Apocynin Is Not an Inhibitor of Vascular NADPH Oxidases but an Antioxidant

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Abstract—A large body of literature suggest that vascular reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases are important sources of reactive oxygen species. Many studies, however, relied on data obtained with the inhibitor apocynin (4‘-hydroxy-3’-methoxyacetophenone). Because the mode of action of apocynin, however, is elusive, we determined its mechanism of inhibition on vascular NADPH oxidases. In HEK293 cells overexpressing NADPH oxidase isoforms (Nox1, Nox2, or Nox4), apocynin failed to inhibit superoxide anion generation detected by lucigenin chemiluminescence. In contrast, apocynin interfered with the detection of reactive oxygen species in assay systems selective for hydrogen peroxide or hydroxyl radicals. Importantly, apocynin interfered directly with the detection of peroxides but not superoxide, if generated by xanthine/xanthine oxidase or nonenzymatic systems. In leukocytes, apocynin is a prodrug that is activated by myeloperoxidase, a process that results in the formation of apocynin dimers. Endothelial cells and smooth muscle cells failed to form these dimers and, therefore, are not able to activate apocynin. Dimer formation was, however, observed in Nox-overexpressing HEK293 cells when myeloperoxidase was supplemented. As a consequence, apocynin should only inhibit NADPH oxidase in leukocytes, whereas in vascular cells, the compound could act as an antioxidant. Indeed, in vascular smooth muscle cells, the activation of the redox-sensitive kinases p38 mitogen-activate protein kinase, Akt, and extracellular signal–regulated kinase 1/2 by hydrogen peroxide and by the intracellular radical generator menadione was prevented in the presence of apocynin. These observations indicate that apocynin predominantly acts as an antioxidant in endothelial cells and vascular smooth muscle cells and should not be used as an NADPH oxidase inhibitor in vascular systems. Hypertension. 2008;51:211-217.

Key Words: apocynin ■ NADPH oxidase ■ Nox1 ■ Nox4 ■ leukocytes ■ reactive oxygen species

Reactive oxygen species (ROS) have a strong impact on vascular homeostasis.1 ROS originate from several sources, including xanthine oxidase, cytochrome P450 mono-oxygenases, mitochondria, and uncoupled NO synthase, as well as from the proteins of the Nox family, the NADPH oxidases. The identification of the individual contribution of these generator systems to oxidative burden has been a focus of an impressive amount of publications. The motivation of these studies is that a site-directed ROS lowering therapy to inhibit individual generator systems should be superior to the presently unsuccessful approaches with antioxidants in preventing ROS-dependent cardiovascular diseases.2 Although it is generally appreciated that molecular techniques involving antisense oligonucleotides, small-interfering RNA, or transgenic animals are to be preferred to pharmacological inhibitors to characterize the contribution of individual ROS generator systems, the latter studies still represent the majority of scientific contributions in the field.

Different from NO synthase, mitochondria, and xanthine oxidase, for which excellent effective and specific inhibitors have been developed, pharmacological inhibition of Nox family reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases still represents a major challenge for all experimental settings. In addition to unspecific drugs affecting NADPH oxidase activation or expression such as statins or angiotensin II receptor blockers, only 2 compounds have been widely used: diphenylene iodonium (DPI) and apocynin (4‘-hydroxy-3’-methoxyacetophenone/acetovanillone).2 Although DPI (together with the lucigenin NADPH redox-cycling artifact) has been very instrumental in establishing the concept of vascular NADPH oxidases, we now know that DPI is not selective at all and acts as an unspecific flavin oxidant. In fact, through this mechanism, DPI has been reported to basically inhibit all vascular sources of ROS, including mitochondria, cytochrome P450 mono-oxygenases, and the NO synthase. Therefore, DPI probably has caused an
overestimation of the biological significance of the NADPH oxidases.

Apocynin has already been characterized as an NADPH oxidase inhibitor in the early 1980s. With the disapproval of DPI as a specific NADPH oxidase inhibitor, apocynin has become exceedingly popular, with >250 publications focusing on aspects of vascular NADPH oxidases only.

This large body of literature is strongly contrasted by the few studies that have characterized the inhibitory action of apocynin. However, already in the original report of apocynin as an NADPH oxidase inhibitor it was noted that the compound requires activation by myeloperoxidase (MPO). Because MPO is not expressed in vascular cells in culture, we concluded that apocynin might not inhibit vascular NADPH oxidases as expressed in endothelial cells or smooth muscle cells and that the action of apocynin might be unrelated to its inhibitory effect on the leukocyte NADPH oxidase.

Materials and Methods

For a detailed Material and Methods section please see http://hyper.ahajournals.org.

Immunoblotting

Standard SDS-PAGE and Western blot were carried out, and proteins were detected using appropriate primary antibodies and infrared fluorescent–labeled secondary antibody for infrared-based fluorometric detection by the Odyssey system (Licor).

Amplex Red Assay for H2O2 Detection

Cells were incubated in buffer containing Amplex Red (50 μmol/L) and horseradish peroxidase (HRP; 2 U/mL). After 60 minutes, oxidation of Amplex Red was measured by a microplate fluorometer (excitation: 540 nm; emission: 580 nm). For the cell-free assay, pyrogallol was used as source of ROS.

ROS Detection With Chemiluminescence

The following enhancers were used: lucigenin (5 and 250 μmol/L for superoxide anions), LO12 (250 μmol/L for ROS in general), and luminol (200 μmol/L)/HRP (1 U/mL for predominant detection of H2O2) using a tube chemiluminescence reader.

High-Performance Liquid Chromatography–Based Assays

Dihydroethidium, ethidium, and oxyethidium, as well as apocynin and its reaction products, were separated by high-performance liquid chromatography using a C18 column, and detection was carried out using fluorescence for oxyethidium and UV absorption for ethidium.

Identification of Apocynin Oxidation Products by Liquid Chromatography/Mass Spectrometry

Analysis was carried out by a ScieX API4000 mass spectrometer operating in multiple reaction monitoring mode. Chromatographic separation was performed on a Gemini C18 column (150×2 mm I.D.; 5-μm particle size; Phenomenex).

Results

Apocynin Inhibits Superoxide Anion in Leukocytes but Not in Transfected HEK293 Cells

A nonredox concentration of lucigenin (5 μmol/L) was used to compare the effect of apocynin on phorbol myristate acetate (PMA)–stimulated superoxide anion (O2–) production in leukocytes and HEK293 cells transiently transfected with the leukocyte NADPH oxidase (Nox2, p47phox, and p67phox). Apocynin dose-dependently inhibited the O2– production in leukocytes after a lag time of several minutes but not in HEK293 cells (Figure 1). This suggests that leukocytes have a property to mediate the inhibitory effect of apocynin, which is missing in HEK293 cells. Indeed, it has been suggested that MPO, which is selectively expressed in leukocytes, is needed to elicit the inhibitory effect of apocynin.

Interestingly, when high concentrations of lucigenin (250 μmol/L) were used, we failed to observe an inhibitory effect of apocynin on PMA-induced O2– production in leukocytes (data not shown). This observation suggests that the lucigenin redox cycling artifact4,5 is sufficient to maintain a chemiluminescence signal in leukocytes despite inhibition by apocynin. These data explain previous observations that the PMA-induced cytochrome C reduction in leukocytes was sensitive to apocynin, whereas the lucigenin chemiluminescence was not.6

![Figure 1. Effect of apocynin on superoxide production in leukocytes and transiently transfected HEK293 cells. Original tracings (A and B) and statistical analysis (C and D) of the O2– signal detected by lucigenin (5 μmol/L)–enhanced chemiluminescence in human leukocytes (A and C) and HEK293 cells transiently transfected with the leukocyte NADPH oxidase (Nox2, p47phox, and p67phox; B and D). NADPH oxidase activity was stimulated with PMA (100 nmol/L) at the time point indicated. n=3; *P<0.05 apocynin present vs absent.](http://hyper.ahajournals.org.)
MPO Is Required to Activate Apocynin

When apocynin is incubated with MPO and H₂O₂, to simulate the process of activation, a product is formed (Figure 2A), which by molecular mass corresponded with an apocynin dimer as detected by liquid chromatography-mass spectrometry (Figure 2B). Accordingly, in HEK293 cells, which do not express MPO, dimer formation did not occur even after overexpression of Nox isoforms or administration of H₂O₂. In contrast, when human MPO was added, the apocynin dimer was readily detectable in those cells overexpressing Nox isoforms or coincubated with H₂O₂. In the absence of H₂O₂ or Nox proteins, HEK293 cells, however, did not produce the apocynin dimer (Figure 2C). Accordingly, the combination of MPO and apocynin inhibited O₂⁻/H₂O₂ production in HEK293 cells transfected as indicated (C; GFP indicates green fluorescent protein; A, Noxa1; O, Noxo1) and in leukocytes, porcine aortic endothelial cells (PAECs), as well as rat aortic vascular smooth muscle cells (RSMCs) (300 μmol/L) as indicated. Leukocytes were incubated with: zymosan (1 mg/mL), N-formyl-methionyl-leucyl-phenylalanine (FMLP: 10 μmol/L), and PMA (100 nmol/L). PAECs were incubated with tumor necrosis factor-α (TNF-α; 100 ng/mL), vascular endothelial growth factor (VEGF; 100 ng/mL), angiotensin II (300 nmol/L), and platelet-derived growth factor AB (PDGF; 50 ng/mL) for 30 minutes. Pos denotes the positive control: cells incubated with MPO and H₂O₂. n=3; P<0.05 vs control condition.

Apocynin Acts as a Radical Scavenger

The previously mentioned results may indicate that the effect of apocynin on the ROS level in vascular cells is unrelated to the NADPH oxidase activity. A potential explanation could be that apocynin acts as a radical scavenger in some detection systems. Therefore, the effect of apocynin on ROS detection was analyzed using different assay systems and NADPH oxidase-independent ROS production by pyrogallol, xanthine/xanthine oxidase, or potassium peroxide. Similar results were obtained using these different generator systems, as described below.

When O₂⁻ was measured using dihydroethidium oxidation, the inhibitory effects of apocynin were seen only at concentrations above 100 μmol/L (Figure 3A). In contrast, apocynin inhibited the lucigenin-enhanced chemiluminescence when high concentrations of lucigenin were used, which are known to undergo redox cycling (Figure 3B). Apocynin also interfered with the detection of peroxides using the Amplex Red/HRP assay (Figure 3C), as well as with LO12 chemiluminescence (Figure 3D), which detects hydroxyl radicals, peroxynitrite, and O₂⁻. In phosphate buffer, apocynin did not directly decompose H₂O₂, which was measured photometrically (data not shown). This suggests that apocynin will predominantly act as a scavenger for peroxide-dependent ROS formed by the different generator systems or as intermediated within cells or during ROS detection.

Apocynin Inhibits Peroxide Detection From All Vascular Nox Isoforms

To determine whether this scavenging property interferes with ROS detection from different NADPH oxidases, the
effect of apocynin was tested in HEK293 cells overexpressing different Nox proteins. Apocynin dose dependently inhibited the NADPH oxidase–mediated ROS signal obtained by LO12 and luminol/HRP chemiluminescence, as well as by Amplex Red/HRP fluorescence. Importantly, this effect was not restricted to NADPH oxidases containing cytosolic activator components, such as the combination of Nox2 with p47phox and p67phox and Nox1 with Noxo1 and Noxa1, but was also present in Nox4 (Figure 4), which is constitutively active and does not require cytosolic activators. The effect of apocynin was reversible, and washout of the inhibitor normalized ROS production in Nox-overexpressing cells to control conditions (data not shown). To exclude that HRP, which may promote apocynin activation, interfered the effect of apocynin, the Amplex Red oxidation assay in HEK293 cells overexpressing Nox1 NADPH oxidase was determined in the absence of HRP. Even under these conditions, apocynin dose dependently inhibited the Amplex Red oxidation (data not shown).

Apocynin Acts as an Antioxidant in Vascular Cells

To determine whether the ROS scavenging properties of apocynin are relevant to ROS scavenging in vascular cells, activation of redox-sensitive kinases was studied in rat smooth muscle cells, directly stimulated with H2O2, or the intracellular ROS generator menadione. To rule out that H2O2 stimulates endogenous NADPH oxidases in these cells, the experiments were performed in the presence or absence of the potent (yet unselective) NADPH oxidase inhibitor DPI (10 μmol/L). Apocynin suppressed the basal phosphorylation of Akt and extracellular signal–regulated kinase 1/2 and impaired the ROS-induced activation of p38 mitogen–activated protein kinase, as well as of Akt and extracellular signal–regulated kinase 1/2 (Figure 5). This effect was unrelated to endogenous NADPH oxidases, because adding DPI had no effect on the inhibitory action of apocynin.

Discussion

In this study, we provide evidence that apocynin predominantly acts as an antioxidant but not as an inhibitor of NADPH oxidases in nonphagocytic cells in culture. Apocynin failed to block the O2− production of Nox1, Nox2, or Nox4 when overexpressed in HEK293 cells. Moreover, in vascular cells, a similar activation of apocynin, as seen in leukocytes, was not observed. Most importantly, however, apocynin turned out to be a scavenger of radicals and directly inhibited the ROS-induced signaling in vascular cells.

The strong need for NADPH oxidase inhibitors has resulted in a marked prominence of the orthomethoxy substituted catechol apocynin. Apocynin was observed to prevent the activation of the leukocyte NADPH oxidase as it blocked the assembly of the oxidase in vitro. The molecular mechanism of action of the compound, however, has been a matter of investigation since its initial discovery. In fact, apocynin does not act as a competitive inhibitor for substrate or cofactors at any of the NADPH oxidase subunits but rather interferes with the assembly process of the oxidase, which is required for the activation at least of Nox2 and Nox1. Apocynin prevents the translocation of p47phox to Nox2 in leukocytes, monocytes, and endothelial cells. The inhibitory action of the compound, however, occurs after a lag time only, as also observed in the present study. It was, therefore, assumed that apocynin has to covalently modify components of the NADPH oxidase and/or undergoes activation. The latter process appears to involve MPO, because apocynin does not inhibit the oxidase in cells devoid or deficient of
MPO,\textsuperscript{7} and agents such as zymosan that promote the release of MPO\textsuperscript{10} enhance the efficacy of apocynin.\textsuperscript{3} It is assumed that apocynin is activated by H$_2$O$_2$ and MPO to form an apocynin radical, which then oxidizes thiols in the NADPH oxidase.\textsuperscript{7} Indeed, thiols are critical for the function of p47phox,\textsuperscript{11} and thiol oxidizing agents have been shown to block NADPH oxidase activation.\textsuperscript{12} In line with this concept, it was observed that supplementation of thiol provided either as glutathione or cysteine prevents the inhibitory effect of apocynin on the NAPDH oxidase.\textsuperscript{7,13}

An alternative explanation for the lag time of the inhibitory effect of apocynin was that through the step of an apocynin radical an apocynin dimer is formed.\textsuperscript{9} In fact, it has been suggested that this dimer is the active inhibitory compound that may block NADPH oxidase activity in endothelial cells.\textsuperscript{9} This concept, however, has been challenged, because the dimer does not inhibit the leukocyte NADPH oxidase activity as measured by oxygen uptake and because the dimer, different to apocynin itself, is a powerful scavenger of O$_2$\textsuperscript{1100}.

Although the apocynin dimer might not be the active inhibitory compound, dimer formation is a consequence of the activation of apocynin.\textsuperscript{9} Indeed, we also observed in the present study that apocynin dimerizes in stimulated leukocytes. This dimer could be used as a marker for apocynin activation and as an index for the inhibitory action of apocynin on the NADPH oxidase. Based on this assumption, we determined whether dimers occur in vascular cells or HEK293 cells overexpressing NAPDH oxidase subunits. Importantly, neither in the supernatant nor in the cytosol of endothelial cells or smooth muscle cells were apocynin...
dimers detected. This effect was most likely a consequence of the lack of MPO, as well as the fairly low ROS formation in these cells, because only the combined supplementation of H$_2$O$_2$ and MPO led to detection of dimers in the cells. Likewise, when HEK293 cells overexpressing large amounts of NADPH oxidase were studied, the apocynin dimer only became detectable when MPO was supplemented. Thus, the inhibitory effect of apocynin was restricted to cells that generated large amounts of ROS and expressed MPO.

Such a profile is most applicable to leukocytes and will render apocynin a relative selective inhibitor of the leukocyte NADPH oxidase, at least ex vivo. The in vivo situation might be different, because MPO, released from white blood cells, can adhere to the glycocalix and can even be taken up by endothelial cells. This mechanism is sufficient at least to limit NO bioavailability in vivo, and it might, therefore, be speculated that it could promote apocynin activation in endothelial cells in vivo, too. In fact, a large number of studies using apocynin as an NADPH oxidase inhibitor have used prolonged treatment protocols. Under such conditions, even a small continuous activation of apocynin could be important, because the compound should act in an irreversible manner. The in vitro activation of apocynin might, however, be limited by the presence of antioxidants; the concentration of reduced thiols in noninflamed tissue and in the blood should be sufficiently high to scavenge a large proportion of the apocynin radical formed by MPO.

With such a concept in mind, we studied the effects of apocynin on 3 different NADPH oxidase isofoms transiently expressed in HEK293 cells. Importantly, apocynin failed to inhibit any of these enzymes, even at high concentrations. Certainly, the previous discussion on the role of MPO can serve to explain this observation. Moreover, it is imperative to realize that predominantly Nox2 depends on p47phox, the subunit that is considered the target of apocynin. Nox4 is constitutively active even in the absence of cytosolic subunits, and Nox1 can be activated not only by p47phox but also and much more efficiently by the combination of Noxa1 and Noxo1. Whether the apocynin radical is also able to block the constitutive activity of these proteins is currently unknown.

The obvious selectivity of apocynin for the NADPH oxidase expressed in leukocytes requires an alternative concept for the pronounced positive properties of the compound in vitro, as well as in vivo. It is possible that the effects of apocynin at least in vivo are not related to NADPH oxidase inhibition and ROS signaling at all. The most probable mechanistic explanation, however, is that apocynin acts as an antioxidant. Indeed, in the present study we provide clear evidence that the drug interferes with several different ROS detection assays, which detect either H$_2$O$_2$ or hydroxyl radicals. In contrast, apocynin only interferes with O$_2^-$ detection when excessive concentrations of the compound are used. Neither lucigenin chemiluminescence in intact cells or with ROS generators nor the dihydroethidium assay was affected by apocynin concentrations $\leq$100 $\mu$mol/L, and this observation is in line with previous studies using cytochrome C reduction to measure O$_2^-$ formation. In contrast, ROS detection by the LO12 chemiluminescence assay, which detects peroxyxyl radicals, peroxynitrite, and O$_2^-$ was markedly inhibited even when ROS were generated NADPH oxidase independently. Likewise, the luminol and the Amplex Red assay, which both detect H$_2$O$_2$ through the HRP-mediated conversion of H$_2$O$_2$, were strongly inhibited by apocynin. Because apocynin can react with HRP, an alternative explanation could be that the compound interferes with the detection systems in these assays. It was noted, however, that, at least for MPO, apocynin does not inhibit the activity of the enzyme. Therefore, it has to be concluded that apocynin acts as a scavenger predominantly for reaction products of H$_2$O$_2$, although it might also significantly scavenge other O$_2^-$ at concentrations $>$300 $\mu$mol/L.

H$_2$O$_2$ has a strong impact on cellular homeostasis and affects a large number of signaling cascades. Akt and mitogen-activated protein kinases are among the best-characterized enzyme systems affected by H$_2$O$_2$. Through this mechanism H$_2$O$_2$ acts as a second messenger for important vascular stimuli, like thrombin. In the present study we observed that apocynin is able to suppress the activation of the mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 and p38, as well as Akt, in response to H$_2$O$_2$. Although H$_2$O$_2$ has been suggested to activate the NADPH oxidase in vascular cells, this effect appears to be unrelated to oxidative activation, because a similar observation was made with menadione, an intracellular generator of ROS and redox cycle, and in cells treated with the highly effective and selective NADPH oxidase inhibitor DPI. These data establish that, at least in cultured cells, the predominant action of apocynin is the scavenging of ROS and not NADPH oxidase inhibition.

Perspectives
In the present study we provide evidence that the inhibitory action of apocynin for NADPH oxidases is restricted to MPO-expressing leukocytes and that the compound does not inhibit NADPH oxidases in MPO-free vascular cells. We also demonstrated that the prominent effect observed with apocynin in cultured cells could be a consequence of ROS scavenging. Although there is little doubt that vascular NADPH oxidases are important effectors of vascular homeostasis, the improper use of apocynin has potentially lead to an overestimation of the physiological relevance of this enzyme. Landmark observations, such as the blood pressure–lowering effect of apocynin in hypertensive animals and the renal protective action of the compound, which were attributed previously to an inhibition of vascular NADPH oxidases, may have to be revisited or reinterpreted.

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Disclosures

None.

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Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant


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Online data supplement

Extended Material and methods section

Material

Myeloperoxidase was obtained from Planta Natural Products and hydrogen peroxide as well as ethyl acetate from Merck. TNFα, VEGF and PDGF-AB were purchased from Peprotech. Apocynin, menadione and luminol were from Fluka. LO12 was purchased from Wako chemicals and Dihydroethidium and Amplex Red from Invitrogen (Molecular Probes). All other chemicals we obtained from Sigma.

Cell culture

HEK293 cells and rat aortic smooth muscle cells (RSMC) were cultured in DMEM with 4.5 g/L glucose, supplemented with 8 % fetal calf serum and gentamycin (50µg/mL) as well as essential amino acids (0.1mmol/L) and sodiumpyruvate
Porcine aortic endothelial cells (PAEC) were cultured in Ham`s F12 with 8% fetal calf serum and gentamycin (50µg/mL).

Isolation of human leukocytes

Leukocytes (PMN) were isolated from the blood of healthy donors by a discontinuous percoll gradient centrifugation as described previously. Leukocytes were resuspended in HBBS buffer (pH 7) plus magnesium sulfate (0.8 mmol/L) and calcium chloride (1.4 mmol/L).

Immuno-blotting and immune-precipitation

Protein extraction was performed using triton x-100 (1%) lysis buffer (pH 7.4) with the following composition: (concentrations in mmol/L), Tris-HCl (50), sodium chloride (150), sodium pyrophosphate (10), sodium fluoride (20), nonidet NP40 (1%), desoxycholate (0.5%), proteinase inhibitor mix, phenylmethylsulfonyl fluoride (1), orthovanadate (2), caliculyn A (0.1), okadaic acid (0.01). Proteins were separated by SDS-PAGE and following Western blot detected using appropriate primary antibodies and infrared-fluorescent labeled secondary antibody for infrared-based fluorimetric detection by the Odyssey system (Licor). Primary antibodies against AKT, p-AKT, ERK 1/2 and p-ERK 1/2 were obtained from Cell signalling and anti phospho-p38 MAP kinase was obtained from Biosource. Infrared-dye-conjugated secondary antibodies were from Licor. By densitometry, the intensity of the individual phosphorylated proteins was normalized to that of the non-phospho-proteins. The maximal phosphor-signal on a blot was set to 100% and all other values were normalized accordingly.

Transient transfection of HEK293 cells

HEK293 cells were seeded into 6-well plates and transfected with 2 µg plasmid DNA encoding human Nox1, Nox2, p47phox, p67phox, rat Nox4, mouse Noxa1 or Noxo1 and GFP using lipofectamine according to the manufacturers instructions.

Amplex Red assay for hydrogen peroxide detection in HEK 293 cells

Cells were grown on 12-well plates to 90% confluence in phenol-red free medium. Then cells were incubated in HEPES-modified Tyrode`s solution (300 µL) containing
Amplex Red assay for hydrogen peroxide detection in cell free systems
Pyrogallol (10 µmol/L) was dissolved in HEPES-modified Tyrode’s solution (300 µL) containing Amplex Red (50 µmol/L) and horse-radish peroxidase (2 U/mL). After 60 min the supernatant was transferred to 96-well plates and hydrogen peroxide-dependent oxidation of Amplex Red was measured by a microplate fluorimeter (excitation 540 nm, emission 580 nm).

ROS detection with chemiluminescence
Radical formation was determined by chemiluminescence using lucigenin (5 and 250 µmol/L for superoxide anions), LO12 (250 µmol/L for ROS in general including superoxide anions, hydrogen peroxide, peroxynitrite and hydroxyl radicals) or luminol (200 µmol/L) / horse-radish peroxidase (1 U/mL for predominant detection of hydrogen peroxide) with EDTA-detached cells in 500 µl HEPES-modified Tyrode’s solution. Chemiluminescence was calculated as counts per minute using a chemiluminescence reader (LB9505 Berthold).

HPLC-based superoxide anion detection
Dihydroethidium (DHE) (5 mmol/L stock solution in DMSO) was dissolved in HPLC-grade water (pre-treated with argon) to a final concentration of 50 µmol/L. DHE was incubated with Apocynin (10, 100, 1000 µmol/L), Pyrogallol (1, 10 µmol/L) or with a combination of both compounds.

DHE and its reaction products ethidium (E) and oxyethidium (OHE) were separated by HPLC on a C_{18} reverse-phase column (Nucleosil EC, C_{18} 5.0 µm, 4 x 250 mm, Macherey-Nagel) with H_{2}O + 0.1% trifluoracetic acid / acetonitrile as mobile phase. 20 µL sample were injected. DHE, E and OHE were separated by a linear increase from 45% to 65% acetonitrile within 10 min followed by 5 min 100% acetonitrile 10 min 45 % acetonitrile with a flow rate of 0.5 mL/min. Detection of fluorescence at
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595nm (emission) and 510 nm (exitation) was used to monitor oxyethidium. Dihydroethidium was monitored by UV absorption at 350 nm.

**Analysis of Apocynin monomer and dimer products with HPLC system**

Apocynin (1mmol/L) was incubated in HBBS (pH 7) plus magnesium sulfate (0.8 mmol/L) and calcium chloride (1.4 mmol/L) without (5 min at 25°C) or with cells (30 min at 37°C), MPO (0.1 µmol/L) or hydrogen peroxide (1 mmol/L) and different stimuli simultaneously as indicated in the result section. Liquid-liquid extraction was performed twice using 200µl ethyl acetate (1:1). After evaporation of the solvent with a vacuum pump (speed vac Plus sc110A, Savant), pellets were reconstituted with 50µl methanol/water (1:1, v/v) and analyzed by HPLC.

Apocynin mono- and dimers were separated using 50µl sample injected into the HPLC. Mono- and dimers were separated by an isocratically gradient with 15 min 40% CH₃CN followed by 5 min 100% CH₃CN and 5 min 40 % CH₃CN with a flow rate of 0.7 mL/min (Nucleosil EC, C₁₈ 5.0 µm, 4 x 250 mm, Macherey-Nagel).

**Identification of apocynin oxidation products by LC/MS**

Liquid-liquid extracted apocynin was analysed by a Sciex API4000 mass spectrometer operating in multiple reaction monitoring (MRM) mode. Chromatographic separation was performed on a Gemini C18 column (150x2 mm I.D., 5 µm particle size; Phenomenex).

**Statistics**

All values are mean ± SEM. Statistical analysis was carried out using ANOVA followed by LSD post hoc testing. Densitometry was performed using the odyssey-software. A p-value of less than 0.05 was considered statistically significant.

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