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Effects of methoxylation of apocynin and analogs on the inhibition of reactive oxygen species production by stimulated human neutrophils

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Abstract

Owing to their multiple side effects, the use of steroidal drugs is becoming more and more controversial, resulting in an increasing need for new and safer anti-inflammatory agents. In the inflammatory process, reactive oxygen species produced by phagocytic cells are considered to play an important role. We showed that apocynin (4′-hydroxy-3′-methoxy-acetophenone or acetovanillone), a non-toxic compound isolated from the medicinal plant *Picrorhiza kurroa*, selectively inhibits reactive oxygen species production by activated human neutrophils. Apocynin proved to be effective in the experimental treatment of several inflammatory diseases such as arthritis, colitis and atherosclerosis. These features suggest that apocynin could be a prototype of a novel series of non-steroidal anti-inflammatory drugs (NSAIDs). So far, apocynin is mainly used in vitro to block NADPH oxidase-dependent reactive oxygen species generation by neutrophils. In order to get a better insight in what chemical features play a role in the anti-inflammatory effects of apocynin, a structure–activity relationship study with apocynin analogs was performed. We show here that especially substances with an additional methoxy group at position C-5 display enhanced anti-inflammatory activity in vitro. Our approach may lead to the development of more effective anti-inflammatory agents which are safe and which lack the side effects of steroids. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Apocynin; Neutrophil; NADPH oxidase; Reactive oxygen species; Respiratory burst

1. Introduction

Reactive oxygen species, produced by stimulated polymorphonuclear neutrophils, play an important role in host defence against invading microorganisms. Upon triggering, neutrophils start to consume a large amount of oxygen which is converted into reactive oxygen species, a process which is known as the respiratory or oxidative burst (Babior, 1978; Babior, 1995). Although reactive oxygen species formation by neutrophils may be a physiological response which is advantageous to the host, the process is certainly also disadvantageous since it may give rise to excessive tissue damage (Weiss, 1989; Malech and Gallin, 1987). Therefore, compounds that can interfere with reactive oxygen species production may be useful tools to prevent tissue destruction. In our search for inhibitors of reactive oxygen species production, we isolated apocynin (4′-hydroxy-3′-methoxy-acetophenone) from the roots of *Picrorhiza kurroa* by means of activity-guided isolation (Simons, 1989). Apocynin is a potent inhibitor of the superoxide-anion ($\cdot O_2^-$)-generating NADPH oxidase of stimulated human neutrophils (IC_{50}: 10 μM) (Simons et al., 1990; Simons, 1989). Additional interesting aspects of apocynin are its very low toxicity (LD_{50}: 9 g/kg upon oral administration to mice) (Gajewska et al., 1981) and the fact that it does not interfere with the other killing capacities of neutrophils, such as phagocytosis and intracellular killing (Stolk et al., 1994a).

In the recent literature, there is growing interest in apocynin as an anti-inflammatory agent. Although its full

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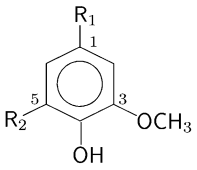
Table 1
Structures of apocynin, vanillin, and vanillic acid and their C-5 methoxylated derivatives

		R_1	R_2
Apocynin	(APO)	-$COCH_3$	H
Vanillin	(VAN)	-CHO	H
Vanillic acid	(VAC)	-COOH	H
Acetosyringone	(ACS)	-$COCH_3$	-OCH_3
Syringaldehyde	(SAL)	-CHO	-OCH_3
Syringic acid	(SAC)	-COOH	-OCH_3



spectrum of activity is not fully understood yet, in many laboratories apocynin is used under a wide variety of experimental conditions as a tool to inhibit neutrophil NADPH oxidase activity, thereby preventing the production of oxygen radicals (Zhou and Lai, 1994; Nishikawa et al., 1996; Suzuki et al., 1992; Van der Goes et al., 1998; Laperre et al., 1999; Salmon et al., 1998). Furthermore, structure–activity relationship studies have been performed to test a number of apocynin analogs and several suggestions have been put forward with regard to the impact of different substitutions on the benzene ring (Stuppner et al., 1995; Dorsch et al., 1994).

The possible beneficial effects of inhibitors of oxygen radical production in inflammatory processes prompted us to extend the number of apocynin analogs in order to substantiate the effect of the different functional groups in apocynin and related compounds.

Here, we report the ability of several analogs of apocynin that differ at positions C-1 and C-5 (Table 1) to inhibit reactive oxygen species production by human neutrophils, induced by two different stimuli: opsonized zymosan or phorbol 12-myristate 13-acetate (PMA) and measured as luminol or lucigenin-enhanced chemiluminescence.

2. Materials and methods

2.1. Reagents

Apocynin, vanillin, and vanillic acid were obtained from Carl Roth (Karlsruhe, Germany). Before use, apocynin was purified by recrystallization from water. Acetosyringone, syringaldehyde, and syringic acid were obtained from Fluka Chemika (Buchs, Switzerland). Zymosan A, phorbol 12-myristate 13-acetate (PMA), 5-amino-2,3-dihydro-1,4-phthalazine-dione (luminol), bis-*N*-methylacridinium nitrate (lucigenin), hypoxanthine, xanthine oxidase, superoxide dismutase, propidium iodide, 5-carboxy fluorescein diacetate (CF DA) were all purchased from Sigma (St. Louis, MO, USA). Hank's balanced salt solution (HBSS) was obtained from Life Technologies (Paisley, Scotland).

2.2. Measuring reactive oxygen species production

Neutrophils were isolated from venous blood from healthy volunteers (Bloedbank Midden-Nederland, Utrecht, The Netherlands) as described by Verbrugh et al. (1978). In white 96-well, flat-bottom microtiter plates (Costar, Badhoevedorp, The Netherlands), compounds were serially diluted to final volumes of 50 μl. To each well, 50 μl of a neutrophil suspension ($1 \cdot 10^7$ cells/ml) and 50 μl luminol (120 μM) or lucigenin (400 μM) solutions were added. The neutrophils were triggered by adding 50 μl of opsonized zymosan A (final concentration: 200 μg/ml) or PMA (final concentration 10 nM), and chemiluminescence was monitored every 2 min for 0.5 s during a 30-min period using a Titertek Luminoskan luminometer (TechGen International, Zellik, Belgium). Peak levels were used to calculate the activity of test samples in relation to their corresponding controls (identical incubations without test sample). Experiments were performed in Hank's balanced salt solution (HBSS) buffered at pH 7.35 with $NaHCO_3$ and supplemented with 0.1% (w/v) gelatin to avoid cell aggregation (HBSS-gel). Opsonized zymosan was obtained by incubation of washed commercial zymosan A with 1:10 diluted human pooled serum at 37 °C for 30 min. After washing, the opsonized product was resuspended in HBSS (final concentration: 0.8 mg/ml). PMA was dissolved in dimethyl sulfoxide (DMSO), stored at −20 °C, and diluted in HBSS to a final concentration of 40 nM immediately before use.

2.3. Inhibition of myeloperoxidase release

Neutrophils were forced to release myeloperoxidase by incubating them (50 μl of 10^7 cells/ml in HBSS) with 100 μl opsonized zymosan (0.83 mg/ml in HBSS) at 37 °C for 15 min in U-well microtiter plates (Greiner Labortechnik, Nürtingen, Germany). After centrifugation ($250 \times g$; 4 °C; 5 min), 100 μl of the supernatants was transferred to 96-well flat-bottom microtiter plates (Greiner Labortechnik) and myeloperoxidase activity was assessed by a modified version of the method described by Henson et al. (1978). Briefly, supernatants were mixed with 200 μl of 50 mM potassium phosphate buffer (pH 6.0) containing 3,3′-dimethoxybenzidine (0.7 mM) and H_2O_2 (0.17 mM), and absorbance at 450 nm was read every 2 min during a 20-min period using an automatic enzyme-linked immunosorbant assay (ELISA) reader (SLT Labinstruments, Salzburg, Austria). Test samples were added to incubation mixtures containing neutrophils and opsonized zymosan or supernatants to determine the effects on both myeloperoxidase release and/or myeloperoxidase activity. Since myeloperoxidase release starts rapidly and slows down after a few minutes, we calculated our data as the slope of the time curve in the linear part of the ascending limb. Furthermore, non-specific release of myeloperoxidase, e.g. as consequence of cell rupture, was excluded

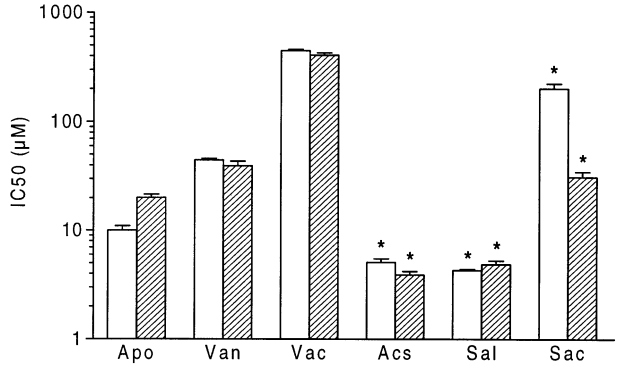


Fig. 1. Inhibitory effects of test substances on luminol (open bars) or lucigenin (hatched bars)-enhanced chemiluminescence response of opsonized zymosan-triggered human neutrophils ($n=9$). Neutrophils were stimulated and reactive oxygen species production was measured every 2 min for 30 min. Chemiluminescence was detected as relative light units using luminol or lucigenin as enhancing agents. For abbreviations of compounds, see Table 1. Values are depicted as mean IC_{50} values ± standard errors of the mean (S.E.M.). * Significantly different from the corresponding molecule without the additional methoxy group, $P<0.05$.

by determining the viability of the neutrophils with and without opsonized zymosan incubation.

2.4. Scavenging of superoxide anion

In white 96-well, flat-bottom microtiter plates, test compounds were serially diluted in phosphate-buffered saline (pH 7.4) to a final volume of 50 μl. Hypoxanthine (50 μl; 4 mM), lucigenin (50 μl; 0.4 mM), and either buffer (PBS; 25 μl) or superoxide dismutase (25 μl; 80 U/ml) were added. Superoxide anion production was initiated by the addition of 25 μl of xanthine oxidase (80 mU/ml) and the chemiluminescence signal generated was monitored every min for 0.5 s during a 15-min period using a Titertek Luminoskan luminometer. Activities of test compounds were calculated using the superoxide dismutase-inhibitable part of the chemiluminescence signal.

2.5. Cytotoxicity

A stock solution of 5-carboxyfluorescein diacetate (CFDA; 10 mg/ml) in acetone was prepared and stored at − 20 °C. Prior to use, this stock solution was diluted 1:1000 in the appropriate buffer. Propidium iodide (1.5 mg) was dissolved in 10 ml of phosphate-buffered saline containing 2.5% quenching ink, 5% w/v EDTA, and 8 mg bovine serum albumin. Neutrophils were labeled (20 °C, 15 min) with the vital stain CFDA (10 μg/ml), washed, and resuspended to a concentration of 10^7 cells/ml. 100-μl amounts of this cell suspension were incubated with equal volumes of graded amounts of sample at 37 °C for 15 min, whereafter the cells were washed and stained with 25 μl of propidium iodide/ink solution for the detection of dead cells. The percentage of dead cells was determined using a fluorescence microscope (Fluovert, Leitz, Wetzlar, Germany).

2.6. Statistical analysis

Student's paired *t*-test was used to evaluate the statistical significance of differences. Differences with *P* values <0.05 were considered statistically significant.

3. Results

3.1. Inhibition of luminol- and lucigenin-enhanced chemiluminescence by opsonized zymosan- and PMA-stimulated human neutrophils

The effects of substitution with a methoxy group at C-5 of apocynin, vanillin, and vanillic acid on opsonized zymosan- or PMA-induced chemiluminescence by human neutrophils were studied. For opsonized zymosan stimulation, it was clearly shown that methoxylation at C-5 (resulting in acetosyringone, syringaldehyde, and syringic acid, respectively) led to a significantly increased inhibitory activity on luminol- as well as lucigenin-enhanced chemiluminescence (Fig. 1; Table 1).

With PMA as stimulus, however, increased activities were observed for luminol-enhanced, but not for lucigenin-enhanced, chemiluminescence (Fig. 2).

3.2. Inhibition of myeloperoxidase release

Myeloperoxidase is an enzyme released from azurophilic granules by stimulated neutrophils and converts H_2O_2 into hypohalites (McRipley and Sbarra, 1967). These hypohalites are very reactive metabolites and can only be detected with luminol as enhancer (Dahlgren and Stendahl, 1983). This suggests that deactivation and/or decreased release of myeloperoxidase may be involved in the inhibitory effects on luminol-enhanced chemiluminescence. To exclude that test substances inactivate myeloperoxidase and/or inhibit its

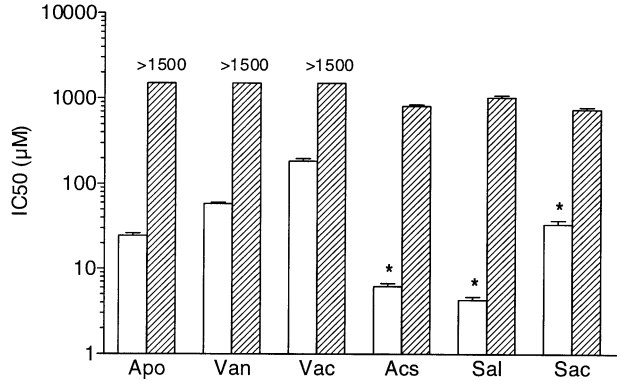


Fig. 2. Inhibitory effects of test substances on luminol (open bars)- or lucigenin (hatched bars)-enhanced chemiluminescence response of PMA-triggered human neutrophils ($n=9$). * Significantly different from the corresponding molecule without the additional methoxy group, $P<0.05$.

release, a myeloperoxidase inhibition assay was performed (Table 2). Syringic acid and vanillic acid were the only substances that interfered with myeloperoxidase-mediated effects. The other compounds did not affect myeloperoxidase release and/or activity. No differences in neutrophil viability between opsonized zymosan-incubated and control mixtures were observed.

3.3. Scavenging of superoxide anion

Scavenging is the term used to describe the interference of a test substance with reactive oxygen metabolites rather than with the production thereof. To distinguish between inhibition and scavenging, a superoxide anion scavenging assay was carried out in which superoxide anions are generated in a cell-free hypoxanthine/xanthine-oxidase system. All compounds tested were devoid of superoxide anion scavenging activity (data not shown) indicating that they act at the level of the O_2^--generating NADPH oxidase complex. Furthermore, none of the compounds exhibited direct quenching effects on luminol or lucigenin signals.

3.4. Calculated lipophilicity

The lipophilicity (log *P* value) of the compounds was calculated using the method of Hansch and Leo (1979). Log *P* values of vanillin, vanillic acid, apocynin, syringaldehyde, syringic acid and acetosyringone were 1.05, 1.22, 0.89, 0.88, 1.04 and 0.84, respectively. No correlation between the activity and the log *P* values of the test substances was found, which excludes that the activity could be due to membrane-permeability only.

3.5. Cytotoxicity

To exclude that the measured inhibitory effects of test substances can be attributed to cytotoxic activity, the 5-carboxyfluorescein/propidium iodide toxicity assay was used. The samples were tested in concentrations up to 500 μM. None of the compounds showed any signs of being toxic (data not shown).

Table 2
Inhibitory effects on myeloperoxidase release and/or activity ($n=6$)

Compounds	IC_{50} (μM)
Apocynin	>1250
Vanillin	>1250
Vanillic acid	165 ± 53
Acetosyringone	>1250
Syringaldehyde	>1250
Syringic acid	44 ± 9.6

To determine the effects on myeloperoxidase release/activity, neutrophils were incubated with the test compounds and stimulated with opsonized zymosan to achieve myeloperoxidase release. Effects of the compounds were determined using 3,3′-dimethoxybenzidine and hydrogen peroxide as substrate. Absorbance at 450 nm was measured every 2 min for 20 min. Values are depicted as mean IC_{50} values ± S.E.M.

4. Discussion

In this study, we show that substitution of a methoxy group at the position C-5 of apocynin, vanillin, and vanillic acid significantly increases their ability to interfere with the generation of reactive oxygen species by human polymorphonuclear neutrophils. The C-5 substitution with a methoxy group increases the electronic density of the aromatic ring, which may increase its anti-oxidant activity. Since none of the compounds showed significant signs of $\cdot O_2^-$ scavenging activity or cytotoxicity, we suggest that these compounds interfere with the signal transduction that mediates neutrophil activation.

Our in vitro findings are consistent with the in vivo results of Dorsch et al., who reported that acetosyringone shows stronger antiasthmatic properties than apocynin in the plethysmographic guinea-pig model with ovalbumin as challenging agent (Dorsch et al., 1994). Although reactive oxygen species are thought to play a minor role in asthma, our findings help explain the increased antiasthmatic properties of C-5 methoxylation.

To quantitate the inhibitory effects of the compounds on the generation of reactive oxygen species after stimulation of neutrophils, we used two stimuli which represent different neutrophil activation pathways. Opsonized zymosan was used as a model system for opsonized microorganisms. Opsonized zymosan consists of the cell walls of baker's yeast coated with IgG, mannose-binding lectin, and $C3_{b(i)}$ complement fragments (Roos et al., 1981). Phorbol 12-myristate 13-acetate (PMA) is a soluble agent that activates neutrophils directly at the level of protein kinase C, which also leads to the activation of the respiratory burst (Burnham et al., 1989).

Although opsonized zymosan and PMA both stimulate the $\cdot O_2^-$-generating NADPH oxidase, their transductional mechanisms within the neutrophil are quite different (McPhail and Snyderman, 1983). One important difference between stimulation with opsonized zymosan and PMA is the fact that opsonized zymosan stimulation of human neutrophils, unlike PMA stimulation, leads to a substantial release of the enzyme myeloperoxidase from primary granules inside the cell (Niessen et al., 1991).

The decreased reactive oxygen species generation by neutrophils incubated with the C-5 methoxylated compounds acetosyringone, syringaldehyde, and syringic acid as compared with their C-5 demethoxylated compounds apocynin, vanillin, and vanillic acid may indicate that C-5 methoxylation may play an important role in reactive oxygen species inhibitory activity. Future experiments have to show whether these analogs have a similar mode of action as proposed for apocynin.

Strikingly, when luminol was used as enhancer molecule, this reactive oxygen species-inhibiting effect was more or

less independent of the stimulus used (Fig. 1). However, upon PMA stimulation and lucigenin enhancement, apocynin, vanillin, and vanillic acid did not show any inhibitory activity and neither did the C-5 methoxylated analogs (Fig. 2). The difference between luminol and lucigenin as chemiluminescence enhancers may be explained by the different levels they act at: luminol is known to detect both intra- and extracellular reactive oxygen species production (Dahlgren et al., 1989), whereas the site of action of lucigenin is the extracellular space, most probably since neutrophils are practically impermeable to lucigenin (Dahlgren et al., 1985). Another aspect is that luminol-enhanced chemiluminescence is mainly dependent on the myeloperoxidase–H_2O_2 system, whereas experiments with myeloperoxidase-deficient neutrophils have indicated that lucigenin-dependent chemiluminescence is independent of the myeloperoxidase–H_2O_2 system (Aniansson et al., 1984).

The more likely explanation for the lack of inhibitory activity of apocynin and its analogs against PMA-induced, lucigenin-enhanced chemiluminescence is that apocynin is thought to need metabolic conversion by the combined action of myeloperoxidase and reactive oxygen species to become activated (Simons et al., 1990). In contrast to opsonized zymosan, stimulation of human neutrophils with PMA results in little or no myeloperoxidase release (Niessen et al., 1991), which may explain the absence of inhibitory activity of apocynin upon PMA stimulation. Furthermore, it is known that apocynin and its analogs exhibit more or less H_2O_2-scavenging activity (Stolk et al., 1994a), which could explain the activities of the compounds after PMA stimulation in the luminol-enhanced chemiluminescence assay. Based on the putative inhibition of lucigenin-enhanced chemiluminescence by PMA-stimulated neutrophils, we performed a discriminatory experiment with extrinsic myeloperoxidase. Indeed, apocynin was able to inhibit reactive oxygen species production in this experimental set-up, which is in favor of the theory of metabolic activation and excludes a major role of H_2O_2-scavenging activity. Since myeloperoxidase release only occurs in stimulated cells, this makes apocynin a selective inhibitor of NADPH oxidase-mediated reactive oxygen species production by activated human neutrophils.

Besides being a potent inhibitor of reactive oxygen species generation, apocynin also has other significant anti-inflammatory properties ('t Hart et al., 1990, 1992; Engels et al., 1992; Stolk et al., 1994b), which deserve more thorough basic studies, e.g. the impact of different substitution patterns on these activities.

In our efforts to elucidate the mode of action of apocynin, we are currently testing apocynin and its analogs in several in vitro and in vivo assays (e.g. oxygen consumption in human neutrophils and experimental colitis in rats). These structure–activity relationship studies may contribute to the unravelling of the mechanisms underlying the anti-inflammatory activity of apocynin and to the development of new apocynin-related anti-inflammatory drugs.

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