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Original Contribution

Pro-oxidant activity of apocynin radical

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ABSTRACT

Apocynin has been widely used as an NADPH oxidase inhibitor in many experimental models. However, concern regarding the efficacy, selectivity, and oxidative side effects of the inhibitor is increasing. In this study, our aim was to characterize the pro-oxidant properties of apocynin and the structurally-related compounds vanillin and vanillic acid. Glutathione (GSH), cysteine, ovalbumin, and the coenzyme NADPH were chosen as potential target biomolecules that could be affected by transient free radicals from apocynin, vanillin and vanillic acid. Additionally, trolox and rifampicin were used as models of hydroquinone moieties, which are particularly susceptible to oxidation. Transient radicals were generated by horseradish peroxidase/hydrogen peroxide-mediated oxidation. In the presence of apocynin, oxidation of GSH was increased seven-fold, and the product of this reaction was identified as GSSG. Similar results were obtained for oxidation of cysteine and ovalbumin. Oxidation of the coenzyme NADPH increased more than 100-fold in the presence of apocynin. Apocynin also caused rapid oxidation of trolox and rifampicin to their quinone derivatives. In conclusion, the pro-oxidant activity of apocynin is related to its previous oxidation leading to transient free radicals. This characteristic may underlie some of the recent findings regarding beneficial or deleterious effects of the phytochemical.

Introduction

The NADPH oxidases (NOXs), a family of transmembrane enzymes that catalyze the single-electron reduction of molecular oxygen, are ubiquitous in mammals. The NADPH oxidase isoform present in phagocytes (NOX2) is the enzyme responsible for the respiratory burst, the non-mitochondrial consumption of molecular oxygen. Its product, the superoxide anion radical (O_2•-), is the precursor for many reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), the hydroxyl radical (•OH), and hypochlorous acid (HOCl), that are essential for the oxidative microbicidal function of these cells [1]. Currently, the primary focus of NOXs research is directed at understanding the involvement of these enzymes in the pathophysiological mechanisms of diseases, such as cancer [2,3], atherosclerosis [4], hypertension [5], and neurodegenerative diseases [6]. Indeed, the unbalanced cellular redox status provoked by exacerbated production of ROS and/or defective intracellular antioxidant mechanisms is a key component in the development of these and many other diseases [7,8].

NADPH oxidase is a multicomponent enzyme complex formed by membrane and cytosolic proteins. NOX2 is composed of two transmembrane proteins which together form cytochrome b558 (p22phox and gp91phox/NOX2), three cytosolic proteins (p47phox, p67phox, p40phox), and a GTPase (Rac2) [9]. Though NOX2 is most highly expressed in phagocytes, expression has also been detected in the CNS, endothelium, fibroblasts, cardiomyocytes, skeletal muscle, hepatocytes, and hematopoietic stem cells [9]. Activation of NADPH oxidase is characterized by the migration of the cytosolic proteins to the membrane, leading to assembly of the enzyme complex and release of the superoxide anion radical into the phagosome or the extracellular medium.

A growing body of evidence underscoring the crucial involvement of ROS in the genesis and/or progression of a myriad of diseases has stimulated the development of drugs targeting NADPH oxidase and the study of their effects on pathophysiological mechanisms [10]. The phytochemical 4-hydroxy-3-methoxyacetophenone, also known as apocynin, is one of the most well characterized NADPH oxidase inhibitors. A recent review detailed many examples of applications of apocynin, including beneficial effects in asthma, neurological diseases, atherosclerosis, and hypertension [11]. Apocynin acts by inhibiting the assembly of NADPH oxidase in monocytes, neutrophils, and endothelial cells [12–14]. Although not completely understood, the mechanism of action of apocynin appears to be related to the formation of its dimer and trimer derivatives, which are produced during oxidation of the drug [13,15]. In this regard, the neutrophil peroxidase, myeloperoxidase (MPO), is thought to be essential for the effects of apocynin, through catalyzing its oxidation [13,16]. Due to this theory, the mechanisms underlying the inhibitory effects of apocynin in cells that lack MPO remain a controversial issue. For instance, Schlüter et al. demonstrated that apocynin inhibited NADPH oxidase in granulocytes,

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but not in cells from rat aortic ring, unless exogenous peroxidase was added to the medium [17]. Similarly, Heumüller at al. demonstrated that apocynin failed to function as an NADPH oxidase inhibitor in endothelial cells and smooth muscle cells. However, the inhibitory capacity of apocynin could be re-established through the addition of MPO [18].

Another issue that has not yet been clarified regarding the use of apocynin is the evidence for intracellular pro-oxidant effects. For example, apocynin markedly decreases the intracellular reduced/oxidized glutathione ratio (GSH/GSSG) in monocytes stimulated by serum-treated zymosan [12]. In glial cells, apocynin induces a significant increase in malonyldialdehyde, lactate dehydrogenase release, and hydrogen peroxide concentration and a decrease in the intracellular glutathione reduced/oxidized (GSH/GSSG) ratio in a dose-dependent manner [19]. In vascular fibroblasts, apocynin stimulates the production of ROS instead of inhibiting production [20].

These putative pro-oxidant properties could also be involved in the mechanism of action of the drug as an NADPH oxidase inhibitor or in the mechanisms of other biological effects that are not yet characterized. Indeed, the generation of redox cycling systems has been implicated in the mechanism of action of many drugs. It is well known that oxidizing species are able to inactivate enzymes, alter the cellular redox status, and, consequently, regulate cell growth, differentiation, and angiogenesis, among other important cellular processes [21]. In this regard, it was recently reported that apocynin inhibits Akt phosphorylation of the hepatocyte growth factor signaling pathway in human lung cancer cells, leading to suppression of metastasis [22]. The authors also demonstrated that apocynin inhibited the enzymatic activity of phosphoinositide 3-kinase. These properties were shared by vanillin, the aldehyde analogous to apocynin, but not by vanillic acid [22]. Similarly, in the current study, we found that apocynin and vanillin, but not vanillic acid, are powerful pro-oxidants.

Herein, we have characterized the chemistry of apocynin and the analogous compounds vanillin and vanillic acid. We found that the transient radical of apocynin can act as a powerful pro-oxidant species. We also found that thiols, the coenzyme NADPH, and hydroquinone-like structures are easily oxidized by apocynin radicals.

Materials and Methods

Chemicals

Apocynin, vanillin, vanillic acid, trolox, horseradish peroxidase (HRP), nicotinamide adenine dinucleotide 2′-phosphate (NADPH), diethylenetriaminepentaacetic acid (DTPA), rifampicin, catalase, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione (GSH), oxidized glutathione (GSSG), *o*-phthalaldehyde (OPA), *N*-ethylmaleimide (NEM), 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), and pyranine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) was prepared by diluting a 30% stock solution and calculating the concentration from the absorption of the solution at 240 nm ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$). All of the reagents used for solutions, buffers, and mobile phases were of analytical grade.

Oxidation of GSH by transient methoxy catechol radicals

A 1 mM solution of GSH was incubated with 100 μM H_2O_2 and 0.1 μM HRP in PBS at 37 °C in the presence or absence (control) of 10 μM methoxy catechols. The reactions were stopped by the addition of 10 μg/ml catalase. Following the reaction, the remaining concentration of GSH was measured using the DTNB method, as previously described [23]. Briefly, the supernatant (0.45 ml) was combined with an equal volume of 300 mM Na_2HPO_4 and 0.1 ml of DTNB solution (0.2 mg/mL DTNB in 1% citrate). The absorbance at 412 nm was calculated relative to a blank containing 0.45 ml PBS, 0.45 mL of 300 mM Na_2HPO_4, and 0.1 ml of DTNB. A standard curve was generated to calculate the concentration of GSH. A UV-Mini 1240 spectrophotometer (Shimadzu, Japan) was used for absorbance measurements.

Measurement of the product GSSG

The reaction mixture described above for measurement of the remaining GSH was also used to quantify the production of oxidized glutathione (GSSG). The concentration of GSSG was determined by HPLC, as previously described [24]. The HPLC method for the determination of GSSG is based on derivatization of GSSG with OPA in alkaline medium, which leads to the formation of a stable, highly fluorescent derivative [25]. Briefly, a 200 μl aliquot of the reaction mixture was incubated at 25 °C with 200 μl of 40 mM NEM for 25 min in the absence of light to interact with GSH present in the sample. To this mixture, 750 μl of 0.1 M NaOH was added. To 20 μl portion of this final mixture, 300 μl of 0.1 M NaOH and 20 μl of 0.1% OPA in methanol were added. The well-capped tubes were then incubated at 25 °C for 15 min in the absence of light, and 20 μl from each sample was injected into the HPLC system. The fluorescent derivative of GSSG was separated by HPLC (Varian ProStar in line with a fluorescence detector set at 350/420 nm). The analyses were carried out isocratically on a Luna C18 reverse-phase column (250×4.6 mm, 5 μm). The mobile phase consisted of 15% methanol in 25 mM sodium hydrogen phosphate, pH 6.0 (v/v), and the flow rate was 1.0 ml/min.

Measurement of oxygen consumption

Consumption of dissolved molecular oxygen during the oxidation of GSH by methoxy catechol radicals was monitored using a YSI 5300A Oxygen Monitor (Yellow Spring, USA). The initial reaction mixture contained 1 mM GSH or 1 mM cysteine, 0.1 μM HRP in PBS, and 10 μM methoxy catechols (control reactions did not contain methoxy catechols). The reaction was initiated by the addition of 100 μM H_2O_2, and oxygen consumption was monitored for 5 minutes at 37 °C.

Oxidation of Ovalbumin's sulfhydryl groups by transient methoxy catechol radicals

Egg white was used as a source of ovalbumin, and the total amount of sulfhydryl (SH) groups was measured using the DTNB technique adapted for determination in ovalbumin [26]. Reagent grade chemicals were used to prepare the following solutions: Tris-glycine buffer (0.1 M Tris-(hydroxymethyl)-aminomethane (Tris), 0.1 M glycine, and 4 mM ethylenediaminetetraacetic acid disodium salt, pH 8.0); 5% sodium dodecyl sulfate in Tris-glycine buffer (denoted SDS-Tris-glycine); Reagent DTNB (4 mg/ml DTNB in Tris-glycine buffer, pH 8.0). A 1 % (w/v) solution of egg white was prepared in SDS-Tris-glycine and centrifuged for 5 minutes at 1000×*g*. The supernatant from this fractionation was used for the assays described below.

For determination of the total amount of SH groups, 500 μl of supernatant was mixed with an equal volume of SDS-Tris-glycine buffer and 10 μl of DTNB reagent. The reaction mixture was incubated for 15 min at 40 °C in a water bath to facilitate protein unfolding and promote the access of all sulfhydryl groups to DTNB. Finally, the absorbance of the solution at 412 nm was measured relative to a blank containing SDS-Tris-glycine buffer [26]. To test the reactivity of the methoxy catechol radicals with SH groups, the protein supernatant described above was incubated with 10 nM HRP and 10 μM H_2O_2 in the absence (control) or presence of 10 μM methoxy catechols for 15 min. Then, the amount of remaining SH groups was quantified as described above. A UV-Mini 1240 spectrophotometer (Shimadzu, Japan) was used for absorbance measurements in these assays.

Oxidation of NADPH by transient methoxy catechol radicals

A 100 μM solution of NADPH was incubated with 10 μM H_2O_2 and 0.01 μM HRP in 10 mM phosphate buffer, pH 7.0, with 50 μM DTPA at 37 °C in the absence (control) or presence of 10 μM methoxy catechols. The rate of NADPH oxidation was measured at 340 nm [27]. The blank for absorbance measurements consisted of phosphate buffer and 10 μM methoxy catechols. An HP8452 diode array spectrophotometer (Agilent, USA) was used to measure absorbance in these assays.

Oxidation of Trolox by transient methoxy catechol radicals

A 100 μM solution of trolox was incubated with 50 μM H_2O_2 and 0.01 μM HRP in 10 mM phosphate buffer, pH 7.0, with 50 μM DTPA at 37 °C in the absence (control) or presence of 5 μM methoxy catechols. The rate of trolox oxidation was measured at 272 nm [28]. The blank for absorbance measurements consisted of phosphate buffer and 5 μM methoxy catechols. An HP8452 diode array spectrophotometer (Agilent, USA) was used to measure absorbance in these assays.

Oxidation of rifampicin by transient methoxy catechols radicals

A 100 μM solution of rifampicin was incubated with 100 μM H_2O_2 and 0.01 μM HRP in 100 mM phosphate buffer, pH 5.5, at 25 °C in the absence (control) or presence of 50 μM methoxy catechols. The rate of rifampicin oxidation was measured at 472 nm [29]. The blank for absorbance measurements consisted of phosphate buffer. An HP8452 diode array spectrophotometer (Agilent, USA) was used to measure absorbance in these assays.

DPPH radical scavenging activity

The relative antioxidant potency of the methoxy catechols was compared by characterizing their capacity to act as reducing agents of the 1-diphenyl-2-picrylhydrazyl radical (DPPH). The methoxy catechols (50 μM) were incubated with 100 μM DPPH dissolved in ethyl alcohol for 60 minutes at room temperature in the absence of light. Absorbance at 517 nm was measured relative to a blank consisting of ethyl alcohol, and trolox was used as a reference antioxidant. The scavenging activity of the methoxy catechols on DPPH was calculated as follows:

$$\%\ \text{scavenging activity} = Ac - Ai\ /\ Ac \times 100,$$

where *Ac* is the absorbance of DPPH, and *Ai* is the absorbance of the samples where methoxy catechols were added.

Pyranine-based procedure for the evaluation of the reactivity of methoxy catechols with peroxyl radicals, generated by AAPH thermolysis

This experiment was performed as described by Lissi *et al.* with minor modifications [30]. The fluorescent compound pyranine (5 μM) was incubated with 20 mM AAPH in PBS at 37 °C in the absence (control) or presence of the methoxy catechols. Fluorescence bleaching of pyranine was monitored using an excitation wavelength (λ_{ex}) of 460 nm and an emission wavelength (λ_{em}) of 510 nm on a Spectramax M2 Microplate Reader (Molecular Devices, USA). The final reaction volume was 300 μl. The lag phase (induction time) obtained when antioxidants were present was used to compare the relative reactivity of the methoxy catechols with AAPH-derived peroxyl radicals.

Results

In this study, we compared the antioxidant and pro-oxidant properties of apocynin and the structurally related compounds vanillin and vanillic acid (Fig. 1). Vanillic acid was chosen for comparison with apocynin and vanillin because the *p*-substituted carboxylate group causes an opposite electronic effect in the aromatic ring [31] thought to affect susceptibility to oxidation [32,33]. In order to elucidate the chemical properties of the apocynin radical, which has been proposed to be a transient species formed when activated cells are incubated with apocynin, our experimental approach was to assess the reactivity of apocynin with biomolecules. The transient free radical was produced through the oxidation of apocynin by hydrogen peroxide in a reaction catalyzed by HRP.

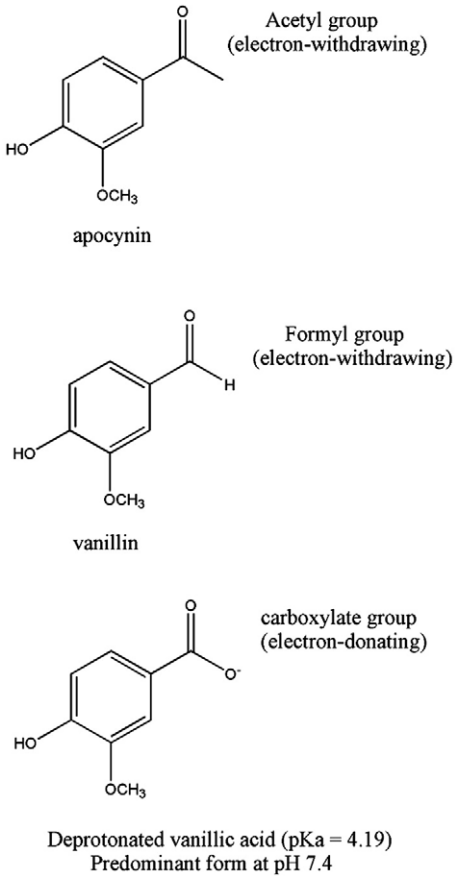

Fig. 1. Structure of the compounds characterized in this study and the electronic effects of their substituents [32,33].

Initially, the reactivity of the apocynin, vanillin and vanillic acid radicals were compared by characterizing their capacity to oxidize GSH. As a control, oxidation was also conducted in the absence of the methoxy catechols. The results in Fig. 2a show that GSH was poorly oxidized by HRP/H_2O_2. However, the addition of a catalytic amount of apocynin to the reaction mixture significantly increased consumption of GSH. Similar results were obtained for vanillin, but not for vanillic acid (Fig. 2a). Since oxidized glutathione (GSSG) is the expected product of GSH oxidation, the concentration of GSSG in the reaction medium was also measured. GSSG concentration was analyzed by HPLC after derivatization with *o*-phthalaldehyde (OPA) in an alkaline medium. It is important to emphasize that prior to derivatization the remaining GSH, which can also react with OPA, was removed from the reaction medium with *N*-ethylmaleimide (NEM) [24]. Consistent with the previous results, the presence of apocynin or vanillin in the reaction mixture caused a significant increase in the production of GSSG, but vanillic acid had only a minor effect (Fig. 2b).

Another experimental procedure used to study the oxidation of GSH by phenoxyl radicals is to measure the consumption of dissolved

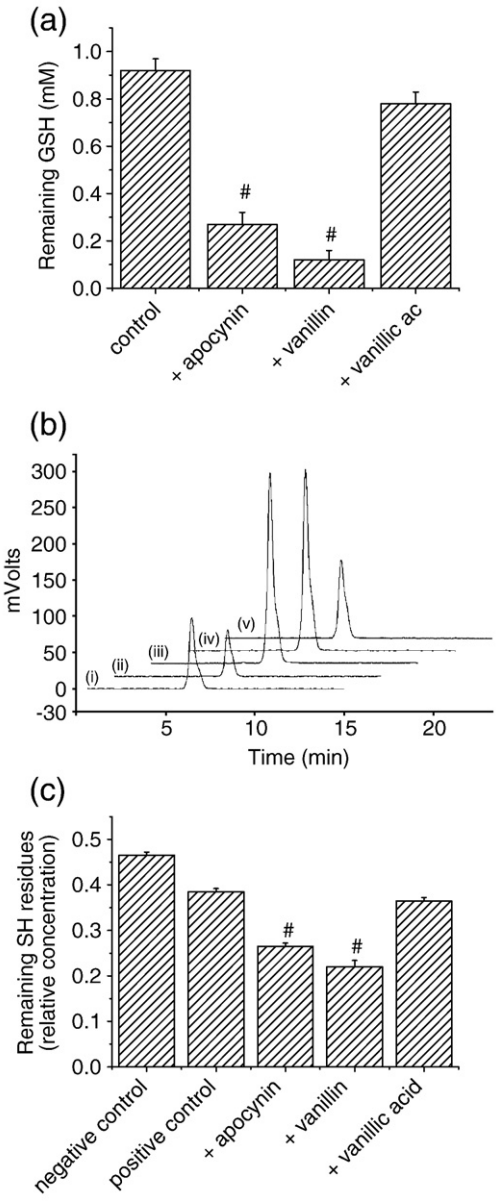

Fig. 2. Oxidation of sulfhydryl groups by methoxy catechol radicals. (a) Oxidation of GSH mediated by methoxy catechol radicals. The control reaction mixture consisted of 1 mM GSH, 0.1 μM HRP, 100 μM H_2O_2. Apocynin, vanillin, and vanillic acid were added to the indicated reactions at a concentration of 10 μM. (b) Production of GSSG during oxidation of GSH mediated by methoxy catechol radicals. The GSSG product was derivatized with 0.1% OPA and analyzed by HPLC with fluorescent detection. (i) Chromatogram of 100 μM pure GSSG. (ii) Chromatogram of the reaction without methoxy catechols. (iii) Chromatogram of the reaction in the presence of apocynin. (iv) Chromatogram of the reaction in the presence of vanillin. (v) Chromatogram of the reaction in the presence of vanillic acid. (c) Oxidation of sulfhydryl groups (SH) in ovalbumin mediated by methoxy catechol radicals. The reaction mixture (positive control) consisted of 1 % (w/v) ovalbumin, 0.01 μM HRP, 10 μM H_2O_2. Apocynin, vanillin, and vanillic acid were added to the indicated reactions at a concentration of 10 μM. The negative control was 1% (w/v) ovalbumin alone. Results are presented as the mean and SD of triplicate experiments. #Statistically significant difference relative to control ($p<0.05$, One Way Analysis of Variance and Tukey multiple comparison test).

molecular oxygen in the reaction mixture. Depletion of molecular oxygen is induced through its one-electron reduction by the diglutathionyl anion radical ($GSSG^{\bullet-}$) [34]. The results depicted in Fig. 3 demonstrate that apocynin and vanillin radicals, but not vanillic acid radicals, can initiate this redox cycle.

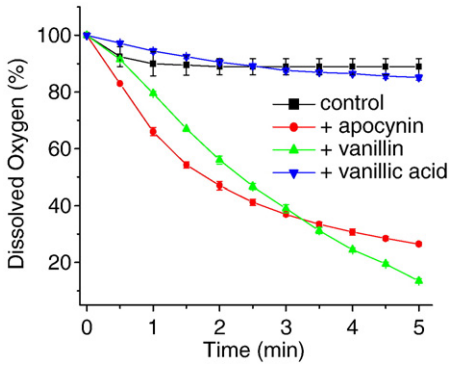

Fig. 3. Consumption of dissolved oxygen during the oxidation of GSH mediated by methoxy catechol radicals. The control reaction mixture consisted of 1 mM GSH, 0.1 μM HRP, 100 μM H_2O_2. Apocynin, vanillin, and vanillic acid were added to the indicated reactions at a concentration of 10 μM. See Materials and Methods for further details. Results are presented as the mean and SD of triplicate experiments.

The capacity of apocynin and vanillin radicals to act as oxidizing agents was also observed when the amino acid cysteine was used as a reductant instead of GSH (data not shown). Similar to results for GSH, apocynin and vanillin radicals were able to oxidize cysteine residues in ovalbumin, which was used as a protein model. Vanillic acid caused only a minor effect (Fig. 2c). This observation indicates that the pro-oxidant capacity of apocynin and vanillin to oxidize thiol groups can be extended to oxidation of macromolecules.

Another biomolecule that could be a potential target for oxidizing species like the apocynin radical is the coenzyme NADPH, particularly in neutrophils treated with the chemical. Therefore, we compared the capacity of transient apocynin, vanillin, and vanillic acid radicals to oxidize NADPH (Fig. 4). Oxidation of NADPH in the absence of methoxy catechols was used as a control. Loss of the reactant NADPH was monitored by the decrease in absorbance at 340 nm. The results in Fig. 4 show that NADPH was also susceptible to oxidation by apocynin and vanillin radicals. The pro-oxidative effect was also observed when 200 μM apocynin was used instead of 10 μM (data not shown). At higher concentration, our experimental settings did not allow a precise measurement, but the reaction rate was always increased.

The hydroquinone/quinone is a ubiquitous intracellular couple. Since this redox-sensitive molecular target is present in many enzymatic systems, we next assessed the effects of apocynin on the conversion of hydroquinone to quinone. As a model for quinone-based systems, we assessed the effects of apocynin on the conversion of trolox to trolox quinone. The formation of the quinone was monitored by the increase in absorbance at 272 nm. The clear isosbestic point of 286 nm indicated the transformation of trolox to trolox quinone [28]. Trolox was poorly oxidized to trolox quinone in the presence of HRP/H_2O_2; however, the addition of a catalytic amount of apocynin to the reaction mixture provoked a significant increase in the oxidation rate (Figs. 5a, b and c). In contrast to the previous experiments, vanillin had no pro-oxidant effects on the oxidation of trolox.

Another hydroquinone/quinone transformation that can be easily assessed using UV-visible spectrophotometry is the oxidation of rifampicin to rifampicin quinone [29]. In order to confirm the results for trolox, we subjected rifampicin to oxidation by HRP/H_2O_2 and tested the effects of the methoxy catechols. The reactions were followed by bleaching of rifampicin at 472 nm [29]. Similar to the results above, the presence of apocynin increased the oxidation rate. Vanillin and vanillic acid were less effective (Figs. 5d and e).

All of the results above suggest that apocynin and vanillin can act as pro-oxidants when their transient free radicals are generated intracellularly. However, as phenolic compounds, the antioxidant properties of these molecules must be also considered. Therefore, we

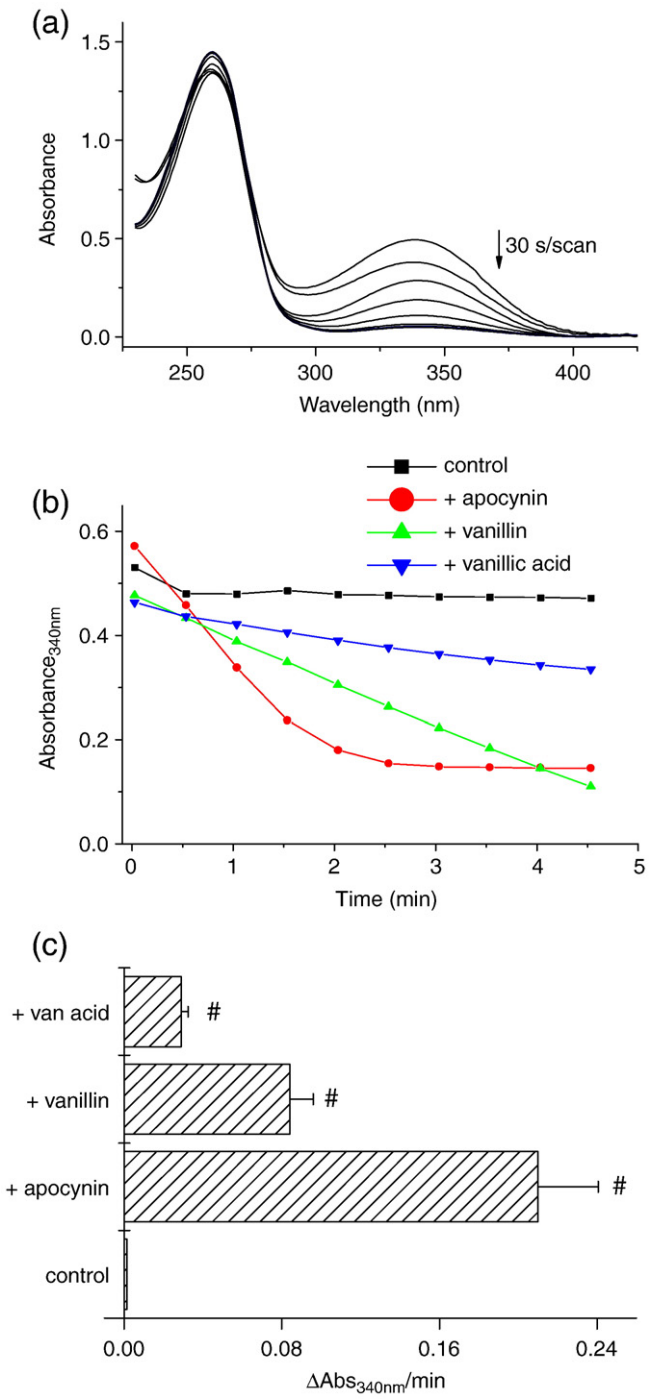

Fig. 4. Oxidation of NADPH by methoxy catechol radicals. (a) Oxidation of NADPH mediated by the apocynin radical. The control reaction mixture consisted of 100 μM NADPH, 0.01 μM HRP, 10 μM H_2O_2. Apocynin was added to the indicated reactions at a concentration of 10 μM. Scans were obtained at 30 s intervals. (b) Kinetic profile of NADPH oxidation. (c) Relative rate of NADPH oxidation. The control reaction mixture consisted of 100 μM NADPH, 0.01 μM HRP, and 10 μM H_2O_2. Methoxy catechols were added to the indicated reactions at a concentration of 10 μM. Results are presented as the mean and SD of triplicate experiments. #Statistically significant difference relative to control ($p<0.05$, One Way Analysis of Variance and Tukey multiple comparison test).

also compared the antioxidant capacities of apocynin, vanillin, and vanillic acid. The antioxidant trolox was used as a reference substance. The relative antioxidant potency of the methoxy catechols was assessed by comparing by their capacity to reduce the 1-diphenyl-2-picrylhydrazyl radical (DPPH). Results in Table 1 show that apocynin and vanillin were poor antioxidants. Interestingly, vanillic acid was a better antioxidant than both vanillin and apocynin. However, all of the methoxy catechols were poor antioxidants when compared to trolox.

The antioxidant properties of the methoxy catechols on peroxyl radicals were also characterized. In these experiments, the peroxyl radical generated by thermolysis of AAPH oxidizes the fluorescent compound pyranine. The presence of an antioxidant provokes a lag phase in the fluorescence bleaching of pyranine [30]. Fig. 6 shows examples of the effects of apocynin, vanillin, vanillic acid, and trolox (the reference antioxidant) on the oxidation of pyranine by peroxyl radicals. These results confirm that the methoxy catechols are poor antioxidants compared to trolox. However, within the methoxy catechol group, vanillic acid was a more efficient antioxidant than apocynin or vanillin.

The exacerbation in the oxidation of the biomolecules described above was totally dependent on the initial production of transient free radicals. Indeed, when the reactions were conducted in the absence of peroxidase, the methoxy catechols were ineffective as pro-oxidants (data not shown).

Discussion

Despite some controversy, apocynin is the most commonly used inhibitor of NADPH oxidase in phagocytic and non-phagocytic cells. However, concern is growing regarding the side effects and specificity of the inhibitor [17,18]. Therefore, the recently reported evidence of pro-oxidant activities of apocynin in the intracellular medium represents an important issue regarding the chemistry of apocynin that might be related to its mechanism as an NADPH oxidase inhibitor and/or to toxic effects [19,20]. In order to clarify this issue and characterize the potential pro-oxidant roles of apocynin, we focused on the interaction between apocynin radicals and potentially susceptible oxidizable biomolecules or xenobiotics that could represent a model for the action of apocynin.

First, it must be emphasized that pro-oxidant activity is a general term used when a substance is able to initiate or exacerbate, by one of many different mechanisms, a deleterious oxidative process. Moreover, it is not uncommon for a classic antioxidant substance to possess pro-oxidant characteristics [35–37]. For phenolic antioxidants, an important pro-oxidant pathway is through the oxidizing capacity of their transient free radicals [27]. In support of this pathway, a recent study has demonstrated that phenolic substances with an oxidation potential lower than 0.45 V act as antioxidants, whereas compounds with higher values (Epa>0.45 V) act as pro-oxidants [38]. Therefore, an important question is whether the phenolic transient free radical is able to cause a posterior oxidation, or, as expected for an antioxidant, to generate a final unreactive product.

In general, our results indicate that apocynin radical is an authentic example of a phenolic compound with exacerbated pro-oxidant characteristics. Similar results were also obtained for vanillin, but not for vanillic acid. The only difference between these compounds is the *p*-substituent group in the aromatic ring. The acetyl and formyl groups are electron-withdrawing groups, and the carboxylate is an electron-donating group (Fig. 1). These electronic effects have a direct influence on the reduction potential of phenolic compounds. In general, electron-withdrawing groups increase the reduction potential, and electron-donating groups decrease the reduction potential [33]. Hence, if the reduction potential of phenolic compounds determines whether the compounds possess antioxidant or pro-oxidant characteristics [38], then apocynin and vanillin are likely to be better pro-oxidants than vanillic acid, consistent with the results of this study. Observations regarding the pro-oxidant characteristics of these molecules were further supported by comparing the relative antioxidant potency of these molecules. In this case, vanillic acid was

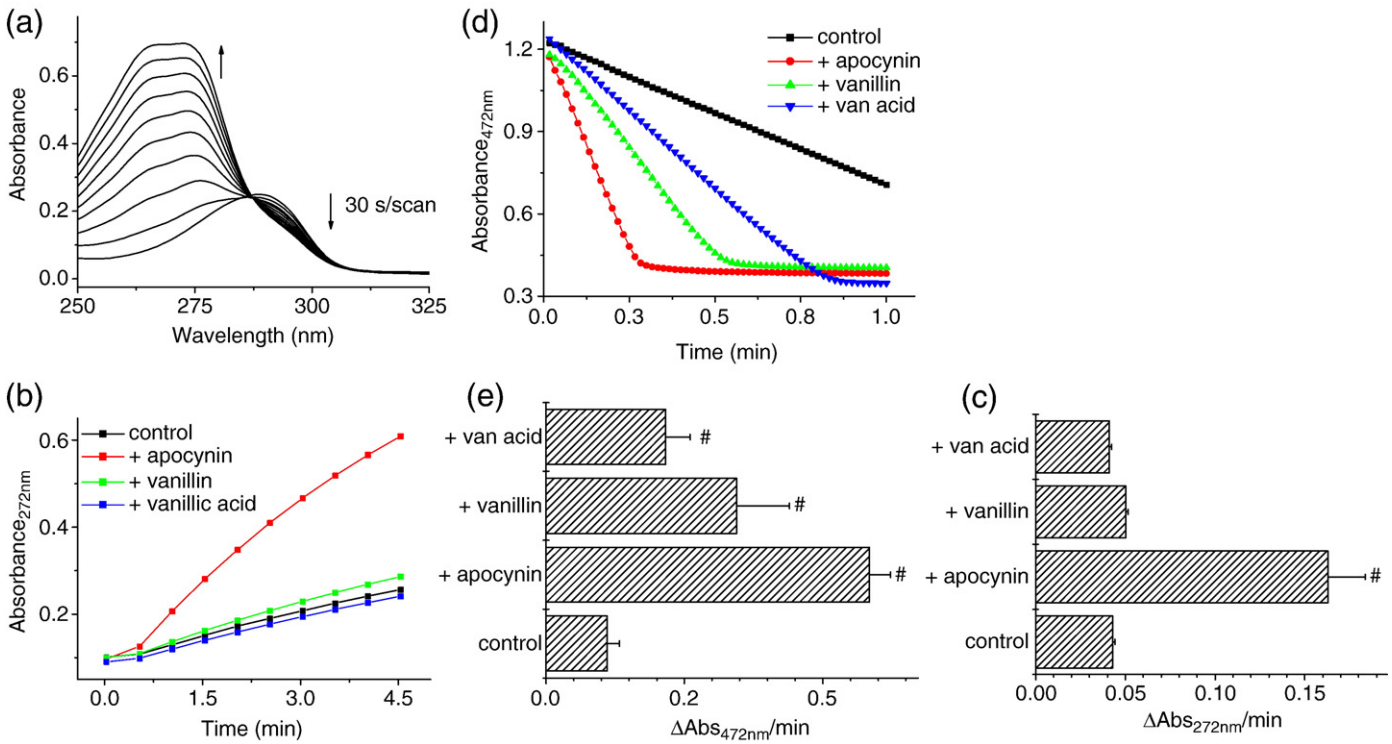

Fig. 5. Oxidation of hydroquinone moieties by methoxy catechol radicals. (a) Oxidation of trolox mediated by the apocynin radical. The control reaction mixture consisted of 100 μM trolox, 0.01 μM HRP, and 10 μM H_2O_2. Apocynin was added to the indicated reactions at a concentration of 5 μM. Scans were obtained at 30 s intervals. (b) Kinetic profile of trolox oxidation. (c) Relative rate of trolox oxidation. The control reaction mixture consisted of 100 μM trolox, 0.01 μM HRP, and 10 μM H_2O_2. Methoxy catechols were added to the indicated reactions at a concentration of 5 μM. Results are presented as the mean and SD of triplicate experiments. #Statistically significant difference relative to control ($p<0.05$, One Way Analysis of Variance and Tukey multiple comparison test). (d) Kinetic profile of rifampicin oxidation. (e) Relative rate of rifampicin oxidation. The control reaction mixture consisted of 100 μM rifampicin, 0.01 μM HRP, and 50 μM H_2O_2 in 0.1 M phosphate buffer pH 5.4. Methoxy catechols were added to the indicated reactions at a concentration of 50 μM. Results are presented as the mean and SD of triplicate experiments. #Statistically significant difference relative to control ($p<0.05$, One Way Analysis of Variance and Tukey multiple comparison test).

more effective as an antioxidant than apocynin or vanillin. However, apocynin, vanillin, and vanillic acid are all poor antioxidants compared to trolox, which is frequently used as a reference antioxidant. Therefore, these data suggest that the action of apocynin as an NADPH oxidase inhibitor cannot be attributed to its antioxidant properties.

The capacity of apocynin and vanillin radicals to oxidize thiols is quite impressive. GSH, cysteine, and cysteine residues in ovalbumin were all efficiently oxidized by apocynin and vanillin radicals. Indeed, 10 μM apocynin was able to promote the oxidation of 650 μM GSH, while in the absence of apocynin only about 90 μM GSH could be oxidized. The tripeptide GSH is the major endogenous antioxidant in mammalian cells, and the GSH/GSSG ratio is a direct reflection of the cellular redox status [39]. From these studies, there is no doubt that the formation of the apocynin radical inside cells would lead to GSH depletion, as previously described [12,19]. Moreover, the oxidation of thiols can trigger a redox cycle that culminates with the production of superoxide anion [34], potentially amplifying pro-oxidant effects.

Table 1
DPPH free radical scavenging activity of methoxy catechols and trolox

	DPPH Scavenging (%)
Apocynin	6.9 ± 1.5
Vanillin	6.8 ± 0.7
Vanillic Acid	23.2 ± 4.8
Trolox	77.3 ± 0.9

The concentrations of the antioxidants were 50 μM. Data are presented as mean and SD of triplicate experiments.

The apocynin and vanillin radicals are also capable of oxidizing quinone moieties, which are ubiquitous in cells and include the mitochondrial electron carrier coenzyme Q (CoQ). Apocynin radicals had a high capacity to act as oxidizing agents of trolox and rifampicin, used in this study as models of quinone structures. These observations suggest that endogenous quinones would also likely be sensitive to the action of apocynin, leading to further alteration of the cellular redox status.

Our finding that the apocynin radical is able to oxidize NADPH, the endogenous substrate of the NADPH oxidase enzymatic complex, may have important implications for the mechanism of apocynin as an NADPH oxidase inhibitor. In fact, depletion of its substrate would block NADPH oxidase from production of the superoxide anion. Moreover, this chemical interaction could also contribute to imbalance in the cellular redox status. It is well understood that the oxidation of NADPH involves the intermediate NADP• (NADP radical), which is also able to initiate a pro-oxidant process by reducing molecular oxygen to the superoxide anion [27].

It must be emphasized that the pro-oxidant activity of apocynin is related to its previous oxidation leading to transient free radicals. In other words, apocynin itself does not present this activity. However, the growing body of evidence showing the intracellular pro-oxidative activity of apocynin [12,19,20] suggests that the generation of apocynin free radicals inside the cells must be expected and, as a consequence, the alteration in the cellular redox status. Since this redox imbalance is able to trigger intracellular signal transduction pathways, such as gene activation, proliferation, apoptosis etc, we must to conclude that the use of apocynin in complex biological systems do not necessarily involve specific inhibition of NOX enzymes.

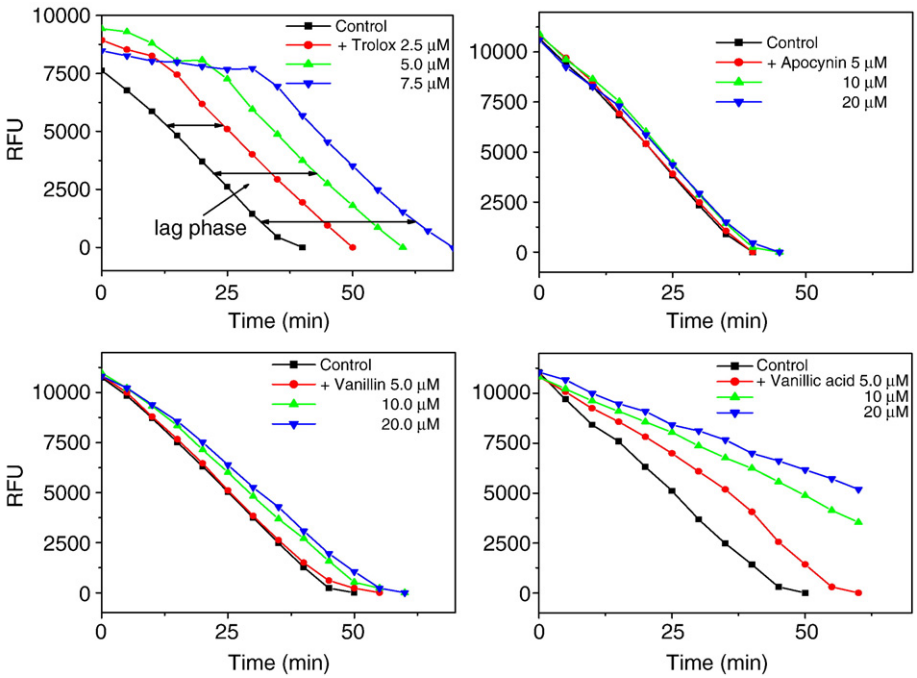

Fig. 6. Time course of peroxyl radical-induced fluorescence bleaching of pyranine and lag phases caused by the addition of antioxidants. The control reaction mixture consisted of 5 μM pyranine, 20 mM AAPH in PBS at 37°.

In summary, our findings clearly show the pro-oxidant capacity of apocynin radical, which could lead to an intracellular redox imbalance. Therefore, the next logical question is whether the pro-oxidant capacity of apocynin is a harmful side effect, or whether it is involved in the apocynin mechanism of action as an NADPH oxidase inhibitor or in other biological effects. A conclusive answer to this question is not currently known; however, generation of oxidizing species is the basis for the action of many drugs, including chemotherapeutics. Interestingly, recently it was demonstrated that apocynin and vanillin, but not vanillic acid, can suppress metastasis in cancerous cells [22]. Moreover, vanillic acid is a poor inhibitor of NADPH oxidase in neutrophils compared to vanillin and apocynin [40]. Thus, we suggest that the pro-oxidant characteristics of apocynin radical must be taken into account in future studies regarding the benefits or deleterious effects of apocynin and vanillin. Fig. 7 presents the potential targets of the apocynin radical in cells.

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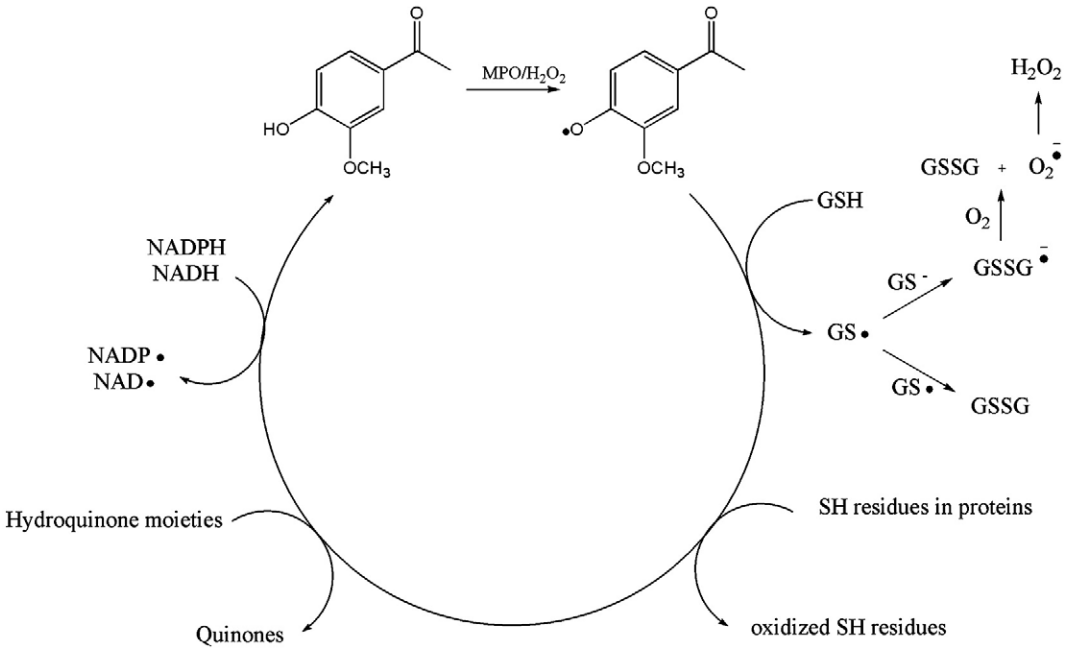

Fig. 7. Pro-oxidant effects of apocynin. The putative molecular targets of the apocynin radical.

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