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The reactivity of *ortho*-methoxy-substituted catechol radicals with sulfhydryl groups: Contribution for the comprehension of the mechanism of inhibition of NADPH oxidase by apocynin

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ABSTRACT

Redox processes are involved in the mechanism of action of NADPH oxidase inhibitors such as diphenyleneiodonium and apocynin. Here, we studied the structure-activity relationship for apocynin and analogous *ortho*-methoxy-substituted catechols as inhibitors of the NADPH oxidase in neutrophils and their reactivity with peroxidase. Aiming to alter the reduction potential, the *ortho*-methoxy-catechol moiety was kept constant and the substituents at para position related to the hydroxyl group were varied. Two series of compounds were employed: methoxy-catechols bearing electron-withdrawing groups (MC-W) such as apocynin, vanillin, 4-nitroguaiacol, 4-cyanoguaiacol, and methoxy-catechol bearing electron-donating groups (MC-D) such as 4-methylguaiacol and 4-ethylguaiacol. We found that MC-D were weaker inhibitors compared to MD-W. Furthermore, the radicals generated by oxidation of MC-W via MPO/H_2O_2, but not for MC-D, were able to oxidize glutathione (GSH) as verified by the formation of thiyl radicals, depletion of GSH, and recycling of the *ortho*-methoxy-catechols during their oxidations. The capacity of oxidizing sulfhydryl (SH) groups was also verified when ovalbumin was incubated with MC-W, but not for MC-D. Since the effect of apocynin has been correlated with inactivation of the cytosolic fractions of the NADPH oxidase complex and its oxidation during the inhibitory process develops a special role in this process, we suggest that the close relationship between the reactivity of the radicals of MC-W compounds with thiol groups and their efficacy as NADPH oxidase inhibitor could be the chemical pathway behind the mechanism of action of apocynin and should be taken into account in the design of new and specific NADPH oxidase inhibitors.

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1. Introduction

Reactive oxygen species (ROS) play a significant role in the pathogenesis of a myriad of inflammatory and cardiovascular diseases, such as, diabetes, atherosclerosis, asthma, Alzheimer's disease, psoriasis, rheumatoid arthritis, and also in the aging process [1–3]. In this concern, one of the predominant cellular sources of ROS is a family of multisubunit enzymes known as the NADPH oxidases. These enzymes catalyze the univalent reduction of molecular oxygen, thereby forming superoxide (O_2^-). This one-electron reduced form of molecular oxygen is the primary source of ROS, and from it, several oxidizing substances as hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\bullet$), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$) can be generated [4].

NADPH oxidase develops a primordial role in the phagocytic cells, since the generation of ROS is the base for their microbicidal activity. However, the presence of several homologous proteins in nonphagocyte tissues has provided evidence that this oxidase system has many other physiological functions, but unfortunately, it has been also linked to many other deleterious effects. For instance, cardiovascular NADPH oxidases have been shown to play important roles in blood pressure regulation, as well as pathophysiological events, including hypertension and atherosclerosis [5,6]. In the kidney, ROS generated by NADPH oxidase play essential roles in normal renal function, but also have been implicated in pathological conditions related to abnormal kidney function as diabetic nephropathy [7,8]. NADPH oxidase is also expressed in the central nervous system and there are substantial evidences of the involvement of ROS in the normal brain aging, as well as disease states, such as Alzheimer's disease, ischaemic injury, and stroke [9,10]. NADPH oxidase is a multienzyme complex comprising the membrane-bound cytochrome b558, three cytosolic factors (p47phox, p67phox, p40phox), and the small GTPase Rac2. When the cells are activated by stimuli such as immunocomplex, opsonized particles, arachidonic acid or phorbol myristate acetate, a cascade of events takes place, resulting in the migration of these cytosolic components to the membrane and assemble of the enzyme complex, which starts producing superoxide [11].

Considering the presence of the NADPH oxidases in the several tissues and their clear linkage with inflammatory diseases, several substances with antioxidant property have been tested as inhibitors of the generation and/or scavengers of the ROS produced via NADPH oxidase activation. Regarding to that, the *ortho*-methoxy-catechol (4-hydroxy-3-methoxyacetophenone), known as apocynin, plays a special role [12]. Indeed, it is the only phytochemical that has been widely used as a NADPH oxidase inhibitor in many experimental models using phagocytic and nonphagocytic cells (over 300 medline-indexed publications). A very important finding, concerning its mechanism of inhibition on NADPH oxidase, was the discovery that apocynin must be oxidized to perform its role and its products could be the real active species [13]. Recently, we demonstrated that during the peroxidase-mediated oxidation of apocynin, its radical intermediate is able to oxidize glutathione (GSH) [14]. Since apocynin inhibits the migration of the cytosolic fraction p47phox to the membrane, and this property has been linked to the inactivation of essential sulfhydryl (SH) residues of this protein [15], we suggested that the necessity of oxidation and the reactivity of apocynin radicals with thiol groups could be behind the mechanism of action of apocynin. Here, we advanced and studied the correlation between the reactivity of *ortho*-methoxy-catechol, analogous to apocynin, with SH groups and their relative potency as NADPH oxidase inhibitor in activated neutrophils. We found that there is a close relationship between the reactivity of the radicals of the *ortho*-methoxy-catechols with thiol groups and their efficacy as NADPH oxidase inhibitors.

2. Materials and method

Chemicals: 4-hydroxy-3-methoxyacetophenone (apocynin), 4-hydroxy-3-methoxybenzaldehyde (vanillin), 2-methoxy-4-nitrophenol (4-nitroguaiacol), 4-hydroxy-3-methoxybenzonitrile (4-cyanoguaiacol), 2-methoxy-4-methylphenol (4-methylguaiacol), 4-ethyl-2-methoxyphenol (4-ethylguaiacol), GSH, zymosan, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), and lucigenin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Myeloperoxidase (EC 1.11.1.7) was purchased from Planta Natural Products (Vienna, Austria) and its concentration was determined from its absorption at 430 nm ($\varepsilon_{430\ nm}$ = 89,000 $M^{-1}\ cm^{-1}$ per heme). Hydrogen peroxide was prepared by diluting a 30% stock solution purchased from Peroxidos do Brasil (Sao Paulo, Brazil) and calculating its concentration using its absorption at 240 nm ($\varepsilon_{240\ nm}$ = 43.6 $M^{-1}\ cm^{-1}$). All the reagents used for solutions and buffers were of analytical grade.

2.1. Isolation of human neutrophils

Neutrophils were isolated from the blood of healthy donors by Ficoll-Paque centrifugation, dextran sedimentation, and hypotonic lysis of red cells [16]. After isolation, neutrophils were resuspended in 10 mM phosphate buffer (pH 7.0) containing 10 mM potassium chloride and 140 mM sodium chloride, plus 1 mM calcium chloride, 0.5 mM magnesium chloride, and 1 mg/ml glucose (supplemented PBS). Opsonized zymosan was prepared as described by Simoes et al. [17]. The blood samples were taken from healthy volunteers. The study was approved by the faculty research ethics committee (Comite de Etica em Pesquisa- FCFAR/UNESP no. 03/2006).

2.2. Lucigenin-dependent chemiluminescence elicited by activated neutrophils

Neutrophils (2×10^6 cells/ml) were preincubated at 37 °C in supplemented PBS with 10 μmol/l lucigenin and the methoxy-catechols for 10 min. The reaction was started by adding 1 mg/ml opsonized zymosan and the chemiluminescence was measured for 30 min. The reactions were carried out in a BioOrbit 1251 luminometer (Turku, Finland). The final reaction volume was 0.5 ml. The integrated light emission was taken as the analytical parameter. The tests where methoxy-catechols were absent were used as control for the calculation of the relative inhibition for each methoxy-catechol. For the calculation of the IC 50, curves with different concentration of each methoxy-catechol were raised. The IC 50 results are triplicates using neutrophils of three healthy donors.

2.3. Oxygen uptake elicited by activated neutrophils

Neutrophils (4×10^6 cells/ml) were incubated at 37 °C in supplemented PBS with 2 mM methoxy-catechols and 1 mg/ml opsonized zymosan. The oxygen consumption was measured in a Clark-type oxygen electrode (Yellow spring instruments 5300A, Cincinnati, OH, USA). The test where methoxy-catechols were absent was used as a control. The buffer solution was initially saturated with air (200 μM) by stirring the opened cell for 5 min.

2.4. HPLC study of the oxidation of ortho-methoxy-catechols catalyzed by MPO and the effect of GSH

Methoxy-catechols (1 mM) were incubated in 50 mM phosphate buffer, pH 7.0, 25 °C, with 0.5 μM MPO and 0.5 mM H_2O_2 for 10 min in the presence or absence of 1 mM GSH. The reaction was started by adding hydrogen. Products were separated in a high performance liquid chromatograph (Waters 2690 separation module in line with a Waters 996 UV–vis detector set at 254 nm). HPLC analyses were carried out isocratically on a Synergi C18 reversed-phase column (250 mm × 4.6 mm, 4 μm), with 60:40 water/acetonitrile (flow rate 0.7 ml/min) as the mobile phase.

2.5. Oxygen uptake during the oxidation of the ortho-methoxy-catechols catalyzed by MPO

Methoxy-catechols (1 mM) were incubated in 50 mM phosphate buffer, pH 7.0, 25 °C, with 0.5 μM MPO in the presence or absence of 1 mM GSH. The reaction was started by adding 0.5 mM H_2O_2. The reactions were monitored with a Clark-type oxygen electrode (Yellow spring instruments 5300A, Cincinnati, OH, USA).

2.6. GSH depletion during the oxidation of the ortho-methoxy-catechols

The reaction medium was composed of 1 mM methoxy-catechols, 1 mM hydrogen peroxide, 0.5 μM MPO and 1 mM GSH in 50 mM phosphate buffer pH 7.0 at 25 °C and a final volume of 1 ml. After a fixed interval, 20 μg/ml catalase was added to stop the reaction and aliquots were removed to measure the concentration of GSH by the DTNB method [18].

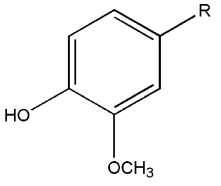
2.7. Ovalbumin's SH groups depletion during the oxidation of the ortho-methoxy-catechols

Egg white was used as a source of ovalbumin and the total SH groups measured using the Ellman's reagent adapted to determination in ovalbumin [19]. The eggs were obtained from a local supermarket. Reagent grade chemicals were used to prepare the following: Tris-glycine buffer (0.1 M tris-(hydroxymethyl)-aminomethane (tris), 0.1 M glycine, and 4 mM ethylenediamine-tetraacetic acid disodium salt, pH 8.0); 5% sodium dodecyl sulfate in tris-glycine buffer (denoted SDS-tris-glycine); Ellman's reagent (4 mg/ml DTNB in tris-glycine buffer). 0.1% solution of egg white was prepared in SDS-tris-glycine and centrifuged for 5 min at 1000 × *g*. For the determination of total SH groups, 500 μl of the protein solution was mixed with 500 μl of SDS-tris-glycine buffer and 10 μl of Ellman's reagent. The reaction mixture was incubated for 15 min at 40 °C in a water bath to allow the protein to unfold and all SH groups to be accessible to DTNB. Finally, the absorbance was measured at 412 nm against a reagent blank [19]. For testing the reactivity of the methoxy-catechol radicals with SH groups, the above protein solution was previously incubated with these compounds (1 mM) in the presence of MPO (0.2 μM), and H_2O_2 (0.1 mM) for 10 min, at room temperature. Then, the remaining SH groups were measured as above.

3. Results

Apocynin and its analogous *ortho*-methoxy-substituted catechols, bearing different substituents at the para position related to the hydroxyl group, were studied as inhibitors of lucigenin-dependent chemiluminescence elicited by opsonized zymosan-activated neutrophils. The substituents that replaced the acetyl group of apocynin were selected as they could exert an electron-donating or electron-withdrawing electronic effect in the aromatic ring [20]. Hence, 4-ethylguaiacol (ethyl) and 4-methylguaiacol (methyl) are examples of *ortho*-methoxy-substituted catechols bearing an additional electron-donating group (MC-D), whereas apocynin (apo), vanillin (van), 4-cyanoguaiacol (cyano), and 4-nitroguaiacol (nitro) are representatives of *ortho*-methoxy-substituted catechols bearing an additional electron-withdrawing group (MC-W) (Fig. 1).

The results in Table 1 show that the MC-D derivatives were significantly weaker inhibitors of the activation of NADPH oxidase when compared to MC-W. These inhibitory effects were not the result of cytotoxic effect on neutrophil as checked by trypan blue exclusion assay (not shown) or direct scavenging action upon superoxide anion, as checked in a cell-free hypoxanthine/xanthine oxidase assay (not shown). To confirm these results, we performed additional experiments whereby the inhibitory potency of the *ortho*-methoxy-substituted catechols was studied via a direct action on the oxygen uptake elicited by stimulated neutrophils. In



where R:	CH_3	4-methylguaiacol (Methyl)	MC-D
	CH_2CH_3	4-ethylguaiacol (Ethyl)	
	$COCH_3$	apocynin (Apo)	MC-W
	COH	vanillin (Van)	
	CN	4-cyanoguaiacol (Cyano)	
	NO_2	4-nitroguaiacol (Nitro)	

Fig. 1 – Molecular structure of the *ortho*-MC-D and *ortho*-MC-W.

Table 1 – Relative potency of the *ortho*-methoxy-substituted catechols derivatives as inhibitors of the activation of NADPH oxidase

Methoxy-catechol	IC 50
Apocynin	8.0 ± 3.0
Vanillin	7.6 ± 1.0
4-Nitroguaiacol	17.7 ± 1.1
4-Cyanoguaiacol	10.9 ± 2.9
4-Methylguaiacol	78.0 ± 10.0
4-Ethylguaiacol	155.1 ± 7.0

The reaction mixture was composed of neutrophils, lucigenin, opsonized zymosan and the methoxy-catechols. See material and methods for details. The results are mean and SEM of triplicates of three experiments using blood of different healthy donors. $^{*}P < 0.05$ relative to the experiment using apocynin (One way analysis of variance and Dunnett multiple comparison test).

agreement with the lucigenin assay, the MC-D derivatives were less effective inhibitors (Fig. 2).

Since the presence of electron-donating or electron-withdrawing substituents in the aromatic ring of phenolic compounds have a direct effect in their reduction potential [21], we studied the reactivity of the methoxy-catechols with peroxidase and the effect of addition of GSH. In these experiments, the methoxy-catechols were incubated with H_2O_2 and MPO for 10 min in the presence or absence of GSH. The remaining concentration of the methoxy-catechols was measured by HPLC. Fig. 3 shows that all *ortho*-methoxy-substituted catechols were promptly oxidized by MPO/H_2O_2, however, when these oxidations were performed in the

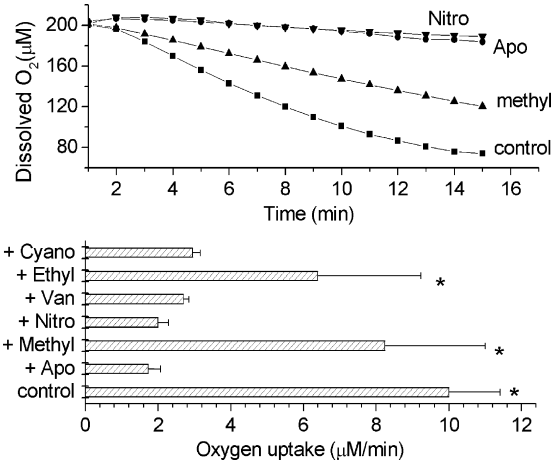


Fig. 2 – Relative potency of the *ortho*-methoxy-substituted catechols derivatives as inhibitors of the activation of NADPH oxidase in activated neutrophils assessed by oxygen uptake. The reaction mixture was composed of neutrophils, opsonized zymosan, and the methoxy-catechols. (Above) Typical profile of oxygen uptake in the presence or absence of the methoxy-catechols. (Below) Inhibition of the oxygen uptake provoked by the methoxy-catechols. See Section 2 for details. The results are triplicates. $^{*}P < 0.05$ relative to the experiment using apocynin (one way analysis of variance and Dunnett multiple comparison test).

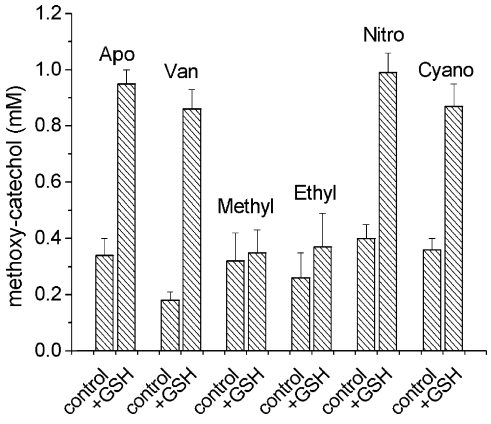


Fig. 3 – The effect of GSH on MPO-catalyzed oxidation of *ortho*-methoxy-substituted catechols. The substrates (1 mM) were incubated during 10 min at 25 °C in 50 mM phosphate buffer, pH 7.0, containing 0.5 μM MPO, 0.5 mM H_2O_2 (controls) or in the presence of 1 mM GSH. The reaction was started by adding hydrogen peroxide. The remaining concentration of the methoxy-catechols was measured by HPLC (see Section 2). Data represent at least three experiments.

presence of GSH, the results were diverse. Indeed, the oxidation of the MC-W derivatives, but not for MC-D, was prevented when GSH was added to the reaction medium. As GSH is a poor substrate for MPO and should not compete with the phenolic compounds [22,23], these HPLC results were indicative that this thiol compound could be able to recycle the methoxy-catechols by reducing back the radical generated during the oxidation of the MC-W derivatives. The confirmation was obtained by measuring the depletion of GSH and the involvement of glutathionyl radicals ($GS^{\bullet}$) during the reactions. The generation of $GS^{\bullet}$ during the oxidation of the methoxy-catechol by MPO/H_2O_2 was evaluated by oxygen uptake assay [24]. Fig. 4 depicts the oxygen uptake provoked by the addition of GSH during oxidation of the methoxy-catechols. As control, the lack of any component, including MPO, H_2O_2, GSH, and methoxy-catechols prevented the oxygen uptake from taking place (not shown). In agreement with the previous results, only for the MC-D derivatives, the addition of GSH did not provoke oxygen consumption. Similar results were observed by measuring the depletion of GSH when methoxy-catechols were oxidized by MPO/H_2O_2 (Fig. 5).

To reinforce our proposal of the involvement of $GS^{\bullet}$ during the peroxidase catalyzed oxidation of the methoxy-catechols, the effect of the pH and concentration GSH was studied regarding the efficiency of oxygen uptake. Fig. 6 shows that the efficiency of oxygen uptake is higher at an alkaline pH, which would favor the formation of glutathionyl anion (GH^{-}) (Scheme 1, reaction 4). Similarly, a higher concentration of GSH would favor the formation of $GH^{\bullet}$ (Scheme 1, reaction 3), instead of dimerization (Scheme 1, reaction 2) [14].

The capacity of the radicals of the MC-W derivatives to react with thiol compounds was extended to SH residues of ovalbumin, which was used here as a model to access the capacity of these methoxy-catechols radicals to oxidize SH

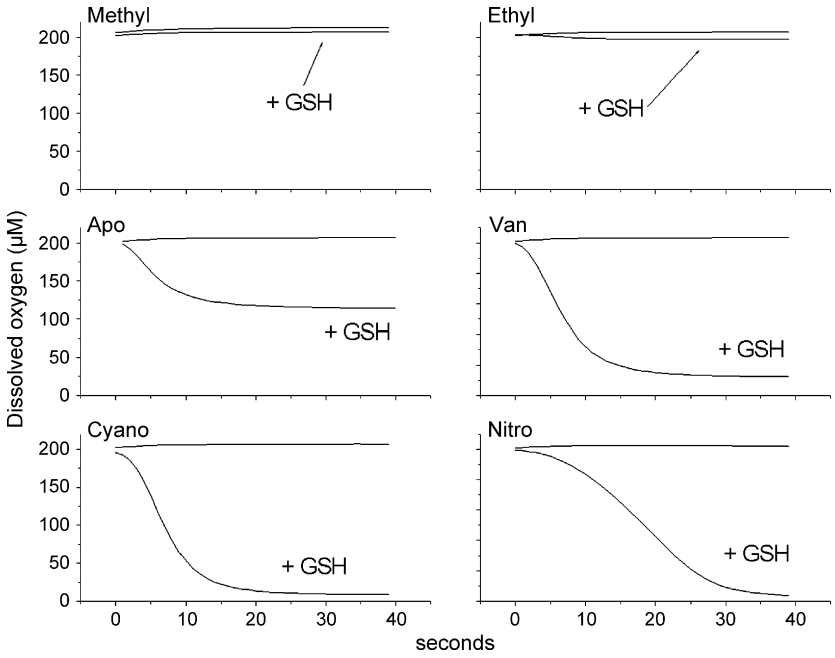


Fig. 4 – Time course of oxygen uptake during oxidation of *ortho*-methoxy-substituted catechols. The compounds (1 mM) were incubated at 25 °C in 50 mM phosphate buffer, pH 7.0, containing 0.5 μM MPO and 0.5 mM hydrogen peroxide in the absence or presence of 1 mM GSH. The reaction was started by addition of the hydrogen peroxide. The buffer solution was initially saturated with air (200 mM) by stirring the opened container for 5 min. Data represent at least three experiments.

groups in proteins (Fig. 7). In these experiments, the compounds were incubated with ovalbumin in the presence of MPO and H_2O_2. Then, the relative amount of reduced SH was quantified using an adapted Ellman's method [19].

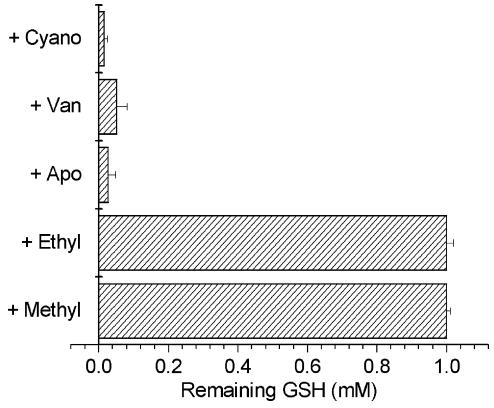


Fig. 5 – Oxidation of GSH during MPO-catalyzed oxidation *ortho*-methoxy-substituted catechols. Methoxy-catechols (1 mM) were incubated during 2 min at 25 °C in 50 mM phosphate buffer, pH 7.0, containing 0.5 μM MPO, 0.5 mM hydrogen peroxide and 1 mM GSH. The remaining concentration of GSH was measured removing aliquots, adding catalase (20 μg/mL) to stop the reaction and the supernatant assayed by the DTNB method [19]. The nitro derivative was not used in this assay, since its native absorbance interferes in the DTNB assay. The results are mean and standard deviation (S.D.) of triplicates.

4. Discussion

A huge number of natural antioxidants are able to scavenge the secondary superoxide's oxidant species such as H_2O_2, HOCl, $HO^{\bullet}$, $ONOO^{-}$. However, these natural antioxidants are, in their majority, unspecific and do not inhibit the production of superoxide. Apocynin seems to be an exception. Indeed, as an antioxidant, it is limited compared to compounds such as quercetin, resveratrol, curcumin, etc., but it is the only natural phenolic compound for which there is substantial evidence of its action as an inhibitor of NADPH oxidase [12]. But, why apocynin? It is a quite simple molecule! What property does it have that is absent in more sophisticated polyphenols? This is the main question addressed here.

The use of apocynin as an inhibitor of the activation of the NADPH oxidase complex is based on the inhibition of the assembly process, as the migration of the p47phox component to the membrane is impeded in its presence [15]. The same property has been reported for *N*-ethylmaleimide and oxidation products of 1-naphthol. The explanation for the effects of these compounds is based on the generation of electrophilic quinones that conjugate with essential thiol residues, present in the cytosolic component p47phox via Michael addition [25,26]. For apocynin, the inhibition of the migration of the cytosolic component p47phox does not match this mechanism. In fact, others and we have demonstrated that apocynin or its dimer oxidation product does not conjugate with GSH [13,14]. Additionally, we found that the apocynin radical generated during its peroxidase-catalyzed oxidation is able to oxidize thiol compounds. Based on this property, we proposed that this could be a pathway by which

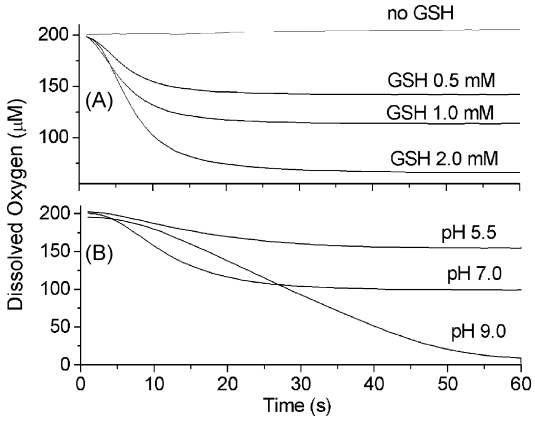


Fig. 6 – Effect of the concentration of GSH and of the pH on efficiency of oxygen uptake during the oxidation of apocynin. (A) The compounds (1 mM) were incubated at 25 °C in 50 mM phosphate buffer, pH 7.0, containing 0.5 μM MPO and 0.5 mM hydrogen peroxide in the absence or presence GSH. (B) The concentration of GSH was kept constant (1 mM). The reactions were started by addition of the hydrogen peroxide. The buffer solution was initially saturated with air (200 mM) by stirring the opened container for 5 min. Data represent at least three experiments.

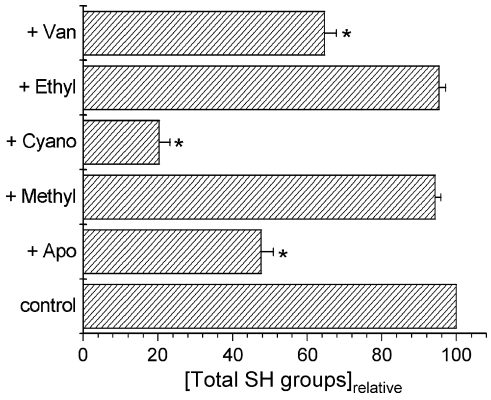
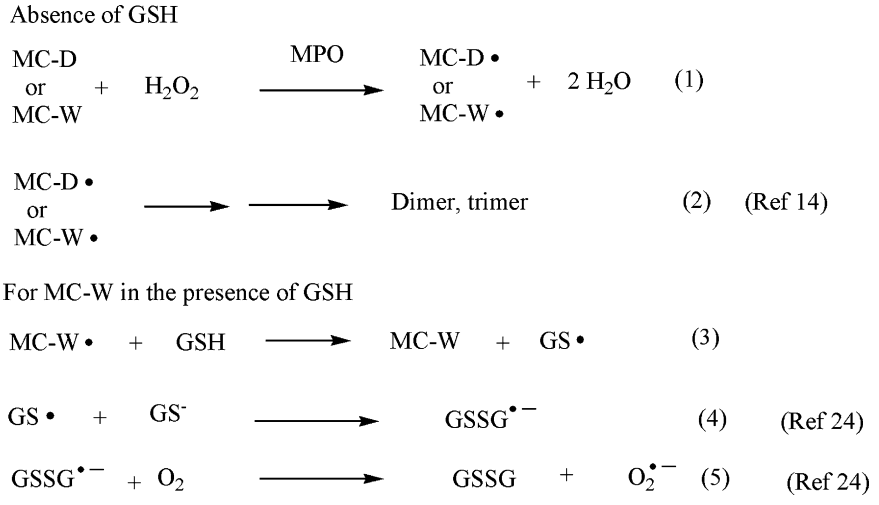


Fig. 7 – Oxidation of ovalbumin's SH groups during MPO-catalyzed oxidation of *ortho*-methoxy-substituted catechols. Methoxy-catechols (1 mM) were incubated during 10 min at room temperature with albumin solution, 0.5 μM MPO, 0.5 mM hydrogen peroxide. The remaining concentration of SH was measured by the adapted Ellman's method. See Section 2 for further details. The nitro derivative was not used in this assay, since its native absorbance interferes in the DTNB assay. The results are mean and SD of triplicates. *P < 0.05 relative to the control (one way analysis of variance and Dunnett multiple comparison test).

apocynin could interact with thiol residues of the cytosolic component of the NADPH oxidase [14]. Corroborating this hypothesis, apocynin markedly decreases the intracellular reduced/oxidized glutathione ratio (GSH/GSSG) in stimulated monocytes [15] and the addition of β-mercaptoethanol prevents the inhibitory effect of apocynin in activated neutrophils [27]. Here, we advance in this proposal, by demonstrating that there is a close relationship between the reactivity of the *ortho*-methoxy-substituted catechols with SH groups and their potency as inhibitors of the assembling of the NADPH oxidase complex. Scheme 1 depicts the putative mechanism for the involvement of GSH during the oxidation of the methoxy-catechols.

We found that the MC-D derivatives, which were not able to oxidize GSH or the cysteine residues of ovalbumin during their MPO-catalyzed reaction, showed weaker capacity as NADPH oxidase inhibitors. We propose that the decreased efficiency of the MC-D might be related to their lower reduction potential compared to the MC-W derivatives [28]. Thus, the radicals of MC-D would not be able to oxidize essential SH groups of the NADPH oxidase complex. In agreement with our hypothesis, only flavonoids with reduction potential higher than 850 mV



Scheme 1 – Proposal for the involvement of GSH during MPO-catalyzed oxidation of the methoxy-catechols. *Ortho*-MC-D and *ortho*-MC-W.

(the redox potential for the couple GS^{-}/GS) were able to oxidize GSH when these compounds were studied regarding their capacity of generating superoxide, via $GS^{\bullet}$, during horseradish peroxidase-catalyzed oxidation [29]. Here, our results demonstrated, clearly, that GSH and the cysteine residues of ovalbumin were able to reduce the radicals generated during the oxidation of MC-W, but not for the MC-D derivatives. In others words, the decrease in the reduction potential, caused by the additional electron-donating group, could impair the action of the MC-D derivatives as NADPH oxidase inhibitors.

In conclusion, we suggest that the capacity of apocynin and analogous methoxy-catechols as inhibitors of the assembling process during NADPH oxidase activation could be related to the oxidation of essential SH groups of the cytosolic fraction of this multienzyme complex. This property must be behind the necessity of oxidation of apocynin during its effect [13]. Considering that all methoxy-catechols studied here are quite similar molecules regarding their liposolubility, the difference observed cannot be explained by the accessibility to the intracellular medium. In the same way, no major difference was observed regarding the reactivity with peroxidase, a criteria that have been reported as relevant to apocynin action as NADPH oxidase inhibitor. Hence, the only difference is the lower reduction potential of the methyl and ethyl derivatives which makes these compounds unable to oxidize SH groups. Thus, we propose that this chemical property of the methoxy-catechol should be taken into account in the design of new and specific inhibitors of the NADPH oxidase complex. Among the other derivatives that were employed here, vanillin deserves further studies. Vanillin is an extremely common flavoring agent. This feature is indication of its low toxicity. Since vanillin was as efficient as apocynin, we suggest that it should be studied as a NADPH oxidase inhibitor in animal models.

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