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Apocynin and Related Methoxy-Catechols as Inhibitors of Neutrophil-NADPH Oxidase in LPS-Activated Whole Blood

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Abstract: Apocynin has been extensively used as a non-toxic inhibitor of the multi-enzyme NADPH oxidase complex in phagocytic and non-phagocytic cells. Here we investigate the potency of apocynin (4-acetylguaiacol) and the related substituted methoxy-catechols, 4-nitroguaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-cyanoguaiacol and eugenol as inhibitors of NADPH oxidase of neutrophils stimulated by Lipopolysaccharide (LPS) in whole blood. These compounds were chosen as they could reveal the structure-activity relationship between the presence of electron-donating and electron-withdrawing substituents groups on the methoxy-catechol moiety and their effect as NADPH oxidase inhibitor. The whole blood experimental model was chosen as it is close to physiological conditions and could reveal the real potency of these drugs. The NADPH oxidase activation was assessed by the Nitro Blue Tetrazolium (NBT) cytochemical assay. We found that apocynin (methoxy-catechol with an acetyl group) was an efficient NADPH oxidase inhibitor in whole blood. The presence of stronger electron-withdrawing substituents groups, represented by the compounds 4-nitroguaiacol and 4-cyanoguaiacol diminished significantly their potency as NADPH-oxidase inhibitor. However, the presence of electron-donating groups represented by 4-methylguaiacol, 4-ethylguaiacol and eugenol were poor inhibitors compared with the other methoxy-catechols. A correlation was noted between the electron-donating and electron-withdrawing character of the substituent groups and the power to inhibit NADPH oxidase.

Key words: Apocynin, methoxy-catechols, NADPH oxidase, respiratory burst, neutrophil

INTRODUCTION

Apocynin (4-hydroxy-3-methoxyacetophenone) has been extensively used as a non-toxic inhibitor of the multi-enzyme NADPH-oxidase complex in phagocytic and non-phagocytic cells (Muranaka *et al*., 2005; Lafeber *et al*., 1999; Barbieri *et al*., 2004; Al-Shabrawey *et al*., 2005). As such, accumulating *in vivo* experimental studies have shown the promising potential of this molecule as a drug for inflammatory disorders. In this connection, apocynin inhibited ovalbumin-induced airway hyperresponsiveness in sensitized mice (Muijsers *et al*., 2001) prevented the increase in excretion of H_2O_2, lipid peroxidation products and proteins in diabetic rats (Asaba *et al*., 2005) and prevented and reversed adrenocorticotropic hormone-induced hypertension in the rat (Zhang *et al*., 2005).

Considering that apocynin could be a useful drug against systemic inflammation where the peripheral blood leukocytes are stimulated (Martins *et al*., 2003) here we evaluated the power of apocynin and related substituted methoxy-catechols to inhibit the NADPH oxidase of neutrophils in a whole-blood assay. This experimental model was used as it resembles the physiological condition and could thus reveal the real potency of these drugs. As apocynin is supposed to be metabolized by the neutrophil peroxidase (myeloperoxidase) to exert its effect (Johnson *et al*., 2002) we studied the correlation between the potential reactivity of these methoxy-catechols with peroxidase and their potency as inhibitors of NADPH oxidase.

MATERIALS AND METHODS

Chemicals: Apocynin, vanillin, vanillic acid, methyl vanillate, 4-nitroguaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-cyanoguaiacol, eugenol, N,N-Dimethylformamide (DMF), lipopolysaccharide (LPS, *Escherichia coli* 026:B6) and Nitro Blue Tetrazolium (NBT) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, USA). All other reagents used for buffer preparations were of analytical grade.

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Solutions: LPS (1 mg mL^{-1}) and NBT (0.1%) were dissolved in PBS. Stock solutions (1 M) of methoxy-catechols were prepared in DMF, stored at 4°C and used within one week.

NBT cytochemical assay: Unless otherwise indicated, the drugs were pre-incubated with 300 μL whole blood and 1 μg mL^{-1} LPS for one hour at room temperature. Then, 300 μL NBT (0.1%) was added and the mixture incubated for 30 min at room temperature. The reaction mixtures were homogenized at 10 min intervals. The blood smears were prepared and stained with Leishman's solution. One hundred neutrophils were counted and scored as NBT-positive cells when well-defined dark spots of formazan precipitate were found in the cytoplasm (Brown, 1993). Negative controls (spontaneous activation) were produced by omitting the stimuli and positive controls by omitting the methoxy-catechols. The final concentration of the solvent (DMF) used to dissolve the methoxy-catechols in the reaction mixture was 0.5%. The same amount of DMF was present in the controls.

RESULTS

We tested apocynin and related substituted methoxy-catechols as inhibitors of the neutrophil multi-enzyme NADPH oxidase complex in whole blood. NADPH oxidase activation was assessed by the cytochemical assay based on the reduction of Nitroblue Tetrazolium (NBT) by the superoxide anion with consequent precipitation of a formazan product into the cytoplasm of the neutrophils (Brown, 1993). Neutrophils were scored positive when dark spots of precipitate were clearly seen in the cytoplasm. The neutrophil counting and scoring were performed by three different analysts, using blood samples from different donors.

Figure 1a shows that the activation level of the NADPH oxidase was a function of the reaction time. In these experiments whole blood was mixed with LPS and NBT without pre-incubation and the blood smears were prepared in the indicated time. We also tested the effect of pre-incubation of the blood with LPS (Fig. 1b). Taking into account the results above, a pre-incubation with LPS for one hour and a reaction time of 30 min after addition of NBT were fixed for subsequent studies. Figure 2 shows the percentage of NBT-positive neutrophils for stimulated and non-stimulated whole blood and the dose-response effect of apocynin.

Besides apocynin, several polyphenols have been described as inhibitors of the oxidative burst of isolated neutrophils in different experimental models and at various potencies (Selloum *et al*., 2001; Deby *et al*., 2005).

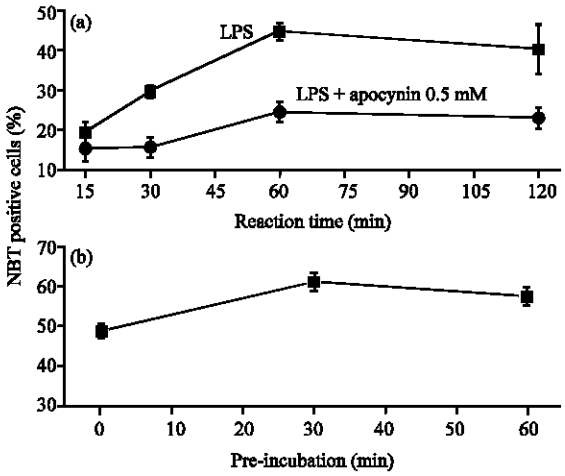

Fig. 1: (a) Time-dependent activation of NADPH oxidase in whole blood. 300 μL whole blood, 300 μL NBT (0.1%) and 1 μg mL^{-1} LPS in the presence or absence of 0.5 mM apocynin. The blood smears were prepared at the indicated time. (b) Effect of pre-incubation with LPS. The whole blood was pre-incubated with 1 μg mL^{-1} LPS at the indicated time. Then 300 μL NBT (0.1%) was added and the blood smears prepared after an additional 30 min. The results are mean and SEM of triplicates

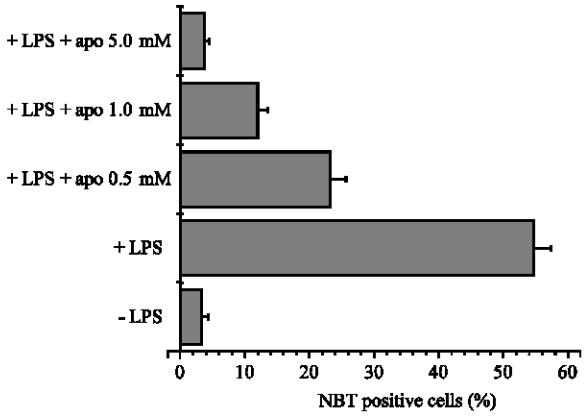

Fig. 2: Apocynin as inhibitor of NADPH oxidase. Percentage of NBT-positive neutrophils for LPS-stimulated and unstimulated whole blood and the dose-response effect of apocynin. Data represent the mean and SEM of 6 experiments using blood from different donors

For apocynin, it is reported that it has to be catalytically oxidized by peroxidases to exert its inhibitory effect (Johnson *et al*., 2002). In this connection, it should be mentioned that the reactivity of phenol derivatives with peroxidase/H_2O_2 depends strongly on the presence of

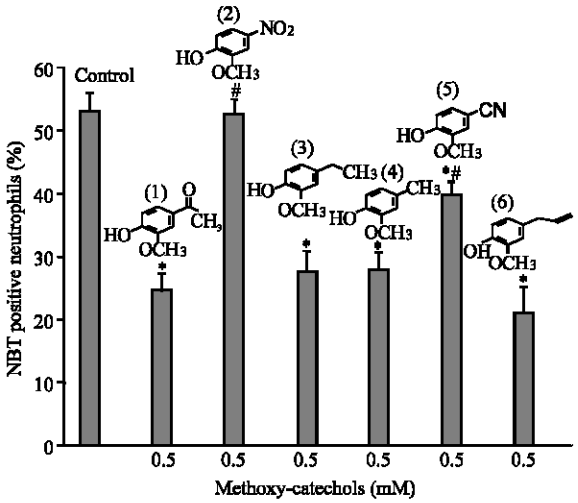

Fig. 3: The presence of electron-donating or electron-withdrawing substituents on methoxy-catechols and their effect in the inhibitory potency of NADPH oxidase in whole blood. LPS-stimulated neutrophils (control), (1) apocynin, (2) 4-nitroguaiacol, (3) 4-ethylguaiacol, (4) 4-methylguaiacol, (5) 4-cyanoguaiacol and (6) eugenol. Data represent the mean and SEM of 6 experiments using blood from different donors. * $p< 0.05$ compared to control, # $p< 0.05$ compared to apocynin (One Way ANOVA). See Methods section for further details

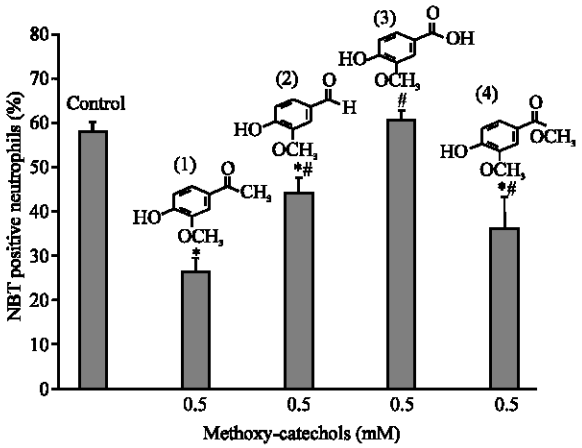

Fig. 4: Effect of the hydrophobicity of the methoxy-catechols on the inhibitory potency of NADPH oxidase in whole blood. LPS-stimulated neutrophils (control), (1) apocynin, (2) vanillin, (3) vanillic acid and (4) methyl vanillate. Data represent the mean and SEM of 6 experiments using blood from different donors. * $p< 0.05$ compared to control, # $p< 0.05$ compared to apocynin (One Way ANOVA). See Methods section for further details

substituent groups that may increase or decrease the reduction potential of these molecules (Job and Dunford, 1976). In brief, electron-donating groups on the benzene ring tend to provoke an increase in its reactivity with the peroxidase system. On the other hand, electron-withdrawing substituents tend to weaken this reactivity (Dunford and Adeniran, 1986). To make the comparison more quantitative we used the physico-chemical Hammett constants for *para* substituents (σ_p), which are positive for electron-withdrawing groups and negative for electron-donating groups (March, 1992). Based on these data, a test was performed for correlation between the NADPH-oxidase inhibitory potency of the methoxy-catechols and their reactivity with peroxidase/H_2O_2, by selecting a panel of methoxy-catechols in which the acetyl group of apocynin was replaced by groups that are supposed to alter their reactivity. In agreement with expectations, it was found that the nitro derivative, with the strongest electron-withdrawing group (NO_2, σ_p = +0.81), was completely inactive as an inhibitor of NADPH oxidase and the cyano derivative (CN, σ_p = +0.62) was significantly less potent than apocynin ($COCH_3$, σ_p = +0.47). For methyl, ethyl and eugenol derivatives (CH_3, CH_2CH_3, $CH_2CH{=}CH_2$, σ_p = -0.13), which have electron-donating groups, the inhibitory power was similar to that of apocynin (Fig. 3).

Apocynin ($COCH_3$ σ_p = +0.47) was also compared with vanillin (CHO σ_p = +0.47), vanillic acid (COOH σ_p = +0.44) and methyl vanillate ($COOCH_3$ σ_p = +0.47). In these cases, the comparison might be related to the hydrophobicity of the compounds, since these groups have similar Hammett's constants. Figure 4 shows that vanillic acid was inactive. Although similar, apocynin was a significantly better inhibitor than vanillin or methyl vanillate.

DISCUSSION

The reduction of tetrazolium compounds or ferricytochrome c by the superoxide anion are among the most frequently used techniques for the specific monitoring of the generation of this reactive species (Dahlgren and Karlsson, 1999). The only problem with the NBT assay is the insolubility of the colored formazan product. In fact, this feature limits its use in quantitative assays since the formazan produced by isolated stimulated cells must be dissolved by adding solvents (Oez *et al.*, 1990). Nevertheless, it is exactly this property that makes the NBT technique a very specific and useful

cytochemical test to monitor NADPH oxidase activity of neutrophils in unstimulated and LPS-stimulated whole blood (Glasser and Fiederlein, 1990). In this case, a dark formazan precipitate can easily be visualized after normal hematological staining (Brown, 1993). Another advantage of this whole-blood/NBT technique, when assaying the effects of xenobiotics on neutrophils, is the close approximation to physiological conditions. Under these experimental conditions, the xenobiotic has to act in a complex medium, where reaction with endogenous metabolites or uptake by erythrocytes or other cells could all neutralize its effect on neutrophils. This characteristic could explain the diminished potency of apocynin as NADPH oxidase inhibitor in the whole blood model compared with neutrophil-isolated assays. Indeed, here the concentration of apocynin that provoked the inhibition of about 50% in the NADPH oxidase activity was about one hundred higher compared to studies were the neutrophil were isolated, 500 and 20 μM, respectively (Van den Worm *et al*., 2001).

Apocynin can be considered a prodrug as it has to be metabolized by the leukocyte enzyme Myeloperoxidase (MPO) to exert its effect. It has been demonstrated that the dimer product formed when apocynin is catalytically oxidized by peroxidase has a stronger effect than apocynin itself (Johnson *et al*., 2002). Similar findings were described for 1-naphthol, whose inhibition of NADPH oxidase was linked to the production of 1,4-naphthoquinone by reaction with H_2O_2 from the oxidative burst (Hart *et al*., 1990). These substances are supposed to react with essential thiol groups on the subunits of the multi-enzyme complex, preventing their assembly into the active complex. Our results are consistent with these ideas as the nitro and cyano derivatives were less effective inhibitors, which could be an indication of their lower reactivity with the neutrophil peroxidase. Unfortunately, there are no reported kinetic data on the reactivity of substituted methoxy-catechols with peroxidases that could corroborate our proposal, but a comparison can be made between p-methoxyphenol (OCH_3 σ_p = -0.28) and p-nitrophenol (NO_2 σ_p = +0.81). While the latter is completely unreactive, the p-methoxy derivative has an apparent rate constant of 5.4×10^6 $M^{-1}s^{-1}$ for the reaction with HRP/H_2O_2 (Dunford and Adeniran, 1986). However, the reactivity of the methoxy-catechol might not be the only factor, since no significant difference was observed between apocynin ($COCH_3$ σ_p = +0.47) and methyl, ethyl and eugenol derivatives (CH_3, CH_2CH_3, $CH_2CH = CH_2$ σ_p = -0.13), which are supposed to be more reactive than apocynin with peroxidase (Dunford and Adeniran, 1986).

We also found that the hydrophobicity of the methoxy-catechols seems to be a factor affecting their ability to inhibit NADPH oxidase. As shown here, vanillic acid was significantly less effective than its ester, methyl vanillate. This makes sense, as the transport of the acid form into the cells could be hindered by its relatively low lipid solubility, compared to the ester derivative. Additional experiments must be performed to elucidate why apocynin is a better inhibitor than vanillin or methyl vanillate, as these molecules have very similar hydrophobicity and reactivity.

CONCLUSION

We have found that the NBT cytochemical assay can be very useful in an *ex-vivo* LPS-stimulated whole-blood model to assess the effects of xenobiotics on NADPH oxidase activity. The potency of the methoxy-catechols seems to depend on their reactivity with peroxidase and hydrophobicity. Given that apocynin is a non-toxic substance and able to exert its effect on whole blood, it could be useful as a potential therapeutic agent for systemic inflammatory pathologies. This is the case for septic shock where peripheral blood leukocytes are stimulated (Martins *et al*., 2003).

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REFERENCES

Al-Shabrawey, M., M. Bartoli, A.B. El-Remessy, D.H. Platt, S. Matragoon, M.A. Behzadian, R.W. Caldwell and R.B. Caldwell, 2005. Inhibition of NAD(P)H oxidase activity blocks vascular endothelial growth factor overexpression and neovascularization during ischemic retinopathy. Am. J. Pathol., 167: 599-607.

Asaba, K., A. Tojo, M.L. Onozato, A. Goto, M. Quinn, T. Fujita and C.S. Wilcox, 2005. Effects of NADPH oxidase inhibitor in diabetic nephropathy. Kidney Int., 67: 1890-1998.

Barbieri, S.S., V. Cavalca, S. Eligini, M. Brambilla, A. Caiani, E. Tremoli and S. Colli, 2004. Apocynin prevents cyclooxygenase 2 expression in human monocytes through NADPH oxidase and glutathione redox-dependent mechanisms. Free Radic. Biol. Med., 37: 156-165.

Brown, B.A., 1993. Hematology: Principles and Procedures, (6th Edn.), Lea and Febiger, Philadelphia, London, pp: 141-143.

Dahlgren, C. and A.J. Karlsson, 1999. Respiratory burst in human neutrophils. Immunol. Methods, 232: 3-14.

Deby-Dupont, G., A. Mouithys-Mickalad, D. Serteyn, M. Lamy and C. Deby, 2005. Resveratrol and curcumin reduce the respiratory burst of Chlamydia-primed THP-1 cells. Biochem. Biophys. Res. Commun., 333: 21-27.

Dunford, H.B. and A.J. Adeniran, 1986. Hammett rho sigma correlation for reactions of horseradish peroxidase compound II with phenols. Arch. Biochem. Biophys., 251: 536-542.

Gerasimov, I.G. and D. Ignatov, 2004. Neutrophil activation *in vitro*. Tsitologia, 46: 155-158.

Hart, B.A., J.M. Simons, G.T. Rijkers, J.C. Hoogvliet, H. Van Dijk and R.P. Labadie, 1990. Reaction products of 1-naphthol with reactive oxygen species prevent NADPH oxidase activation in activated human neutrophils, but leave phagocytosis intact. Free Radic. Biol. Med., 8: 241-249.

Glasser, L. and R.L. Fiederlein, 1990. The effect of various cell separation procedures on assay of neutrophil function. Am. J. Clin. Pathol., 93: 662-669.

Job, D. and H.B. Dunford, 1976. Substituent effect on the oxidation of phenols and aromatic amines by horseradish peroxidase compound I. Eur. J. Biochem., 66: 607-614.

Johnson, D.K., K.J. Schillinger, D.M. Kwait, C.V. Hughes, E.J. McNamara, F. Ishmael *et al*., 2002. Inhibition of NADPH oxidase activation in endothelial cells by ortho-methoxy-substituted catechols. Endothelium, 9: 191-203.

Lafeber, F.P., C.J. Beukelman, E. van den Worm, van J.L. Roy, M.E. Vianen, J.A. van Roon, H. van Dijk and J.W. Bijlsma, 1999. Apocynin, a plant-derived, cartilage-saving drug, might be useful in the treatment of rheumatoid arthritis. Rheumatology (Oxford), 38: 1088-1093.

March, J., 1992. Advanced Organic Chemistry: Reactions Mechanism and Structure. (4th Edn.), John Wiley and Sons, New York, pp: 141-143.

Martins, P.S., E.G. Kallas, M.C. Neto, M.A. Dalboni, S. Blecher and R. Salomao, 2003. Upregulation of reactive oxygen species generation and phagocytosis and increased apoptosis in human neutrophils during severe sepsis and septic shock. Shock, 20: 208-212.

Muijsers, R.B., I. van Ark, G. Folkerts, A.S. Koster, A.J. van Oosterhout, D.S. Postma and F.P. Nijkamp, 2001. Apocynin and 1400 W prevents airway hyperresponsiveness during allergic reactions in mice. Br. J. Pharmacol., 134: 434-440.

Munakata, W., Q. Liu, T. Shimoyama, M. Sawaya, T. Umeda and K. Sugawara, 2003. Ecabet sodium attenuates reactive oxygen species produced by neutrophils after priming with bacterial lipopolysaccharides. Luminescence, 18: 330-333.

Muranaka, S., H. Fujita, T. Fujiwara, T. Ogino, E.F. Sato, J. Akiyama, I. Imada, M. Inoue and K. Utsumi, 2005. Mechanism and characteristics of stimuli-dependent ROS generation in undifferentiated HL-60 cells. Antioxid. Redox Signal, 7: 1367-1376.

Oez, S., E. Platzer and K. Welte, 1990. A quantitative colorimetric method to evaluate the functional state of human polymorphonuclear leukocytes. Blut, 60: 97-102.

Selloum, L., S. Reichl, M. Muller, L. Sebihi and J. Arnhold, 2001. Effects of flavonols on the generation of superoxide anion radicals by xanthine oxidase and stimulated neutrophil. Arch. Biochem. Biophys., 395: 49-56.

Van den Worm, E., C.J. Beukelman, A.J. Van den Berg, B.H. Kroes, R.P. Labadie and H. Van Dijk, 2001. Effects of methoxylation of apocynin and analogs on the inhibition of reactive oxygen species production by stimulated human neutrophils. Eur. J. Pharmacol., 433: 225-230.

Zhang, Y., M.M. Chan, M.C. Andrews, T.A. Mori, K.D. Croft, K.U. McKenzie, C.G. Schyvens and J.A. Whitworth, 2005. Apocynin but not allopurinol prevents and reverses adrenocorticotropic hormone-induced hypertension in the rats. Am. J. Hypertens., 18: 910-916.