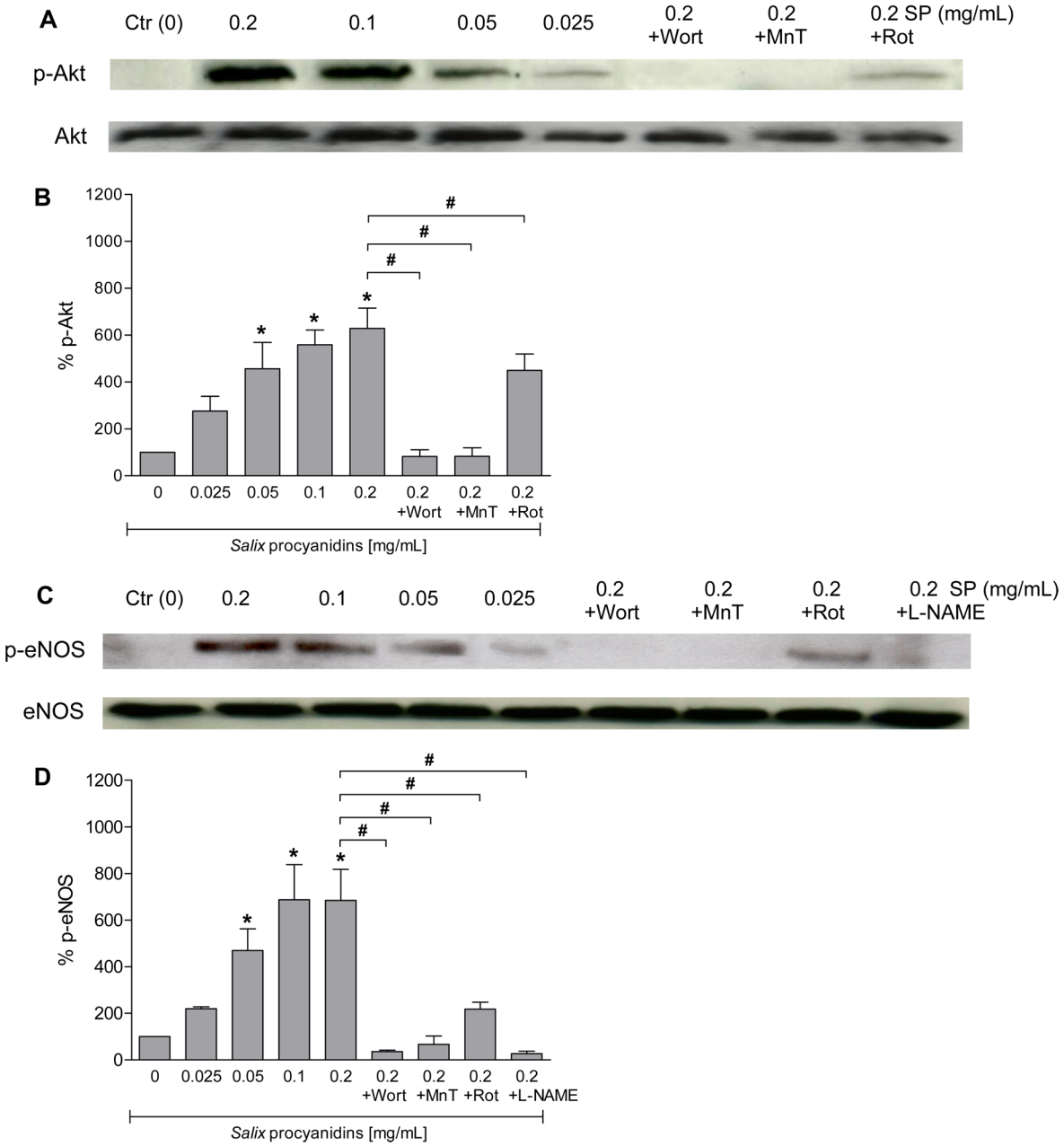

Figure 7. Western blotting analyses in HUVECs. Concentration-dependent effects of *Salix* procyanidins (SP) on the phosphorylation level of Akt at Ser473 and eNOS at Ser1177. Effects of wortmannin (Wort; 30 nM), MnTMPyP (MnT; 100 μM), rotenone (Rot; 10 μM), and L-NAME (200 μM) in the *Salix*-induced phosphorylation of Akt (A) and eNOS (C) are shown. Densitometric analyses from pooled data of three to four different experiments are presented below (B, D). The mean value of the controls (0) was set at 100, and all data were expressed as percentages relative to control value. Values are the mean ± SEM (n = 3−4). *p < 0.05 as compared to controls (0), and $^{\#}p$ < 0.05 as compared to the effect of 0.2 mg/mL *Salix* procyanidins.

more pronounced reduction of the 2,3-*trans* procyanidin-induced relaxation in small compared with large conduit vessels (Figure 9A). However, the general similarity in relaxant responses of both vessel types did not show a significant shift to the EDHF pathway (Figure 9B). A possible reason is that the internal diameter of the small conduit arteries used in the current study was still too large.

In summary, the present study shows that 2,3-*trans* procyanidins of willow bark are endothelium-dependent vasodilators in porcine coronary arteries. The relaxant response is mediated by activation of PI3K/Akt via the NO/cGMP pathway in a redox-sensitive manner and, at least in part, via the EDHF pathway. Small (≤0.5 mm i.d.) and large (2−4 mm i.d.) conduit vessels did not show relevant differences in the response to the 2,3-*trans* procyanidins in the current study. With reference to willow bark, extracts may benefit from the co-occurrence of procyanidins and salicin derivatives, with a pharmacological profile similar to that of aspirin. Interestingly, aspirin has been shown to improve endothelial dysfunction and to elicit NO release.[39,40] Our data thus support the conjecture that 2,3-*trans* procyanidins may contribute to vascular benefits of diverse natural products of plant origin.

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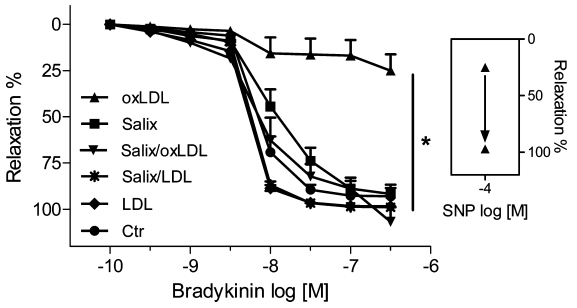

Figure 8. Relaxant response to bradykinin in coronary artery rings in the presence of oxLDL (200 μg/mL), 2,3-*trans* procyanidins (*Salix*, 10 μg/mL), and the combination of 2,3-*trans* procyanidins/oxLDL or 2,3-*trans* procyanidins/LDL or LDL (200 μg/mL) and in their absence as a control (Ctr). Rings were separately incubated with oxLDL or LDL for 4 h at 37 °C. Rings treated with 2,3-*trans* procyanidins (10 μg/mL) were preincubated for 1 h before the addition of lipoproteins. Inset: Relaxant response to SNP (100 μM) following the relaxation to bradykinin (0.3 μM) in rings preincubated with oxLDL. Values are the mean ± SEM (n = 4–6). *p < 0.05 as compared to the respective control value.

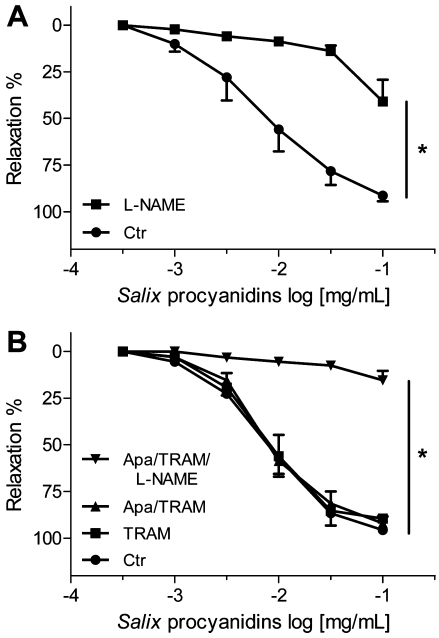

Figure 9. Relaxant response to *Salix* procyanidins in porcine coronary arterial rings of small inner diameter (≤0.5 mm). Experiments were performed in endothelium-intact rings in the presence or absence of L-NAME (200 μM) (A). Role of Ca^{2+}-activated K^+ channel blockers in the combination of apamin (Apa, 100 nM)/TRAM 34 (100 μM)/L-NAME (200 μM), Apa/TRAM 34, and TRAM 34 and in the absence of inhibitors as a control (B). Values are the mean ± SEM (n = 4–6). *p < 0.05 as compared to the respective control value.

EXPERIMENTAL SECTION

Materials. 9,11-Dioxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) was a gift from Upjohn (Kalamazoo, MI, USA), and allopurinol, apocynin, L-NAME, indomethacin, 2′,7′-dichlorofluorescein diacetate, and wortmannin were obtained from Sigma-Aldrich (Taufkirchen, Germany). 2-Aminoethoxydiphenylborane, apamin, charybdotoxin, iberiotoxin, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, rotenone, ryanodine, TRAM 34, and thapsigargin were purchased from Tocris (Bristol, UK). OxLDL and LDL were from Biotrend (Köln, Germany), and MnTMPyP was ordered from Axxora GmbH (Lörrach, Germany).

Procyanidin Sample. Isolation, purification (>95% by HPLC analysis), and characterization of procyanidins from willow bark were reported previously.[6] Briefly, the ethyl acetate-soluble portion (ca. 10% yield related to dry weight), obtained from the parent methanol extract, was repeatedly chromatographed on Sephadex LH-20 using ethanol as eluent to afford a highly purified mixture of oligomeric flavan-3-ols, identified as 2,3-*trans* procyanidins by chemical methods, mass spectroscopy, NMR analysis, and circular dichroism. The polyphenolic sample was composed of dimeric to hexameric 2,3-*trans* procyanidins in the proportions dimers:trimers:tetramers:pentamers/hexamers 1.0:1.4:2.1:6.4. 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; 20 mg/mL) that did not contain Mg^{2+} or Ca^{2+} (composition of Krebs-Henseleit solution see below). For Western blotting, the procyanidin sample 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- and Versuchsanstalt für Tierzucht and Tierhaltung; Teltow-Ruhlsdorf, Germany) and placed in ice-cold preoxygenated KHS (95% O_2 and 5% CO_2; pH 7.4), during transport to the laboratory, 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). Large (left anterior descending and left circumflex; 2–4 mm i.d.) and small (≤0.5 mm i.d.) coronary arteries were removed from the hearts and cleared of fat and adhering tissue. The vessels were stored overnight at 4 °C in prior gassed KHS. Results from preliminary experiments indicated that storing tissue overnight does not impair the contractility of the smooth muscle. On the following day, the vessels were cut into rings (large: 3–4 mm long, small: 2 mm long). 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), attached to a TSE 4711 transducer coupler (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-*trans* Procyanidins. Following an equilibration period of 90 min with a resting tension of 20 mN, the tissue rings were precontracted once with 30 mM KCl and three times with 30 nM 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 cyclooxygenase.

Cell Culture. Human umbilical vein endothelial cells (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 adding the samples, cells were treated with serum-free culture medium.

Electrophoresis and Western Blotting. HUVECs were exposed to the procyanidin sample (25, 50, 100, and 200 μg/mL). Inhibitors were added to the culture flasks 30 min before the addition of the test 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 16000*g* for 15 min, the supernatants were collected and the total protein concentration was determined with the bicinchoninic acid 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, USA) dissolved in BSA-blocking buffer overnight at 4 °C. After four washings 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 the control was set at 100%, and all data were expressed as percentages relative to the control.

Lipoprotein Incubation Assay. Large porcine coronary artery rings were individually incubated in KHS with 2,3-*trans* procyanidins, LDL, oxLDL, or the combinations of LDL plus 2,3-*trans* procyanidins and oxLDL plus 2,3-*trans* procyanidins, respectively, in humidified atmosphere at 37 °C and 5% CO_2 for 4 h. The 2,3-*trans* procyanidin sample (10 μg/mL) was added 1 h before the addition of LDL and oxLDL (200 μg/mL each), respectively. After incubation, tissue rings were mounted in a 20 mL organ chamber as described above. Rings were precontracted with 30 mM KCl and subsequently two times with 30 nM U46619. When the second U46619 contraction had reached a plateau, bradykinin (0.3−1 μM) was added cumulatively to the tissues, and the relaxant response was monitored. In experiments with rings preincubated with oxLDL, sodium nitroprusside (100 μM) was finally added to fully relax the tissues.

Intracellular ROS Assay. After trypsinization, cultured HUVECs were seeded in a black 96-well tissue plate with a clear bottom (Costar, Corning, NY, USA) at 10^3 cells per well. After reaching confluence, cells were washed two times with PBS (Ca^{2+}- and Mg^{2+}-free) and incubated with the procyanidin sample in medium without FCS for 30 min. After a washing step, cells were treated with 5 μM 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 Bonferroni's multiple comparison test. *p* values of <0.05 were considered to be significant.

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Notes

The authors declare no competing financial interest.

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