Investigations on apocynin, a potent NADPH oxidase inhibitor

Onderzoek aan apocynin, een potente NADPH oxidase remmer

(met een samenvatting in het Nederlands)

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- Je moet schieten, anders kun je niet scoren -

(Johan Cruijff)

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General Introduction

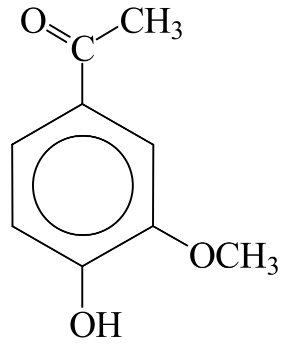
- Alles wat je weet is geen probleem -

(Johan Cruijff, 1995)

History of apocynin

Apocynin, 4'-hydroxy-3'-methoxy-acetophenone, a small molecule with interesting activities, was first described by Schmiedeberg in 1883 and was isolated from the roots of *Apocynum cannabinum* (Canadian hemp) *(1)*. By then, extractions of Canadian hemp were used as official remedies for dropsy and heart troubles *(2)*.

In 1908, Finnemore re-investigated the constituents of Canadian hemp and described a new procedure to isolate apocynin on a larger, more adequate scale *(3)*. He also confirmed that apocynin was identical to acetovanillone which was synthesized and described by Otto in 1891 *(4)*.



Apocynin

Apocynin is an acetophenone with a molecular weight of 166.17 and forms fine needles upon crystallization from water. It possesses a faint vanilla odor and has a melting point of 115°C. The substance is slightly soluble in cold water, but freely soluble in hot water, alcohol, benzene, chloroform, and ether. Although apocynin was first discovered in *A. cannabinum*, its occurrence is not restricted exclusively to the A*pocynaceae* family. In fact, it is a common compound in many plant species *(5–9)* although the quantities in which apocynin is present may vary from species to species. Furthermore, in the wood and paper industry, apocynin is known as one of the degradation products of lignin *(10, 11)*.

In 1971, Basu *et al* reported the isolation of apocynin from the roots of *Picrorhiza kurroa* Royle ex Benth. *(12)*. *P. kurroa* is a small, perennial plant growing at high altitudes in the western Himalayas and which has been used extensively for ages and is still in use in the Ayurvedic system of medicine in India and Sri Lanka. Major fields of application are as a liver tonic, a cardiotonic, and the treatment of jaundice and asthma *(13)*. Although, at that time, no specific properties of apocynin were known which could explain the effectiveness of *P. kurroa*, this compound was considered to be an important constituent contributing to the medicinal potential of this herb. In 1990, in our institute Simons *et al.* subjected the roots of *P. kurroa* to an activity-guided isolation procedure which eventually established the pharmacological potential of apocynin *(14, 15)*. Apocynin proved to be a potent anti-inflammatory agent, based on the selective inhibition of the production of reactive oxygen species (ROS) by activated human polymorphonuclear neutrophils (PMNs). Since PMNs and ROS play an important role in the innate host defense against invading microorganisms, the activity of apocynin can be of significant importance in the treatment of diseases with neutrophils as (pro)inflammatory mediators.

Neutrophils, Reactive Oxygen Species and the NADPH oxidase

In general, vertebrates possess two fundamental mechanisms to respond to infection, the innate and the acquired immune system *(16)*. Innate, or natural immunity is the ability to respond immediately to an infectious challenge, regardless of previous exposure of the host to the invading agent. Elements of the innate system include phagocytic cells, namely polymorphonuclear leukocytes (PMNs) and mononuclear phagocytes (e.g. macrophages), and the complement cascade of circulating soluble pre-enzymic proteins. These elements constitute a relatively nonspecific 'pattern recognition' system which has functional analogues in the immune system of a wide variety of multicellular organisms, including plants *(17)* and insects *(18)*. As such, these evolutionary ancient elements represent a rapid and sensitive surveillance mechanism of host defense when the organism is challenged with an invading microorganism previously 'unseen' by the host's immune system. In contrast to the innate system, adaptive immunity is restricted to vertebrates and represents a precisely tuned system by which host cells define specifically the nature of the invading pathogen or tumor cell *(19)*. Such precision, however, requires time for antigens to be processed and specific lymphocytes and antibodies to be generated. Therefore the adaptive system is slower to respond to new challenges than is the innate system which lacks specificity *(16)*.

Granulocytes arise from pluripotent stem cells located in the bone marrow, and include eosinophils, basophils, and neutrophils. PMNs are the most numerous leukocytes in the human peripheral circulation, and take their name from their typically multilobed nucleus. The daily production of mature PMNs in a healthy adult is in the order of 10^{11} cells. During acute infection or other inflammatory stresses, PMNs are mobilized from the marrow reservoir, containing up to 10 times the normal daily neutrophil requirement *(20)*. PMNs are motile, and very plastic cells which allows them to move to sites of inflammation where they serve as a first line of defense against infectious microorganisms. For this purpose, PMNs contain granules filled with proteolytic and other cytotoxic enzymes *(21, 22)*. Besides releasing enzymes, PMNs are also able to phagocytose and to convert oxygen into highly reactive oxygen species (ROS). Following phagocytosis, ingested microorganisms may be killed inside the phagosome by a combined action of enzyme activity and ROS production.

Upon activation, PMNs start to consume a vast amount of oxygen which is converted into ROS, a process known as the respiratory or oxidative burst *(23, 24)*. This process is dependent on the activity of the enzyme NADPH oxidase. This oxidase can be activated by both receptor-mediated and receptor-independent processes. Typical receptor-dependent stimuli are complement components C5a, C3b and iC3b *(25)*, the bacterium-derived chemotactic tripeptide N-formyl-Met-Leu-Phe (fMLP) *(26)*, the lectin concanavalin A *(27)*, and opsonized zymosan (OPZ) *(28)*. Receptor-independent stimuli include long-chain unsaturated fatty acids and phorbol 12-myristate 13-acetate (PMA) *(29)*. Upon activation, the oxidase accepts electrons from NADPH at the cytosolic side of the membrane and donates these to molecular oxygen at the other side of the membrane, either at the outside of the cells or in the phagosomes containing ingested microorganisms. In this way, a one-electron reduction of oxygen to superoxide anion ($\cdot O_2^-$) is catalyzed at the expense of NADPH as depicted in the following equation:

$$2\,O_2 + NADPH \longrightarrow 2\,\cdot O_2^- + NADP^+ + H^+$$

Most of the oxygen consumed in this way will not be present as $\cdot O_2^-$, but can be accounted for as hydrogen peroxide which is formed from dismutation of the superoxide radical *(30, 31)*:

$$\cdot O_2^- + e^- + 2H^+ \longrightarrow H_2O_2$$

However, hydrogen peroxide (H_2O_2) is bactericidal only at high concentrations *(32)* while exogenously generated superoxide does not kill bacteria directly *(33, 34)* because of its limited membrane permeability. Therefore, a variety of secundary oxidants have been proposed to account for the destructive capacity of PMNs (see Figure 1).

Hydroxyl radicals ($\cdot OH$), formed by the iron catalyzed Fenton reaction, are extremely reactive with most biological molecules although they have a limited range of action *(35)*.

$$H_2O_2 + e^- + H^+ \xrightarrow{Fe^{3+}/Fe^{2+}} H_2O + \cdot OH$$

Singlet oxygen ($^1O_2$) is often seen as the electronically excited state of oxygen and may react with membrane lipids initiating peroxidation *(36)*. Most of the H_2O_2 generated by PMNs is consumed by myeloperoxidase (MPO), an enzyme released by stimulated PMNs *(37-40)*. This heme-containing peroxidase is a major constituent of azurophilic granules and is unique in using H_2O_2 to oxidize chloride ions to the strong non-radical oxidant hypochlorous acid (HOCl) *(41)*. Other substrates of MPO include iodide, bromide, thiocyanite, and nitrite *(42, 43)*.

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^-$$

HOCl is the most bactericidal oxidant known to be produced by the PMN *(44)*, and many species of bacteria are killed readily by the MPO/ H_2O_2 /chloride system *(45)*.

In addition to these ROS, there is considerable interest in nitric oxide (NO) and NO-derived peroxynitrite ($ONOO^-$) as potential cytotoxic agents produced by inflammatory cells *(46-48)*. Peroxynitrite is a potent, relatively stable oxidant *(49)* with properties similar to those of hydroxyl radical. Although murine macrophages are reported to generate NO in response to cytokines *(50)*, studies to detect NO production by stimulated human neutrophils have been contradictionary and mostly negative *(51, 52)*. However, in spite of some reports on nitric oxide synthase (NOS) activity in human neutrophils *(53)*, it is assumed that *in vitro* studies often have been negative since the conditions necessary for induction may not have been completely established *(30)*.

In experimental settings, ROS production by activated phagocytes can be detected using enhancers such as luminol or lucigenin *(54).* For ROS-detection, lucigenin must first undergo reduction, while luminol must undergo one-electron oxidation to generate an unstable endoperoxide, the decomposition of which generates light by photon-emission *(55)*. Luminol largely detects HOCl, which means that luminol detection is mainly dependent on the MPO/H_2O_2 system *(56),* while detection using lucigenin is MPO-independent and more specific for $\cdot O_2^-$ *(57).* Luminol is able to enter the cell and thereby detects intra- as well as extracellularly produced ROS *(58),* while lucigenin is practically incapable of passing the cell membrane and thereby only detects extracellular events *(59)*. However, results should be interpreted with care, because real specificity can never be assumed with any of these light-emission-enhancing compounds *(60)*.

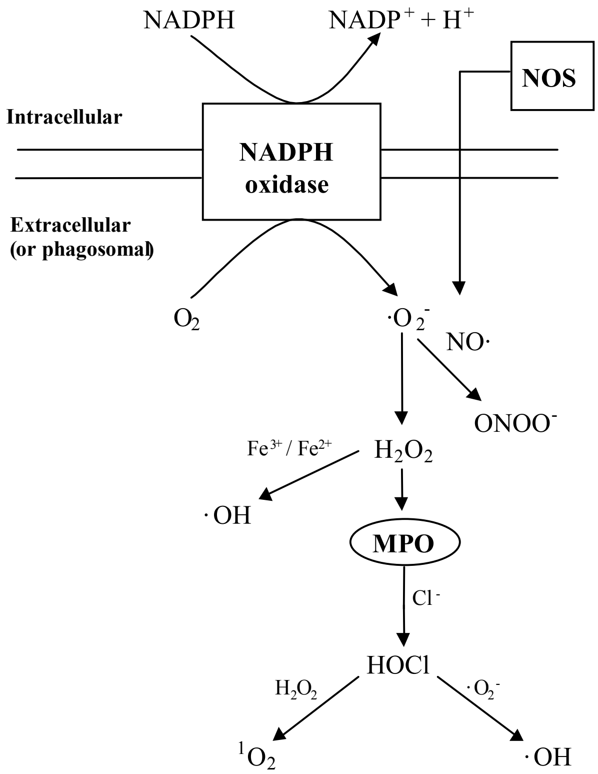


Figure 1. ***Reactive oxidant species production and reactions in stimulated neutrophils.*** *[NOS: nitric oxide synthase, MPO: myeloperoxidase; adapted from Hampton et al.(30)]*

Production of $\cdot O_2^-$ seems to occur within all aerobic cells, to an extent dependent on O_2 concentration. In mitochondria, 1-3% of electrons are thought to form $\cdot O_2^-$. The fact that ROS are also quantitatively significant products of aerobic metabolism is illustrated by the following calculation: a normal adult (assuming 70 kg body weight) at rest utilizes 3.5 mL O_2/kg/min, which is identical to 352.8 l/day or 14.7 mol/day. If 1% makes $\cdot O_2^-$ this gives 0.147 mol/day or 53.66 mol/year or about 1.7 kg of $\cdot O_2^-$ per year. During the respiratory burst, the increase in O_2 uptake can be 10 to 20 times that of the resting O_2 consumption of neutrophils *(61).*

The NADPH oxidase, responsible for ROS production, is a multi-component enzyme system which is unassembled (and thereby inactive) in resting PMNs. However, activation of the phagocyte, *e.g.* by the binding of opsonized microorganisms to cell-surface receptors, leads to the assembly of an active enzyme complex on the plasma membrane *(62, 63)*. The critical importance of a functioning NADPH oxidase in normal host defense is most dramatically illustrated by the recurrent bacterial and fungal infections observed in individuals with chronic granulomatous disease (CGD), a disorder in which the oxidase is non-functional due to a deficiency in one of the constituting protein components *(64-68)*. PMNs from such patients, lacking a functionally competent oxidase, fail to generate $\cdot O_2^-$ upon stimulation. Although the formation of ROS by stimulated PMNs may be a physiological response which is advantageous to the host, it can also be detrimental in many inflammatory states in which these radicals might give rise to excessive tissue damage *(69-71)*.

Essential components of the NADPH oxidase include plasma membrane and cytosolic proteins. The key plasma membrane component is a heterodimeric flavo-cytochrome *b* which is composed of a 91-kDa glycoprotein (gp91^{phox}) and a 22-kDa protein (p22^{phox}) *(72, 73)*. Flavocytochrome *b* serves to transfer electrons from NADPH to molecular oxygen, resulting in the generation of $\cdot O_2^-$. In PMN membranes, a low-molecular-weight GTP-binding protein, Rap1A, is associated with flavocytochrome *b* and plays an important role in NADPH oxidase regulation *in vivo* *(74, 75)*. Furthermore, cytosolic proteins p47^{phox}, p67^{phox}, and a second low-molecular-weight GTP-binding protein, Rac2 are absolutely required for NADPH oxidase activity *(68, 76, 77)* and these three proteins associate with flavocytochrome *b* to form the functional NADPH oxidase *(78-81)*. Additionally, a cytosolic protein, p40^{phox}, has been identified, but its role in oxidase function is not completely defined *(82)*. According to the current model of NADPH oxidase assembly, p47^{phox} and p67^{phox}translocate *en bloc* to associate with flavocytochrome *b* during PMN activation *(81, 83, 84)* (see also Fig. 2). Rac2 translocates simultaneously, but independently of the other two cytosolic components, to associate with the membrane-bound flavocytochrome *b (85, 86)*. Studies of oxidase assembly in PMNs of patients with various forms of CGD suggest that p47^{phox} binds directly to flavocytochrome *b* (79) and at least six regions of flavocytochrome *b* have been identified as putative sites for interaction with p47^{phox}, including four sites on gp91^{phox} and two sites on p22^{phox} *(87-94)*.

Proposed mode of action of apocynin

Apocynin is a selective inhibitor of NADPH oxidase activity and concomitant ROS production (IC50 value: 10 μM) in activated human neutrophils *(14)*. Interestingly, it does not seem to interfere with the PMNs other defense mechanisms, as it does not affect phagocytosis or intracellular killing *(95)*. For this reason apocynin has become an important, widely used, experimental tool to block NADPH oxidase activity.

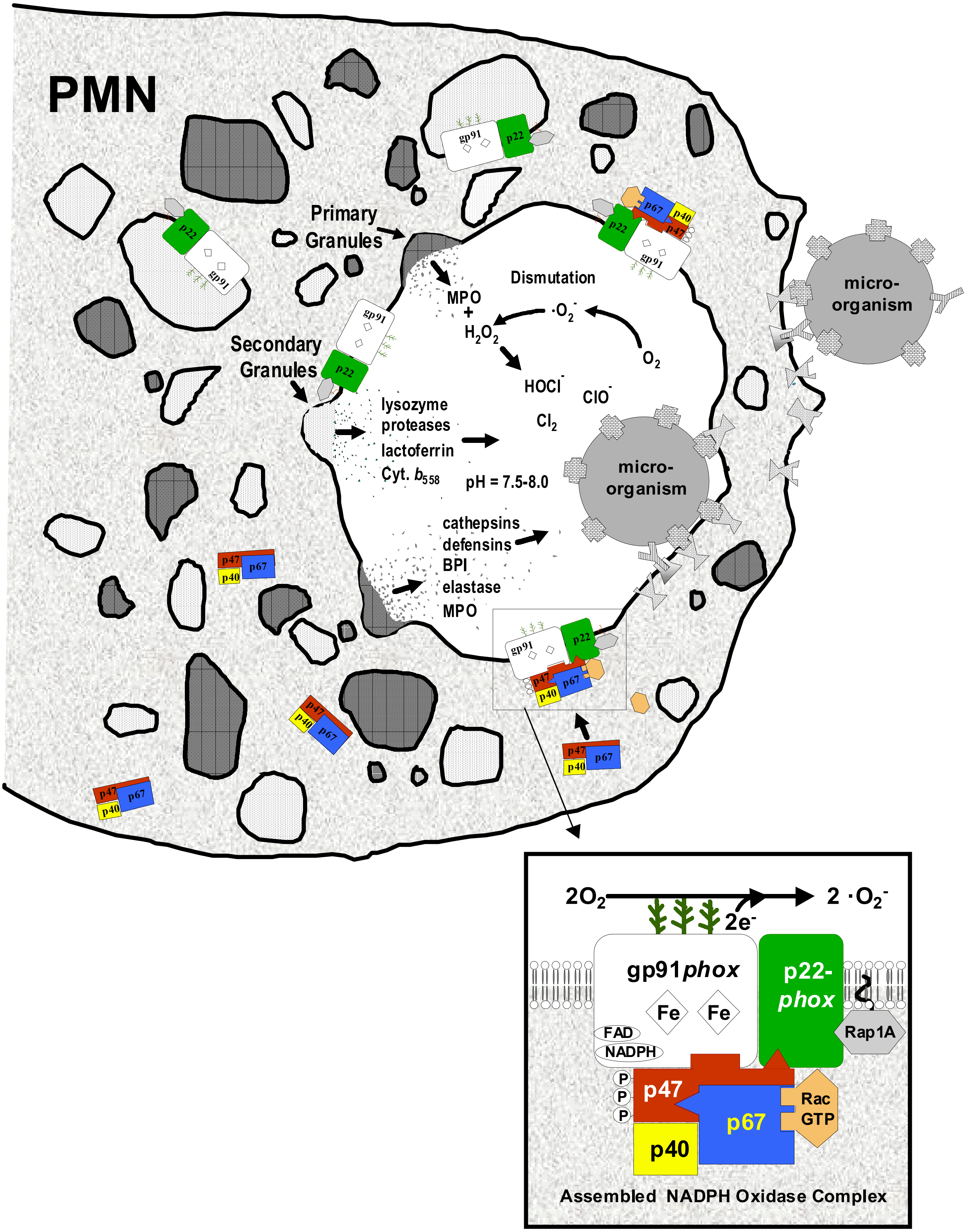


Figure 2. *NADPH oxidase assembly and subsequent ROS production by activated PMNs (picture by courtesy of Dr. F.R. DeLeo)*

However, its mode of action is not completely elucidated and neither is the way in which its anti-inflammatory properties are accomplished, but selective inhibition of ROS production by activated phagocytes may be a valid explanation for the anti-inflammatory activity of apocynin.

In order to further investigate the anti-inflammatory properties of apocynin, many experiments, *in vitro* as well as *in vivo*, have been performed. In 1990, Simons *et al*. hypothesized that apocynin has to be metabolically activated by stimulated neutrophils by means of a ROS and MPO-dependent mechanism *(14)*. This means that the activity of apocynin is merely restricted to *activated* PMNs and thereby to actual sites of inflammation. Evidence in favor of this theory could be their finding that *o*-methoxy-substituted catechols, such as apocynin, do not inhibit $\cdot O_2^-$ release from rat alveolar macrophages, which lack significant MPO activity. In fact, their theory was supported by the findings of Stolk *et al*. *(95)* who performed three different experiments which all pointed to metabolic activation of the molecule: (i) OPZ-stimulated human alveolar macrophages, which are MPO-deficient *(96)*, generate superoxide anion in a way which could not be inhibited by apocynin in concentrations active in experiments with neutrophils; (ii) the OPZ-induced respiratory burst of PMNs that are MPO-deficient, was not inhibited to the same extent as the OPZ-induced burst in normal PMNs; and (iii) human neutrophils, stimulated by PMA, release little or no MPO *(97)*, but do produce superoxide for a prolonged period of time, which could not be inhibited by apocynin.

These findings clearly point to an important interaction between apocynin and MPO and seem to indicate that apocynin is indeed selectively converted into an active metabolite which may be responsible for the anti-inflammatory activity. Additional experiments by the same authors also supported this theory: apocynin (or its active metabolite) inhibits translocation of two essential cytosolic proteins, p47^{phox} and p67^{phox}, to the cell membrane, thereby inhibiting the assembly of NADPH oxidase *(95)*. But, complete inhibition only occurred after seven minutes. This lag time is consistent with the idea that apocynin has to be converted into its active metabolite, before exerting inhibitory activity.

't Hart and Simons *et al.* were the first to propose a conversion mechanism and a possible structure for this active metabolite (a quinone methide), but no scientific evidence was provided to confirm this theory *(98)*. Recently, however, a new structure has been postulated as the probable active metabolite of apocynin. Holland *et al*. suggest that apocynin is metabolically converted into a dimer which they called diapocynin *(99)*. However, the actual existence, chemical properties, and activities of this dimer need further confirmation as no precise chemical data were provided. Recently, Müller *et al*. also reported the isolation of an active metabolite after incubation of apocynin with MPO, but again no characteristics about the structure of this metabolite were presented *(100)*. Still, these reports indicate that the idea of metabolic conversion is more or less accepted. And although the exact mode of action of apocynin is still not fully understood, its activities in different *in vitro* and *in vivo* assays, together with its selectivity and lack of toxicity, are quite impressive. These features make apocynin a valuable lead compound in the search for new, non-steroidal anti-inflammatory compounds.

Toxicity

Side effects of apocynin are not known. Apocynin has very low toxicity (LD50: 9 g/kg) after oral administration in mice *(101)*. Even when treated with apocynin for a three-month period, rabbits do not show any signs of ill-health and other parameters checked were comparable to control-treated animals *(102).* It has also been reported that in an ongoing phase-I clinical study, apocynin is tested for the treatment of lung emphysema.

The patients received, during 4 days, four daily dosages of 3 mL (1 mg apocynin/mL) by inhalation and so far, no side effects, including no adverse gastrointestinal effects, have been observed (J. Stolk and J. Brahim, Department of Pulmonology, Leiden University Hospital, The Netherlands ; *Personal Communication, 1998*). Furthermore, when tested in the *Salmonella typhimurium* mutagenicity assay (Ames test) and the sister chromatid exchange (SCE) test, which tests for DNA-damaging properties, apocynin showed no genotoxic effects at concentrations up to 600 μM *(103)*.

Kinetics of apocynin

Not much is known about the kinetics of apocynin *in vivo,* but interesting metabolic aspects of apocynin were described by Daly *(104)* and Gjertsen *(105)*. They showed that after a period of 20 hours upon *i.p.* administration of 120 mg/kg apocynin to rats, 80% of the apocynin was recovered unchanged in the urine of the animals. Approximately 0.5% was converted into the *para*-isomer, acetoisovanillone. Also traces of 3′,4′-dihydroxy-acetophenone were excreted. A further pathway of apocynin metabolization, although of minor quantitative importance, was ketone reduction to the 1-phenylethanol derivatives 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol and 1-(3′-,4′-dihydroxyphenyl)-ethanol. This pathway had not previously been reported for acetophenone derivatives possessing hydroxyl substituents. This raises the question as to whether one of these compounds may be a candidate for the active metabolite of apocynin. In the next section of this chapter the biological activities of apocynin in connection to inflammatory diseases will be reviewed.

USE OF APOCYNIN IN THE TREATMENT OF INFLAMMATORY DISEASES

Anti-arthritic activity of apocynin

It is known that in the pathogenesis of collagen-induced arthritis (CIA) in rats, neutrophils play an important role, since depletion of this cell type reduces joint inflammation by more than 60% *(106)*. In studies described by 't Hart *et al*., the anti-inflammatory activity of apocynin was tested in a CIA rat model *(107, 108)*. Collagen type II-immunized rats were treated with different doses of apocynin in the drinking water (0.3 - 200 μg/mL) starting 9 days after immunization (this is just before the onset of arthritis, but after the development of a specific immune response). The treatment was terminated 14 days later, at the time when joint swelling in the control group was maximal. Surprisingly, the lowest apocynin concentration protected the animals from joint swelling, whereas increasing the dose up to 200 μg/mL did not improve the effect. Even 100 days after immunization, no flare-up of the joint swelling was observed in apocynin-treated rats. Treatment of rats with low doses of apocynin also reduced plasma IL-6 levels. Interestingly, it was demonstrated that the severity of CIA correlates with increased IL-6 production *(109)*. However, in unpublished results of these authors it was observed that apocynin is not able to cure an already existing inflammation. So, the activity of the molecule seems to be restricted to mechanisms which take place during the onset of the inflammation, which often is the period in which neutrophils play an important role.

Another effect of apocynin which may emphasize its importance in the treatment of rheumatoid arthritis (RA) is that apocynin inhibits inflammation-mediated cartilage destruction in human articular cartilage explants, without having adverse effects on the cartilage itself *(110)*. In these experiments apocynin was added to cultured peripheral blood mononuclear cells (PBMNCs) of RA patients. Cartilage-destructive activity was determined after addition of culture supernatant to tissue samples of the cartilage explants. Additionally, the proliferation of the PBMNCs and their production of tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1) and IL-10 were determined. Also the production of interferon gamma (IFNγ) and IL-4 by T-cells was measured. Apocynin (180 μM) was able to abolish RA PBMNC-induced inhibition of cartilage matrix proteoglycan synthesis, while no effect on inflammation-enhanced proteoglycan release was found. The effect was accompanied by a decrease in IL-1 and TNFα production by the PBMNC. Such findings are in accordance with previously reported experiments *(107, 111)*. No effect on T-cell proliferation was observed, but the production of IFNγ, IL-4, and T-cell derived IL-10 was strongly diminished. Most importantly, apocynin diminished the release of proteoglycans (responsible for the typical consistency) from the cartilage matrix and did not show any direct adverse effects on chondrocyte metabolism. These results underline the importance of further *in vivo* studies to test apocynin's effectiveness in the long-term treatment of chronic inflammatory and degenerative joint diseases.

Apocynin in the treatment of inflammatory bowel disease

ROS have been reported to play an important role in the pathogenesis of inflammatory bowel disease (IBD) *(112, 113)*. In rat models of intestinal inflammation, an increased production of ROS also contributes to tissue injury *(71)*. Palmen *et al.*

examined the effects of apocynin in acute and relapsing experimental colitis in rats, which are models for inflammation in the large intestine *(114, 115)*. In rats, acute colitis was induced by intra-colonic administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) in 30% ethanol (30 mg in 0.25 mL). Relapsing colitis was induced by a subcutaneous injection of TNBS, 5 weeks after the induction of acute colitis. In both the acute and relapsing colitis models, the animals received two intravenous injections of apocynin (4 mg/kg bodyweight) at day 0 and 3 (acute) or day 35 and 38 (relapsing). After sacrificing the rats, the influx of macrophages and PMNs into colon tissue as well as MPO activity and macroscopical damage scores of the colon were determined. In the acute model, apocynin significantly reduced the damage score, MPO activity, and the number of macrophages and PMNs in the colon. Apocynin treatment in relapsing colitis resulted in a striking improvement of the damage score to almost normal values, significantly lower MPO-activity and in decreased numbers of colonic macrophages. These experiments show that, besides inhibition of ROS production, apocynin may also prevent tissue damage in IBD by inhibiting the influx of inflammatory cells into the colon.

Another remarkable effect of apocynin in the treatment of IBD was reported by Rachmilewitz *et al*. *(116)*. Apocynin was tested in a rat model for Crohn's disease, which represents inflammation and damage in the small intestine. They showed that the addition of 120 µg/mL of apocynin to the drinking water resulted in an effective decrease in the extent and severity of jejunal damage. Furthermore, histological data revealed that, after seven days, the villi of the jejunal wall were almost normal, while no granulomas were observed in any of the rats. This effective modulation in the pathogenesis of both small and large intestinal inflammation by apocynin in experimental animals may stimulate further research on the use of apocynin in IBD.

Anti-asthmatic properties of apocynin

As mentioned previously, *P. kurroa* has been used for ages in the treatment of asthma *(13)*. Using different chemical and pharmacological methods, Dorsch *et al.* were able to identify the glucoside of apocynin, androsin, as active compound which prevented allergen- and PAF-induced bronchial obstruction in guinea pigs *(117)*. Also the aglucone, apocynin, was tested for similar activity. It appeared that inhalation of apocynin was more effective than oral uptake. Apocynin was administrated as an aerosol in the plethysmographic guinea pig model, using PAF and/or OVA as challenging agents for the generation of bronchial constriction. In the OVA-model, 0.5 mg apocynin given by inhalation 30 minutes prior to the challenge, resulted in a 76% inhibition of bronchoconstriction after the first challenge while a 64% inhibition was observed after the second challenge. In PAF-challenged animals, 0.34 mg apocynin decreased the bronchoconstriction by 65%. In many experimental animal models for respiratory diseases as well as in *ex vivo* studies, apocynin is used for its beneficial effects *(118-126)*. Because apocynin exhibited neither atropine- or theophylline-like, nor anti-histaminic or broncholytic modes of action, Dorsch *et al.* suggested that the anti-asthmatic effect may be due to interference with cell or mediator systems involved in inflammatory processes. However, apocynin (5 mg/kg body weight, administered orally) was reported to inhibit the increase in airway responsiveness to bradykinin in an ozone-induced airway hyperresponsiveness model in rats without affecting the neutrophil counts in broncho-alveolar lavage fluid *(127)*. Since production of ROS by activated phagocytes are thought to play an important role in airway inflammation *(128, 129)* it is most likely that the effect of apocynin must be attributed to inhibition of ROS production.

Another possible explanation for the effectiveness of apocynin in the treatment of respiratory diseases might be the fact that apocynin inhibits peroxynitrite ($ONOO^-$) formation *(130)*. $ONOO^-$ is the very reactive product of the reaction of nitric oxide (NO) and superoxide anion ($\cdot O_2^-$). For many years, much attention has been paid to the effects of NO in respiratory diseases *(131)*, but recently the focus has been shifted towards reactive nitrogen species (RNS) in general, and to peroxynitrite in particular *(132, 133)*. Peroxynitrite is suggested to induce epithelial damage, mediator release, and consequently hyperresponsiveness *(134)*. This finding may have important clinical implications, since airway inflammation, epithelial damage, and hyperresponsiveness are characteristic features in patients suffering from asthma.

Since apocynin inhibits NO production only at high concentrations *(130, 135, 136)*, it is very likely that its potent $\cdot O_2^-$ inhibition can be accounted for the eventual $ONOO^-$ inhibition. Since ROS appear to have a pivotal role in all pathways leading to the production of RNS, inhibition of superoxide anion production by apocynin may not only largely prevent the formation of peroxynitrite, but also that of other RNS.

Apocynin and atherosclerosis

Atherosclerosis is one of the most common cardiovascular diseases in developed countries and is yet another disease in which ROS are thought to play an important role *(137, 138)*. Among the main causes of the development of atherosclerosis is a high serum level of low-density cholesterol-containing lipoprotein (LDL) *(139)*. In the pathogenesis of atherosclerosis, much research has been directed towards the role of the vascular endothelium. It has been suggested that LDL oxidation by cells of the arterial wall may be a key event in early atherosclerosis *(137, 140)*.

Recently, several reports have been published which claim that endothelial cells may possess a functional NADPH oxidase, capable of producing ROS, similar to that of phagocytes *(141-145)*. Atherogenic levels of LDL have been shown to lead to a significant increase in NADPH oxidase dependent ROS production by the endothelium *(146)*. Importantly, NADPH oxidase activation and concomitant ROS production has been reported to be required for macrophage-mediated oxidation of LDL which increases atherogenicity *(138, 147)*. Although the immediate product of NADPH oxidase ($\cdot O_2^-$) is not reactive enough to induce LDL oxidation, it can be converted into other, more reactive species which are able to directly oxidize the lipoprotein, resulting in the formation and release of peroxidized fatty acids.

Aviram *et al.* tested apocynin in a J-774 A.1 murine macrophage model to investigate its effect on macrophage-mediated oxidation of LDL. They showed that inhibition of the macrophage NADPH oxidase with apocynin (600 μM) inhibited macrophage-mediated oxidation of LDL by 89%, compared with levels in control cells *(148)*.

Experiments with apocynin in endothelial cells showed similar results compared with the effects of apocynin in phagocytes. Holland *et al.* reported that endothelial cells incubated with apocynin (600 μM) and stimulated with the phospholipase A_2 activator thrombin, showed NADPH oxidase inhibition, resulting in a significantly impaired ROS production *(142)*. The same authors also state that endothelial cell incubation with apocynin markedly diminished high LDL-induced increases in cellular H_2O_2 concentrations *(149)*. Furthermore, apocynin was shown to be effective at suppressing atherogenesis *in vivo* in spite of highly elevated serum LDL levels using a rabbit model *(102)*. So, maybe apocynin has revealed a new strategy in the treatment of atherosclerosis, and therefore future treatments should also focus on NADPH oxidase inhibition as an effective way of preventing the endothelium from the initiating events of atherosclerosis.

Effect of apocynin on mediators of inflammation

So far, all effects of apocynin described could be attributed more or less to the inhibition of the NADPH oxidase in activated phagocytes and the consequent inhibition of ROS production. But several reports indicate that apocynin may also have other activities which may contribute to its effectiveness as an anti-inflammatory drug.

Engels *et al.* investigated the effects of apocynin on the production of arachidonic acid-derived inflammatory mediators by guinea pig pulmonary macrophages *(150)*. They showed that apocynin inhibits the formation of the pro-inflammatory compound thromboxane A_2, but stimulates the generation of the anti-inflammatory prostaglandins E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$). They also demonstrated that micromolar concentrations of apocynin (~30 μM) potently inhibited arachidonic acid-induced platelet aggregation, important in thrombosis, possibly through the inhibitory effect on thromboxane formation. The finding that apocynin stimulates PGE_2 production may explain results published by Mattsson *et al.*, who showed that apocynin dose-dependently inhibits tumor necrosis factor-α (TNFα), an important mediator in bacterial septic shock, in lipopolysaccharide (LPS) and peptidoglycan (PG)–stimulated human monocytes *(111)*. Because of the enhanced production of PGE_2 by apocynin, levels of cyclic AMP will increase *(151)*, resulting in a suppression of TNFα production. These results suggest that apocynin not only derives its anti-inflammatory effects from the specific inhibition of ROS production, but that it also affects arachidonic acid-derived mediators, which are also of importance in inflammatory processes.

Reviewing all the previously described activities of apocynin and the frequent use of this compound in modern research, two conclusions can be drawn. Firstly, the frequent use of apocynin in the treatment of inflammatory diseases not only demonstrates its effectiveness, but also stresses the importance of neutrophils and PMN-derived ROS in these disease states. And secondly, for a potent compound used this frequently, it is surprising that its exact mode of action is not completely elucidated yet.

Summarizing all the applications of apocynin and taken into account its low toxicity, selectivity, and lack of known side effects, it can be concluded that apocynin deserves further attention and that studies to elucidate its mode of action may contribute to the development of safe and selective anti-inflammatory drugs which lack the often serious side effects of steroids.

Aim and outline of this thesis

It has been shown in experimental animal models that apocynin is a potent drug in the treatment of inflammatory diseases such as colitis and rheumatoid arthritis. Since its mode of action is not well defined, we tried to get a more precise insight into the mechanisms by which apocynin exerts its activity. Effects on ROS production by stimulated PMNs, as well as effects on other mediators of inflammation were investigated. A better understanding of the mechanism(s) of action of apocynin will contribute to its use as lead-compound in the development of potent and safe non-steroidal anti-inflammatory drugs (NSAIDs).

It has been suggested previously that apocynin is converted into an active metabolite responsible for the eventual activity. In this thesis, experiments providing more information on its inhibitory effects on NADPH oxidase activity in stimulated PMNs are described in Chapter 2. In Chapter 3, some structure-activity relationship studies are presented. Since reactive nitrogen species (RNS) have been shown to play an important role in several inflammatory diseases, effects of apocynin on peroxynitrite formation in murine macrophages are described in Chapter 4. Chapter 5 deals with the isolation, characterization and activity of the possible active metabolite of apocynin. Finally, effects of apocynin, its possible active metabolite, and some apocynin analogs on the production of different cytokines by mononuclear cells and on T cell proliferation are described in Chapter 6.

From the data obtained, we propose a model for the mechanism of action of apocynin which is different from existing theories (Chapter 7).

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Chapter

2

Apocynin, an inhibitor of NADPH oxidase-dependent ROS production in stimulated human neutrophils

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- Vaak hebt iets in het leven waarschijnlijk een noodzaak -

(Johan Cruijff, 1997)

Abstract

Generation of superoxide anion ($\cdot O_2^-$) by the NADPH-dependent oxidase of activated polymorphonuclear neutrophils (PMN) is an essential aspect of the innate immune response to invading microorganisms. In addition to $\cdot O_2^-$, other $\cdot O_2^-$derived reactive oxygen species (ROS) are formed which contribute significantly to host defense.

Apocynin, a compound originally isolated from the medicinal plant *Picrorhiza kurroa*, is a potent inhibitor of the ROS production by activated human PMNs. To gain mechanistic insights into the activity of apocynin we assessed the activation and assembly of the NADPH oxidase of human PMNs stimulated with the particulate stimulus opsonized zymosan (OPZ). The activity of apocynin on extracellular as well as intracellular ROS production following OPZ-stimulation was assessed. To further evaluate effects on NADPH oxidase activity, apocynin-induced inhibition of the oxygen consumption of stimulated human PMNs was also investigated. Effects of apocynin on the assembly of the NADPH oxidase were assessed in a cell-free system and effects on the translocation of $p47^{phox}$, an essential cytosolic component necessary for a functional NADPH oxidase complex, to the cell membrane were also evaluated.

Apocynin inhibited both intracellular and extracellular ROS production. Examining the effects on the assembly of the NADPH oxidase at OPZ-containing phagosomes showed that apocynin interferes with the phagosomal association of the cytosolic protein $p47^{phox}$. This inhibition by apocynin was not instantaneous, but showed a lag time. Inhibition of oxygen consumption by apocynin showed a similar lag time, which may represent the time necessary for metabolic conversion of apocynin into an active metabolite. PMNs incubated with OPZ in the presence of apocynin, showed immediate inhibition upon a second stimulation with OPZ, indicating that apocynin had been converted into its active metabolite inside the cell. In the cell-free system, apocynin did not inhibit the assembly of the NADPH oxidase complex, probably due to the lack of physiological mediators which are present only in intact cells.

These data indicate that apocynin is capable of entering the PMN and may inhibit the assembly of the NADPH oxidase complex by interfering with the translocation of an essential cytosolic protein. Furthermore, these data support the hypothesis that, upon stimulation of PMNs, apocynin is metabolically converted into its active metabolite by the neutrophil granule enzyme MPO in combination with ROS generated by the cells. These data suggest that apocynin is a selective inhibitor of activated human neutrophils and may be a clinically useful, interesting lead-compound for specific non-steroidal respiratory burst inhibitors.

Introduction

Polymorphonuclear neutrophils (PMNs) are phagocytic cells that are readily mobilized to sites of infection and ingest microorganisms such as bacteria by a process known as phagocytosis *(1, 2)*. The ingested microorganisms are killed by reactive oxygen species (ROS) derived from superoxide anion ($\cdot O_2^-$), produced by an activated, phagosome-bound NADPH-dependent oxidase *(3, 4)*. Besides being of great importance in the innate immune system by defending the host against microbial infection *(5)*, the NADPH oxidase also prominently contributes to a variety of inflammatory disorders *(6, 7)*, and excessive production of ROS in some cases can lead to tissue damage and subsequent exacerbation of infection *(8, 9)*.

Apocynin (4′-hydroxy-3′-methoxy-acetophenone) is a small molecule with interesting properties. It has been isolated from the Indian medicinal plant *Picrorhiza kurroa (10)* and appeared to be a potent inhibitor of the NADPH oxidase-mediated production of ROS in activated human PMNs *(11, 12)*. Experimental studies on the effectiveness of apocynin in the treatment of inflammatory diseases in which ROS play an important role, such as arthritis *(13)*, colitis *(14)*, and atherosclerosis *(15)*, already have shown promising results. Despite its general acceptance as a potent inhibitor of the phagocyte NADPH oxidase, neither the active form of apocynin nor its mechanism of action has been defined.

Current models propose that apocynin is converted into an active metabolite by the combined action of myeloperoxidase (MPO), a granule enzyme released from PMNs upon stimulation, and ROS produced by the cell *(16)*. To get a better insight into the mechanisms underlying the activities of apocynin we examined its impact on the assembly and activity of the NADPH oxidase in intact and broken cell assays. Upon stimulation of PMNs, the NADPH oxidase complex, responsible for the conversion of oxygen into ROS, assembles at the membrane of forming phagosomes. The following production of ROS occurs mainly extracellularly or inside the formed phagosomes. Two different probes, ferricytochrome c and dichlorofluorescein (DCF), were used to discriminate between two possible targets for inhibitory effects of apocynin. In contrary to DCF, ferricytochrome c is not capable of entering the cell by passing the cell membrane *(17)*, so only extracellularly produced $\cdot O_2^-$ will be detected using this probe. Comparison of results from these two analytical approaches may give insight into the ability of apocynin to enter the cell and thereby may provide an indication of its site of action in PMNs. Ferricytochrome c only detects superoxide anion ($\cdot O_2^-$), and the rate of $\cdot O_2^-$ generation was measured by the SOD-inhibitable reduction of ferricytochrome c at 550 nm. *(18)*. DCF detects ROS and while DCF is taken up by PMNs, the DCF assay mainly reflects intracellular ROS generation *(19)*. In addition to these assays, effects of apocynin were tested in the oxygen consumption assay. In this assay the decrease of molecular oxygen, used by the activated NADPH oxidase to form ROS, is measured. The advantages of this assay are that it is independent of the type of ROS that is formed, and that interactions between produced ROS and the test-compounds (*e.g.* scavenging) do not interfere with the measurements. Effects of apocynin on translocation of cytosolic oxidase components to the membrane were also investigated by detecting the presence of $p47^{phox}$ in the membrane fractions of phagosomes of OPZ-stimulated human PMNs at different time points. To determine whether effects of apocynin take place at the level of assembly, activity or termination of the NADPH oxidase, apocynin was tested in the cell-free assay. In the cell-free NADPH oxidase reconstitution assay, PMNs are disrupted by nitrogen bomb cavitation and separated into a membrane-enriched fraction (containing the flavocytochrome b complex) and a cytosolic fraction (containing the $p47^{phox}$ and $p67^{phox}$ proteins) *(20)*. These proteins are necessary for a functional NADPH oxidase complex *(21, 22)*. The oxidase is dormant in resting neutrophils but acquires catalytic activity when cells are exposed to appropriate stimuli. In a cell-free system, catalytic activity of the NADPH oxidase in membranes from unstimulated PMNs can be induced by anionic amphiphiles such as sodium dodecyl sulfate (SDS) or arachidonate, in the presence of the cytosolic fraction *(23, 24)*. When the two fractions are combined again in the cell-free assay, a functioning NADPH oxidase complex will be assembled, capable of producing $\cdot O_2^-$. By adding apocynin before, or after addition of SDS, this assay may also discriminate between effects on assembly or effects on the breakdown of the already formed NADPH oxidase complex.

Materials & Methods

Materials

2′,7′-Dichlorodihydrofluorescein diacetate (DCF) was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Superoxide dismutase (SOD) was obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). Apocynin was obtained from Carl Roth GmbH (Karlsruhe, Germany) and was purified by recrystallization from water before use. Myeloperoxidase (MPO) was a gift from Dr. J.P. Weiss, Department of Internal Medicine, University of Iowa College of Medicine, IA, USA. All other reagents were purchased from Sigma (St. Louis, MO, USA), unless specified otherwise.

Neutrophil isolation

Heparinized venous blood and normal human serum (NHS) were obtained from healthy individuals in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa, IA, USA. PMNs were isolated using dextran sedimentation and Hypaque-Ficoll density-gradient separation followed by hypotonic lysis of erythrocytes as described by Boyum *et al.* *(25)*.

Preparation of opsonized zymosan (OPZ)

For each experiment, $5 \cdot 10^8$ particles of zymosan were resuspended in 1.0 mL 100% NHS and incubated under tumbling for 30 min at 37 °C. After centrifugation and washing with DPBS, OPZ particles were resuspended in 5.0 mL of DPBS/g and kept on ice until use. The OPZ particles/PMNs ratio used in ferricytochrome c, DCF, and NADPH oxidase assembly assays was 5:1.

Measuring $\cdot O_2^-$ generation by the reduction of ferricytochrome c

Extracellular O_2^- generation was determined by the SOD-inhibitable reduction of ferricytochrome c as described by DeLeo *et al. (26)*, but with several modifications. Briefly, PMNs ($5 \cdot 10^6$ cells in DPBS/g), ferricytochrome c (100 μM), OPZ ($2.5 \cdot 10^7$ particles) or PMA (1 μg/mL), apocynin, and buffer or SOD (40 μg/mL) were added to each well of a chilled 96-well plate pre-coated with NHS for at least 1 hr at 37 °C, and the plate was centrifuged at 400 × g for 5 min at 4 °C to synchronize phagocytosis. Subsequently, the plate was warmed to 37 °C using a Benchmark microplate spectrophotometer (Bio-Rad, Hercules, CA, USA), and the rate of $\cdot O_2^-$ generation was measured every 15 sec for 15 min (with plate agitation). Activity of samples was calculated from the SOD-inhibitable reduction of ferricytochrome c at 550 nm using an extinction coefficient of 21.1×10^3 M^{-1} cm^{-1} and a platereader pathlenght of 0.8 cm. In control experiments buffer replaced apocynin.

Measuring intracellular ROS production

The production of intracellular ROS was measured as described by DeLeo *et al. (27)*, but with a few modifications. Briefly, cells were suspended in DPBS/g containing 25 μM DCF to 10^7 cells/mL and then equilibrated in the dark for 45 min at room temperature under very gentle shaking. Subsequently, all wells of a 96-well F-bottom microtiter plate were coated with 50 μL of NHS for at least 1 hr at 37 °C. In all experiments, free NHS was removed from plates by two sequential washes with 0.9 % NaCl. PMNs (10^6 cells), buffer or SOD (40 μg/mL), apocynin, and OPZ (10^6 particles) or PMA (1.0 μg/mL) were added to the NHS-coated wells of a chilled 96-well microtiter plate. Cold DPBS/g or sample was added to each well so that the final volume of each well was 200 μl. The plate was centrifuged at 400 × g for 5 min at 4 °C to synchronize phagocytosis. Generation of ROS was monitored continuously (at 485 nm excitation and 538 nm emission) without shaking for 60 min at 37 °C using a BMG FLUOstar 403 microplate spectrofluorometer (BMG Lab Technologies, Durham, NC, USA). Results are expressed as fluorescence/10^6 PMNs.

Oxygen consumption

Oxygen consumption was measured polarigraphically using an oxygen electrode (Radiometer, Copenhagen, Denmark) as described by Weening *et al. (28)*.

In the reaction chamber, 300 μL of a PMN suspension ($3.5 \cdot 10^6$ cells/mL) was equilibrated with 325 μL sample dilution (or buffer for control experiments) at 37 °C. Subsequently, 25 μL OPZ (25 mg/mL) was added and the rate of oxygen consumption monitored for 15 min. Oxygen consumption is expressed as nmoles $O_2/10^6$ PMNs.

NADPH oxidase assembly at OPZ phagosomes

PMNs were incubated with apocynin (100 μM) or buffer, and stimulated with OPZ. Cells were placed on ice at 0, 5, 15 and 30 min to stop phagocytosis. OPZ phagosomes and a plasma-enriched fraction from the OPZ-stimulated cells were isolated using two separate Percoll gradients. Following nitrogen cavitation, cells were centrifuged at 300 x g for 10 min to pellet unbroken PMNs, nuclei, and OPZ phagosomes. Plasma membrane-enriched fractions were isolated from the postnuclear supernatant using a Percoll step gradient as described by Borregaard *et al*. *(29)*. The pellet resulting from the centrifugation was resuspended in 1 mL of relaxation buffer *(29)*. OPZ phagosomes were isolated by density centrifugation through an 8-mL self-forming Percoll gradient with an initial density of 1.065 g/mL at 20.000 x g for 30 min. Phagosomes were collected from the gradient near the buffer/Percoll interface (~1.037 g/mL); uningested OPZ sedimented 3-5 mm below the phagosomes; unbroken cells and debris sedimented near the bottom of the gradient (~1.09 g/mL). OPZ phagosomes and plasma-enriched fractions were each washed twice in relaxation buffer, which included brief sonication using a low setting, and recentrifuged twice. Membrane and phagosome pellets were resuspended in SDS-sample buffer and subjected to 10% SDS-PAGE followed by transfer to nitrocellulose. Immunoblots were probed with a polyclonal antibody to $p47^{phox}$ *(27, 30, 31)* and then quantitated by densitometry using a GS-710 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA, USA).

Fractionation of neutrophils

A plasma membrane-enriched fraction, a specific granule-enriched fraction, and a cytosolic PMN fraction were isolated following nitrogen bomb cavitation and separation on Percoll gradients using the method of Borregaard et al. *(29)*. Membrane fractions were used immediately or supplemented with 0.34 M sucrose and stored at –70 °C for up to 1 month.

Alternatively, crude membrane-enriched fractions and a cytosolic fraction were isolated by sequential centrifugation as described by Fujita *et al*. *(20)*.

Cell-free NADPH oxidase reconstitution assay

The cell-free assay was carried out as previously described by Bromberg *et al.* *(23)* but with several modifications. Briefly, 10 μM FAD, 100 μM ferricytochrome c,

$3 \cdot 10^9$ cell equivalents (CE) membrane-enriched fraction, $5 \cdot 10^6$ CE cytosol fraction, 10 µM GTPγS and 10 µM apocynin were combined in wells of a microtiter plate and supplemented with cell-free assay buffer *(27)* to a final volume of 200 µL and the mixture was incubated for 2 min at room temperature. NADPH oxidase assembly was initiated by the addition of 100 µM SDS. In some experiments, apocynin was added after the addition of SDS to determine whether it inhibited the activity of the formed NADPH oxidase complex. The plate was incubated for another 4-10 min and NADPH (200 µM) was added to all wells. Ferricytochrome c reduction was monitored continuously every 15 sec (with plate agitation) for 10 min at 23 °C using a microplate spectrophotometer (Bio-Rad, Hercules, CA, USA), and $\cdot O_2^-$ production was calculated as described above.

Results

To get a better insight in the mechanism by which apocynin affects the NADPH-dependent ROS production we examined the effects of apocynin on ROS production by PMNs during synchronized phagocytosis of OPZ, monitoring the generation of both intracellular and extracellular ROS.

Measuring $\cdot O_2^-$ generation by reduction of ferricytochrome c

As described previously by others *(12)*, apocynin treatment inhibited OPZ-stimulated superoxide production, as measured by SOD-inhibitable reduction of ferricytochrome c (Fig. 1).

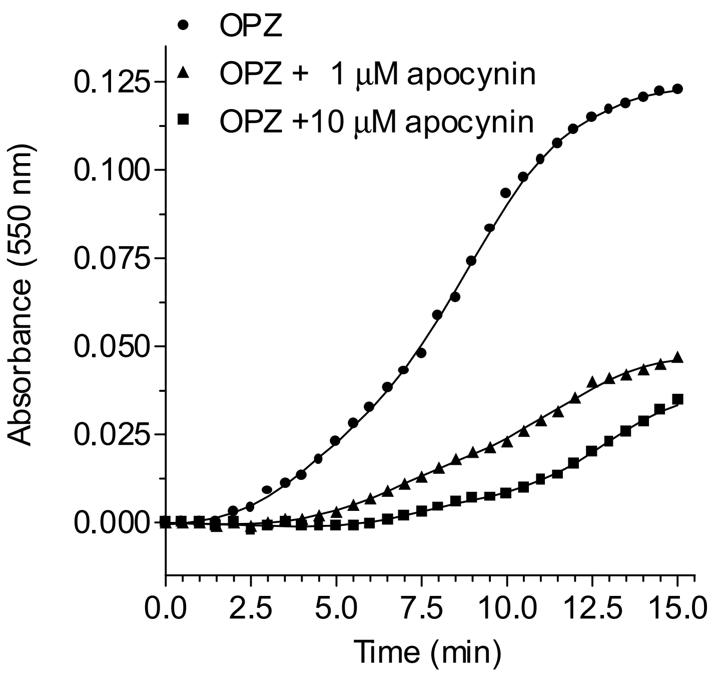

Figure 1. ***Effect of apocynin on the $\cdot O_2^-$-induced reduction of ferricytochrome c in OPZ-stimulated human neutrophils.*** *Plots depict representative curves of four separate experiments.*

This inhibition mainly reflects effects on extracellular $\cdot O_2^-$ production or on $\cdot O_2^-$ escaping from forming phagosomes at the plasma membrane. Comparison of the Vmax of the control experiment (1.63 nmoles $\cdot O_2^-/10^6$ PMNs/min) and that of the apocynin treated PMNs (0.60 and 0.51 nmoles $\cdot O_2^-/10^6$ PMNs/min for 1 μM and 10 μM apocynin respectively) showed that apocynin dose-dependently inhibited the SOD-inhibitable reduction of ferricytochrome c (Fig. 1). Exposure to 1 μM apocynin led to 63 % inhibition, whereas 10 μM apocynin yielded 69 % inhibition of maximum $\cdot O_2^-$ production. Apocynin itself does not interfere with reduction of ferricytochrome c *(12)*. Using PMA as stimulating agent, apocynin did not show any inhibitory effects (data not shown).

Measuring intracellular ROS production

Because ferricytochrome c reduction mostly detects extracellular $\cdot O_2^-$, we used an additional probe to assess ROS production. DCF measures intracellular ROS production and was used to determine whether apocynin exerts its activity intra- or extracellularly. Apocynin dose-dependently inhibited ROS-induced oxidation of DCF compared with control PMNs (Fig. 2).

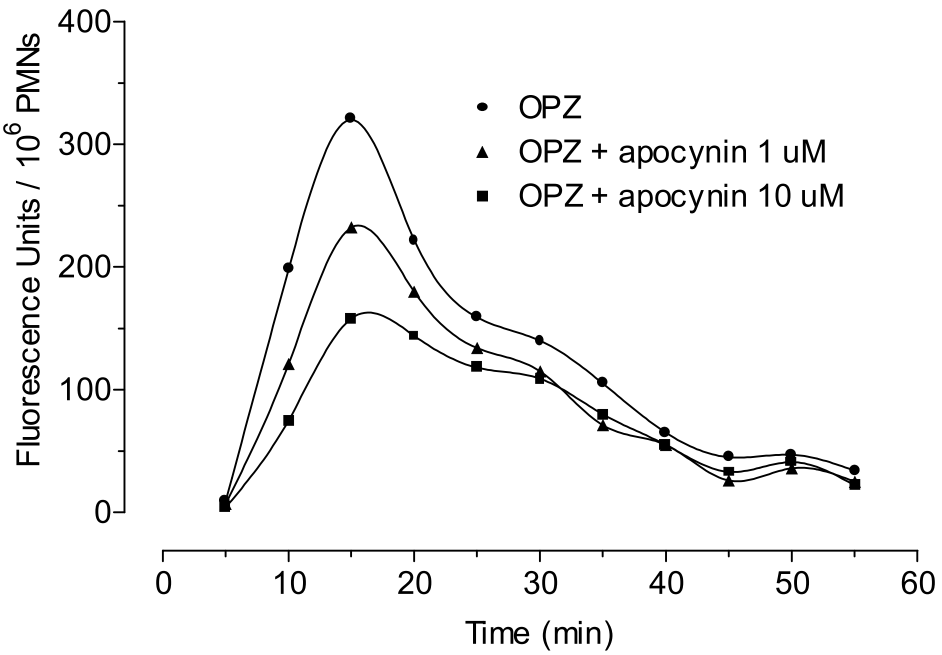

Figure 2. ***Effect of apocynin on the ROS-induced oxidation of DCF in OPZ-stimulated human neutrophils.*** *Plots depict representative curves of three separate experiments.*

Especially during the first 15 min of the measurement this inhibition was very distinct, indicating that inhibition by apocynin may be affecting the onset of the respiratory burst of OPZ-stimulated human PMNs. When cells were stimulated with PMA, no inhibitory effects of apocynin were observed (data not shown). Maximum inhibition of apocynin in this experiment, expressed in terms of percentage and compared with control PMNs, is presented in Fig. 3. Maximum inhibition of the ROS-induced fluorescence occurs at t=15 min.

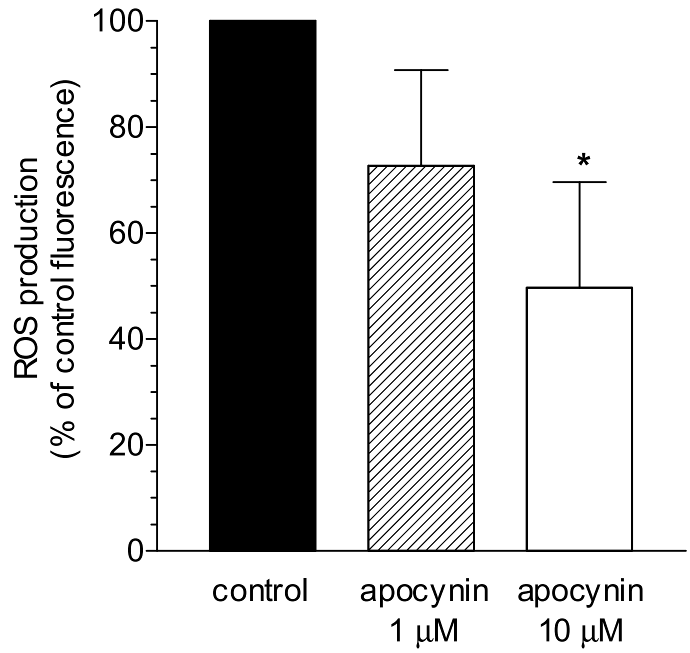

Figure 3. ***Maximum inhibition of apocynin of ROS-induced oxidation of DCF in OPZ-stimulated PMNs***. *Bars represent maximum inhibition at t=15 min and depict the Mean ± Standard Errors of the Mean (n=3).*

** Statistically significant compared with control (Students paired t-test, $p < 0.05$)*

At t=15 min, 1 μM of apocynin inhibited maximum ROS-induced fluorescence by 27.3 ± 18%, whereas 10 μM of apocynin yielded a significant inhibition of 50.4 ± 20% compared with control PMNs.

Oxygen consumption

To confirm that apocynin had a direct effect on the NADPH oxidase and to gain insight into the inhibitory effects of apocynin independent of the type of produced ROS, we assessed respiratory burst activity by measuring the oxygen consumption of human PMNs upon OPZ stimulation. Upon stimulation, PMNs show a dramatic increase in oxygen uptake, a process known as the oxidative burst *(32)*. In OPZ-stimulated PMNs, effects of apocynin on this consumption of oxygen, preceding the production of ROS, were measured. The inhibitory effect of incubation with 10 μM apocynin (curve B) compared with control PMNs (curve A) is depicted in Fig. 4. Remarkably, a lag time of about 5 minutes could be observed, before apocynin exerted its inhibitory activity. No significant differences in the Vmax of control and apocynin-treated PMNs could be observed during the first 5 minutes (15.2 vs. 15.4 nmoles $O_2/10^6$ PMNs/min respectively). However, in the period between 5 and 15 min, a 58% decrease in Vmax of the apocynin-treated cells could be observed compared with control cells (2.5 vs. 5.9 nmoles $O_2/10^6$ PMNs/min respectively). Apocynin did not inhibit oxygen consumption upon stimulation with PMA (data not shown).

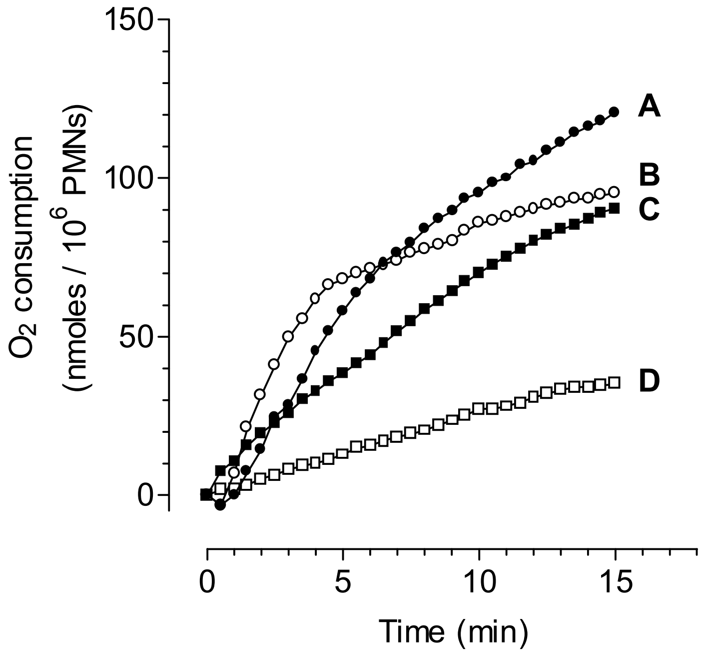

Figure 4. ***Effect of apocynin on the oxygen consumption of OPZ-stimulated human neutrophils.***
Curve A represents oxygen consumption of OPZ-stimulated PMNs without apocynin (control).
Curve B represents oxygen consumption of OPZ-stimulated PMNs in the presence of 10 μM apocynin.
Curve C represents an experiment in which PMNs were incubated with a sub-optimal concentration of OPZ (10 mg/mL) at 37°C. After 7 min, this reaction-mixture was centrifuged (3 min at 250 x g), the pellet was washed twice with cold buffer and resuspended in fresh buffer to its original volume. Finally, cells were stimulated again with OPZ (10 mg/mL), and oxygen consumption was measured (control).
Curve D represents an experiment similar to that depicted by curve C, but in which PMNs were incubated with 10 μM apocynin in the presence of OPZ (10 mg/mL) at 37 °C for 7 min. After being washed and resuspended, PMNs were stimulated again with OPZ and oxygen consumption was measured.

To assess the effect of intracellular conversion of apocynin on the lag time in oxygen consumption inhibition, we performed an experiment in which cells were incubated with a sub-optimal concentration of OPZ in the presence of apocynin for 7 min. Thereafter, cells were washed, resuspended in fresh buffer and oxygen consumption was measured upon a second stimulation with OPZ. Strikingly, an instant inhibition of O_2-consumption in PMNs previously incubated with both apocynin and OPZ was observed (curve D, Vmax: 2.8 nmoles $O_2/10^6$ PMNs/min). A 57% inhibition compared with control PMNs (similar incubation but without presence of apocynin) was observed (curve C, Vmax: 6.6 nmoles $O_2/10^6$ PMNs/min).

NADPH oxidase assembly at OPZ phagosomes

To investigate the mode of action of apocynin as an inhibitor of the OPZ-induced oxidative burst in human neutrophils, the translocation of the cytosolic NADPH oxidase component p47^{phox} upon stimulation was studied. PMNs in the absence or presence of 100 µM apocynin were stimulated synchronously with OPZ. At different time points phagocytosis was stopped, OPZ-containing phagosomes were isolated, and the presence of the cytosolic component p47^{phox} in the phagosome membrane fractions was detected.

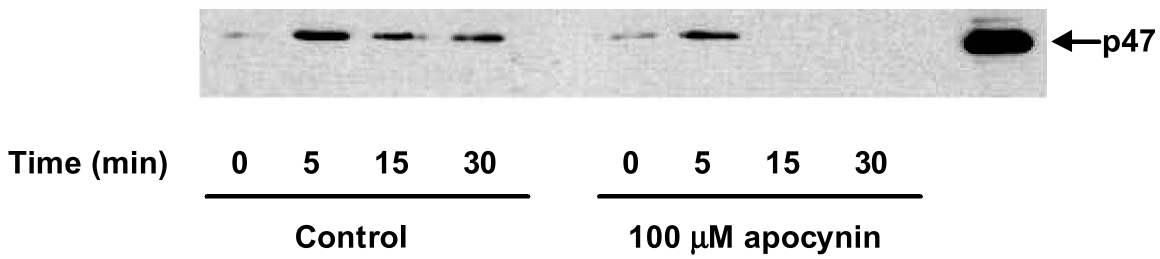

Figure 5. ***Effect of apocynin on the association of cytosolic oxidase component p47^{phox} with OPZ-containing phagosome membranes.*** *Immunoblotting was performed using specific antibodies against p47^{phox} of OPZ-phagosome membranes isolated at 0, 5, 10, 15 and 30 min after OPZ addition to control and apocynin-treated neutrophils.*

Apocynin (100 µM) significantly affected the phagosomal association of the cytosolic compound p47^{phox} to the phagosome membrane (Figs. 5 *&* 6).

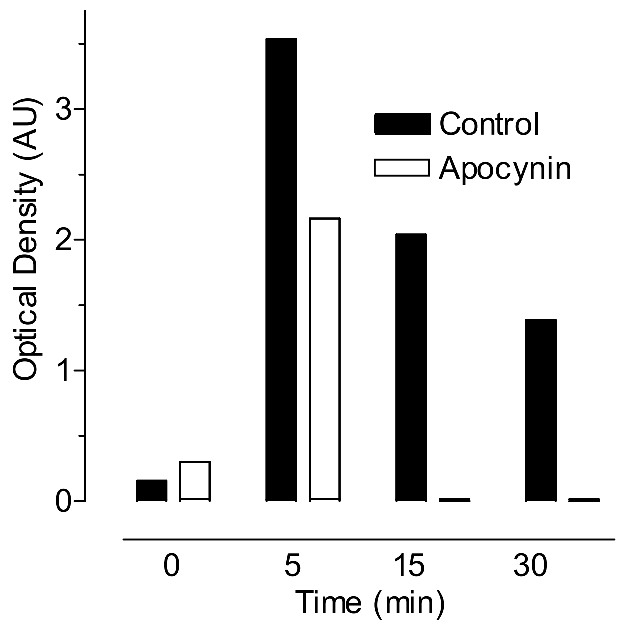

Figure 6. ***Effect of apocynin (100 µM) on the kinetics of p47^{phox} during NADPH oxidase assembly at phagosomal membranes.*** *P47^{phox} was quantitated by densitometry and optical densities are expressed as arbitrary units.*

From t=15 min, no p47[phox] could be detected in the phagosome membrane fractions of the apocynin-treated PMNs, indicating that there is a lag time in inhibitory activity. This lag time is consistent to that observed in the oxygen consumption assay.

Cell-free NADPH oxidase reconstitution assay

Data presented (Figs. 1-6) indicate that inhibition could occur at the level of assembly, activity and/or termination of the respiratory burst.
To determine whether the inhibitory effect of apocynin reflects inhibition of the assembly of the NADPH oxidase or that apocynin affects the degradation of the already formed oxidase complex, we tested apocynin in the cell-free assay. In this assay, essential components are combined *in vitro* in the presence of an amphiphile upon which a functioning NADPH oxidase complex is formed.

Incubation with apocynin (10 μM) did not inhibit the $\cdot O_2^-$-dependent reduction of ferricytochrome c when tested in the cell-free assay, compared with control cells (Fig. 7). The Vmax of the control PMNs was identical to that of the apocynin-treated PMNs (0.60 nmoles superoxide/10^6 PMNs/min).

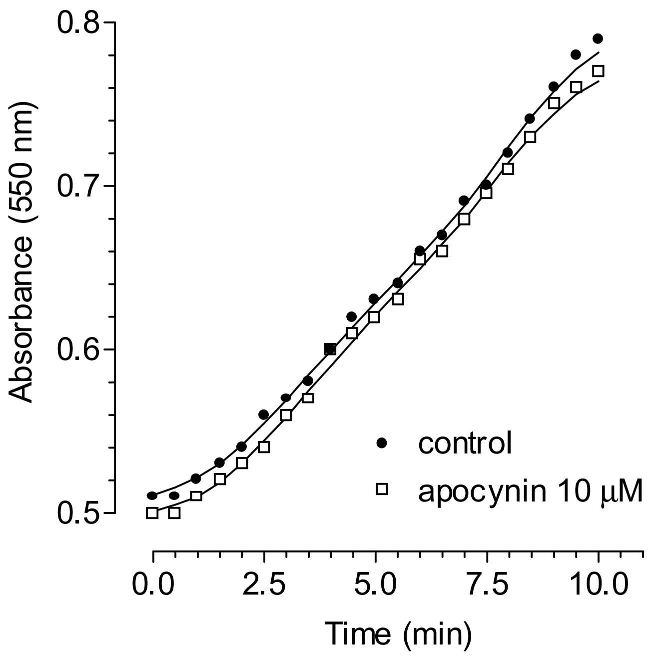

Figure 7. ***Effect of apocynin on the SDS-induced assembly of the NADPH oxidase complex in vitro, measured as the $\cdot O_2^-$induced, SOD-inhibitable reduction of ferricytochrome c.*** *Plots depict representative curves of three separate experiments.*

In an attempt to mimic the possible conversion of apocynin into its active metabolite inside PMNs, the effect of adding MPO to the assay was studied. However, even when a lysate of MPO-containing azurophilic granules was added to the assay, no significant inhibitory activity of apocynin was observed (data not shown). Moreover, a 7 min incubation of apocynin with H_2O_2 (1%) and MPO

(~ 0.01 mg/mL), prior to addition to the assay, did not result in inhibition (data not shown). Incubations were carried out in cell- free assay buffer with pH 7.2 *(27)*.

Moreover, no differences were observed between incubation with apocynin before the addition of SDS, or addition of apocynin after SDS was added to the assay.

Discussion

In order to gain insights into the mechanism by which apocynin inhibits the phagocyte NADPH oxidase we examined the effect of apocynin on the OPZ-triggered PMN respiratory burst. Results obtained in the ferricytochrome c experiments are consistent with the inhibitory effect of apocynin on the ROS production by activated PMNs measured by means of chemiluminescence as described previously by Simons *et al. (11)* and in this thesis. Apocynin proved to be a potent inhibitor of the $\cdot O_2^-$-induced ferricytochrome c reduction. Since ferricytochrome c is not able to penetrate the cell and thereby mainly detects $\cdot O_2^-$ escaping from forming phagosomes or that produced at the plasma membrane, the inhibition most probably reflects effects on nascent phagosomes. As apocynin does not show significant $\cdot O_2^-$ scavenging activity and has no effect on ferricytochrome c itself *(12)*, the effect should be ascribed to specific inhibition of the $\cdot O_2^-$ production.

DCF however, is a probe for intracellular ROS production and is reported to be primarily oxidized by H_2O_2 but also by several other intracellularly produced species of ROS *(33- 36)*, such as hydroxyl radical (·OH), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$) but not $\cdot O_2^-$ *(19)*. PMNs are incubated with dichlorofluorescein diacetate (DCFH-DA), and this non-fluorescent DCFH-DA, which is taken up by the PMNs, usually undergoes deacetylation by esterase enzymes. Oxidation of DCFH-DA by ROS within the cells leads to fluorescent dichlorofluorescein (DCF), which can easily be visualized (strong emission at 525 nm with excitation at 448 nm). This technique became popular as a way of visualizing oxidative stress in living cells *(37)*. Hence this fluorescent imaging is an assay of generalized oxidative stress rather than of production of any particular oxidizing species, and it is not a direct measure of H_2O_2 or $\cdot O_2^-$ levels. Thereby it may provide a more physiological representation of processes inside activated neutrophils. Using DCF as a probe, apocynin showed significant inhibitory activity, which indicates that apocynin passes the cellmembrane and can enter the cell where it exerts its activity.

The fact that apocynin acts as an inhibitor of NADPH oxidase-dependent ROS production in both assays does not only indicate that it can penetrate the cell. It also seems to confirm the hypothesis that apocynin, in the presence of MPO and ROS, probably is converted into its active metabolite. Evidence for this mechanism was provided by Simons *et al.* who showed that addition of sodium azide, an inhibitor of MPO, abolished the inhibitory activity of apocynin in OPZ-stimulated human PMNs

(12). Likewise, Stolk *et al*. described that in PMNs from MPO-deficient patients or in human alveolar macrophages which lack MPO *(38)*, oxygen consumption upon OPZ-stimulation could not be inhibited by apocynin *(39)*. The 'active metabolite' hypothesis seems to be supported by our experiments showing that apocynin exerted its inhibitory activity only upon stimulation with OPZ. When PMA was used as a stimulus, no inhibition was found, neither using ferricytochrome c and DCF as probes nor measuring oxygen consumption, probably due to the fact that no MPO is released upon PMA-stimulation *(40)*.

Stolk *et al*. previously suggested that apocynin inhibits ROS production in PMNs by interfering with assembly of the NADPH oxidase complex *(39)*. However, in their experiments they used cytochalasin B, which adversely affects F-actin polymerization, important for the stabilization of the cytoskeleton *(41)* and necessary for phagocytosis *(42, 43)*. Moreover, they analyzed plasma membrane fractions rather than phagosomes in their experiments *(39)*. Thus, the conditions used by Stolk *et al.* may present a rather non-physiological view of the processes during phagocytosis and NADPH oxidase assembly. Therefore, we investigated the effects on translocation of cytosolic compounds to the actual sites of phagocytosis, the phagosomes, by isolating the phagosomes containing the ingested zymosan particles.

Apocynin altered association of $p47^{phox}$ to the membrane of the phagosome. These data may indicate that apocynin act at the level of assembly of the NADPH oxidase as published by Stolk *et al.*, but accelerated termination of the already formed NADPH oxidase complex can not be excluded. The fact that the inhibition was not instantly could be explained by the fact that apocynin first has to be converted into an active metabolite inside the PMN, before actually affecting the translocation. However, this lag time also fits very well in the hypothesis that apocynin may accelerate the dissociation of the oxidase complex. This lag time also can be observed in the oxygen consumption assay in which apocynin inhibits the uptake of oxygen not until approximately 5 min after stimulation. However, when PMNs were incubated with apocynin and OPZ, centrifuged, washed, and resuspended in fresh buffer, this lag time was absent upon renewed stimulation with OPZ. When the supernatant of the OPZ-stimulated, apocynin-incubated PMNs was added to fresh PMNs, no inhibition was observed upon OPZ stimulation. This experiment may indicate the formation of an active metabolite, formed inside the PMN upon stimulation and excludes the presence of an active metabolite in the supernatant.

Another possible confirmation of this 'conversion theory' may come from the experiments in the broken-cell NADPH oxidase reconstitution assay. Use of this powerful analytical system has yielded much of our current understanding of NADPH oxidase activation and assembly *(23, 24, 44)*. In this system all the necessary components of the NADPH oxidase are re-combined *in vitro*, and addition of an amphiphile leads to a functional oxidase complex capable of producing $\cdot O_2^-$. Apocynin did not inhibit the $\cdot O_2^-$-induced reduction of ferricytochrome c in this system. Therefore, no effects of apocynin on the assembly of the NADPH oxidase complex or on deactivation/termination of the already formed complex could not be determined using this cell-free assay. Even when a lysate of MPO-containing azurophilic granules *(45)*, was added to the assay, no inhibitory activity of apocynin was observed. This effect may be explained by the absence of H_2O_2 in this system, which acts as a substrate for MPO *(46)*. However, after apocynin was incubated with H_2O_2 and MPO for 7 min before addition to the assay, still no significant inhibition was observed. This lack of activity of apocynin might well be due to the fact that this incubation did not mimic intracellular processes. It is well known that in PMNs during phagocytosis pH plays an important role *(46, 47)*, which may affect the activity of the enzyme MPO and the following reactions. Also the lack of other constituents of the intracellular granules of PMNs may play a role.

In summary, we conclude that apocynin is a potent and specific inhibitor of the respiratory burst in stimulated human neutrophils. Although, not providing definitive proof for its mechanism of action, some of our experiments indicate that apocynin has to be converted into an active metabolite and that MPO is necessary in this respect. Especially, the dependency on the presence of MPO and the lag time observed in the oxygen consumption assay and the p47phox translocation assay, support this conversion theory. However, using this experimental set up, it can not be excluded that apocynin may (also) interfere with the deactivation or accelerated dissociation of the assembled NADPH oxidase complex. Further experiments should be designed in such a way that discrimination between inhibition of assembly and accelerated termination of the NADPH oxidase complex may be possible.

Although these data may expand the understanding of the mechanism of action by which apocynin affects the NADPH oxidase activity, some aspects of apocynin certainly need further study. This underlines the importance of further investigation of specific details concerning the exact mode of action in relation with the use of apocynin in several inflammatory diseases in which ROS production plays an important role. Especially the elucidation of the identity of the active apocynin metabolite will be one of the first goals for further research.

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Chapter 3

Apocynin: a lead-compound for new respiratory burst inhibitors ?

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- Vaak wordt een uitslag verward met de situatie -

(Johan Cruijff, 1996)

Abstract

Due 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 (ROS) 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 ROS production by activated human neutrophils. Apocynin proved to be effective in the experimental treatment of several inflammatory diseases like 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 ROS 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 steroid-replacing anti-inflammatory agents.

Introduction

Reactive oxygen species (ROS), produced by stimulated polymorphonuclear leukocytes (PMNs), play an important role in host defence against invading microorganisms. Upon triggering, PMNs start to consume a large amount of oxygen which is converted into ROS, which process is known as the respiratory or oxidative burst *(1, 2)*. Although ROS 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 *(3, 4)*. Therefore, compounds that can interfere with ROS production may be useful tools to prevent tissue destruction. In our search for inhibitors of ROS production, we isolated apocynin (4'-hydroxy-3'-methoxy-acetophenone) from the roots of *Picrorhiza kurroa* by means of activity-guided isolation *(4)*. Apocynin is a potent inhibitor of the superoxide-anion ($\cdot O_2^-$)-generating NADPH oxidase of stimulated human neutrophils (IC50: 10 μM) *(5, 6)*. Additional interesting aspects of apocynin are its very low toxicity (LD50: 9 g/kg upon oral administration to mice) *(7)* and the fact that it does not interfere with the killing capacities of PMNs *(8)*.

In recent literature, there is growing interest in apocynin as an anti-inflammatory agent. Although its full spectrum of activities 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 *(9-14)*. 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 at the ring of the molecule *(15, 16)*.

Possible benificial 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.

		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	$-OCH_3$

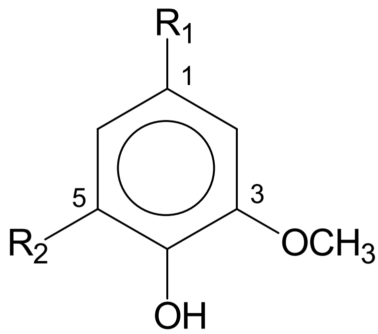

Table I. ***Structures of apocynin, vanillin, and vanillic acid and their C-5 methoxylated derivatives.***

Here, we report the activity of several analogs of apocynin differing at positions C-1 and C-5 (Table I), for their ability to inhibit ROS production by human neutrophils, induced by two different stimuli (OPZ or PMA) and measured as luminol- or lucigenin-enhanced chemiluminescence.

Materials & methods

Reagents

Apocynin, vanillin, and vanillic acid were obtained from Carl Roth GmbH (Karls-ruhe, 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 myristate acetate (PMA), 5-amino-2,3-dihydro-1,4-phthalazine-dione (luminol), bis-N-methylacridinium nitrate (lucigenin), hypoxanthine, xanthine oxidase, superoxide dismutase (SOD), propidium iodide (PI), 5-carboxy fluorescein diacetate (CFDA) were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hank's balanced salt solution (HBSS) was obtained from Life Technologies (Paisley, Scotland).

Measuring ROS production

Neutrophils were isolated from venous blood of healthy volunteers (Bloedbank Midden-Nederland, Utrecht, The Netherlands) as described by Verbrugh *et al. (17)*. 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 PMN 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 (OPZ; final concentration: 200 µg/mL) or PMA (final concentration 10 nM) and chemiluminescence was monitored every 2 min for 0.5 sec 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). OPZ was obtained by incubation of washed commercial zymosan A with 1:10 diluted human pooled serum (HPS) 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 DMSO, stored at -20 °C, and diluted in HBSS to a final concentration of 40 nM immediately before use.

Inhibition of myeloperoxidase-release

PMNs were forced to release myeloperoxidase (MPO) by incubating them (50 µL of 10^7 cells/mL in HBSS) with 100 µL OPZ (0.83 mg/mL in HBSS) at 37 °C for 15 min in U-well microtiter plates (Greiner Labortechnik, Nürtingen, Germany). After centrifugation (250 x g; 4 °C; 5 min), 100 µL of the supernatants were transferred to 96-well flat-bottom microtiter plates (Greiner Labortechnik) and MPO activity was assessed by a modified version of the method described by Henson *et al. (18)* 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 ELISA reader (SLT Labinstruments, Salzburg, Austria). Test samples were added to incubation mixtures containing PMNs and OPZ or supernatants to determine the effects on both MPO release and/or MPO activity. Since MPO 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 MPO, *e.g.* as consequence of cell rupture, was excluded by determining the viability of the neutrophils with and without OPZ incubation.

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 (SOD; 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 sec during a 15-min period using a Titertek Luminoskan luminometer. Activities of test compounds were calculated using the SOD-inhibitable part of the chemiluminescence signal.

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 (PI; 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. PMNs 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 sample amounts at 37 °C for 15 min, whereafter the cells were washed and stained with 25 µL of PI/ink solution for the detection of cellular death.

The percentage of dead cells was determined using a fluorescence microscope (Fluovert, Leitz, Wetzlar, Germany).

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.

Results

Measuring ROS production

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

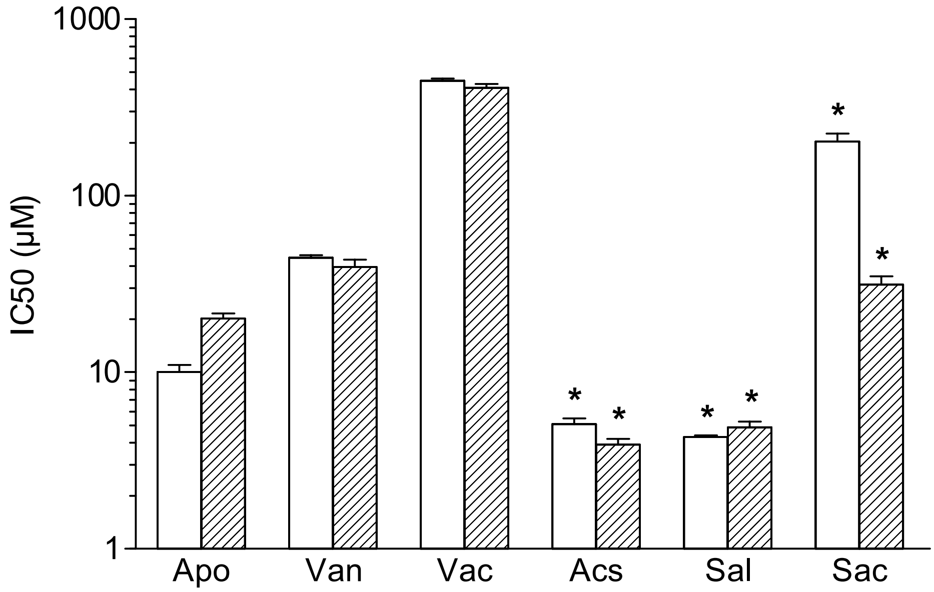

Figure 1. ***Inhibitory effects of test substances on luminol- (open bars) or lucigenin- (hatched bars) enhanced chemiluminescence response of OPZ-triggered human neutrophils (n=9).*** *For abbreveations, see table I. Values are depicted as mean IC50 values ± Standard Errors of the Mean (SEM). * Significantly different from the corresponding molecule without additional methoxy group, P < 0.05.*

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

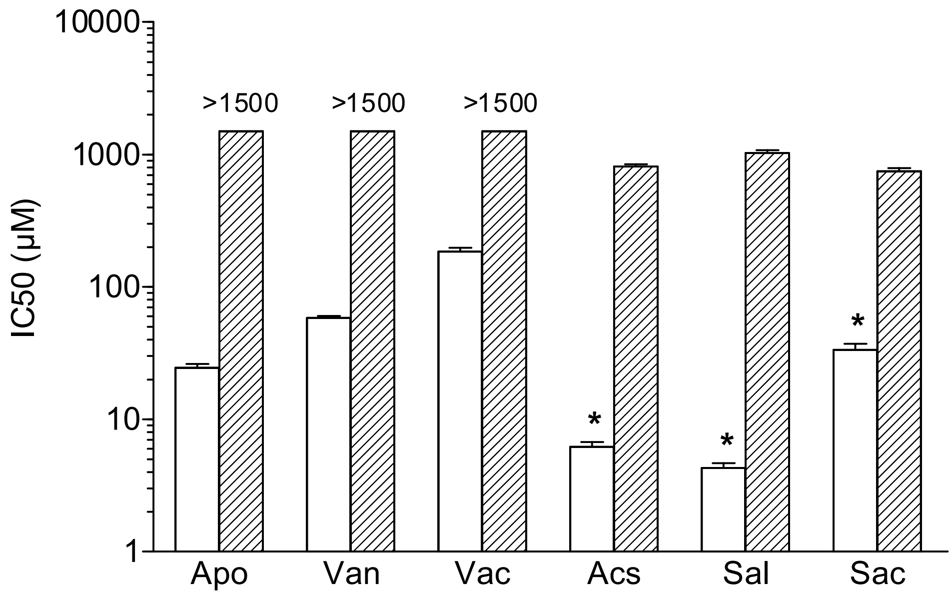

Figure 2. ***Inhibitory effects of test substances on luminol- (open bars) or lucigenin- (hatched bars) enhanced chemiluminescence response of PMA-triggered human neutrophils (n=9).***
** Significantly different from the corresponding molecule without additional methoxy group, P < 0.05.*

Inhibition of myeloperoxidase

MPO is an enzyme released from azurophilic granules by stimulated neutrophils, converting H_2O_2 into hypohalites *(19)*. These hypohalites are very reactive metabolites, which can only be detected with luminol as enhancer *(20)*. This implicates that deactivation and/or decreased release of MPO may be involved in inhibitory effects on luminol-enhanced chemiluminescence. To exclude that test substances inactivate MPO and/or inhibit its release, an MPO-inhibition assay was performed (Table II). Syringic acid and vanillic acid were the only substances interfering with MPO-mediated effects. The other compounds did not affect MPO release and/or activity. No differences in neutrophil viability between OPZ-incubated and control mixtures were observed.

Compounds	IC50 (μM)
Apocynin	> 1250
Vanillin	> 1250
Vanillic acid	165 ± 53
Acetosyringone	> 1250
Syringaldehyde	> 1250
Syringic acid	44 ± 9.6

Table II. ***Inhibitory effects on MPO release and/or activity (n=6).***

Scavenging of superoxide anions

Scavenging is the term used to describe the interference of a test substance with produced rather than the production of, in this case, reactive oxygen metabolites. To distinguish between inhibition and scavenging, the 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.

Calculated Lipophilicity

The lipophilicity (log P value) of the compounds was calculated using the method of Rekker *(21)*. 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 (data not shown).

Cytotoxicity

To exclude that the measured inhibitory effects of test substances are to be attributed to cytotoxic activities, 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 sign of toxicity (data not shown).

Discussion

In this study, we show that substitution of apocynin, vanillin, and vanillic acid with a methoxy-group at position C-5 significantly increases their ability to interfere with the generation of reactive oxygen species (ROS) by human polymorphonuclear cells (PMNs). The C-5 substitution by 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 signal-transduction that mediates neutrophil activation.

Our *in vitro* findings are consistent with *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 *(16)*. Although ROS 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 ROS after stimulation of PMNs, we used two stimuli which represent different PMN-

activation pathways. Opsonized zymosan (OPZ), was used as a model system for opsonized microorganisms. OPZ consists of cell walls of baker's yeast coated with IgG, mannose-binding lectin, and $C3_{b(i)}$ complement fragments *(22)*. Phorbol myristate acetate (PMA) is a soluble agent activating PMNs directly at the level of protein kinase C (PKC) which also leads to the activation of the respiratory burst *(23)* (Fig 3).
Although OPZ and PMA both stimulate the $\cdot O_2^-$-generating NADPH oxidase, their transductional mechanisms within the neutrophil are quite different *(24)*.

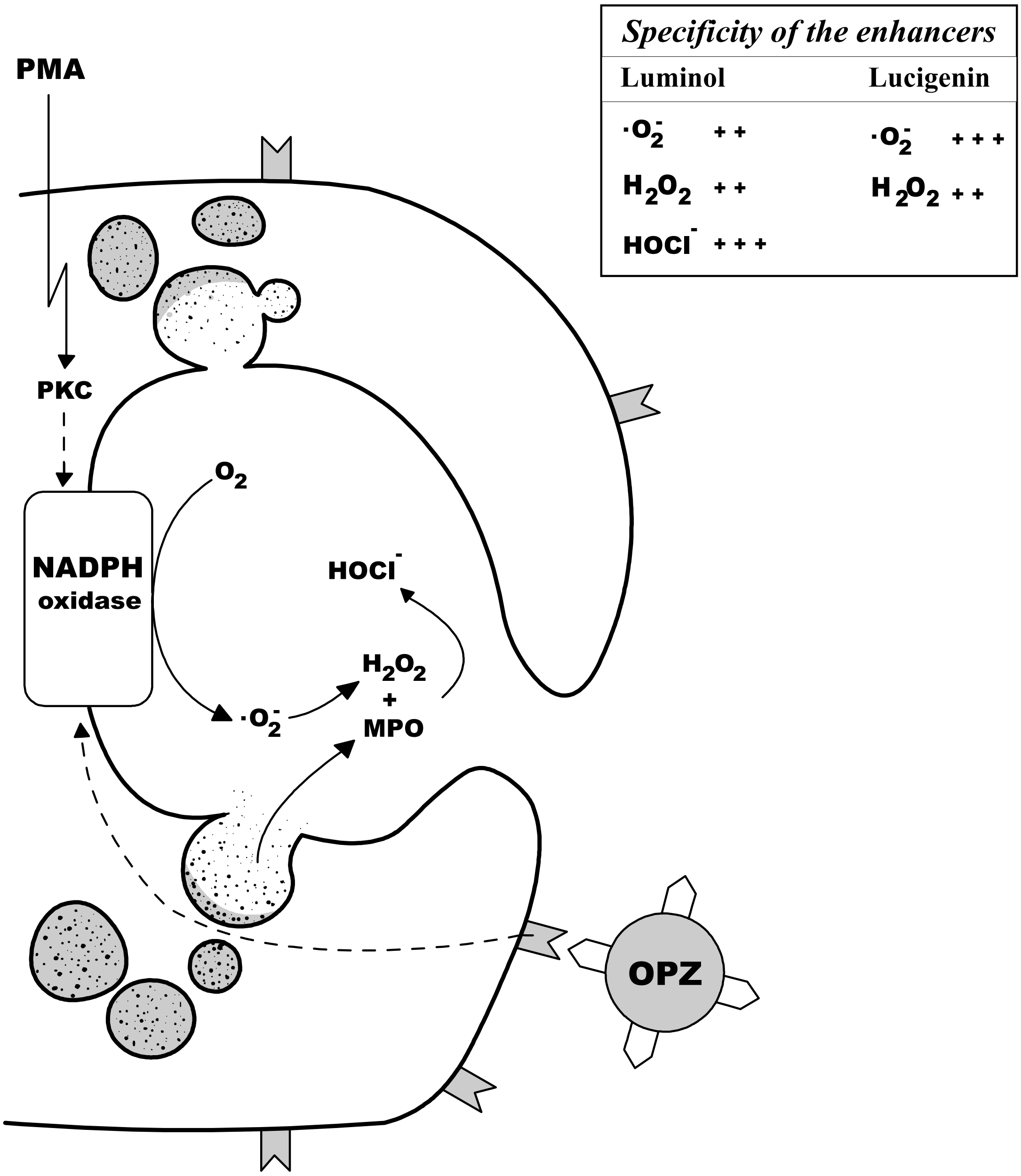

Figure 3. *Schematic representation of processes during the respiratory burst of neutrophils upon stimulation with OPZ or PMA, and specificity of the chemiluminescence probes luminol and lucigenin.*

The exact differences governing respiratory burst-triggering by these two stimuli, however, are not fully understood yet.

The decreased ROS generation by PMNs 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 ROS-inhibitory activity. Future experiments have to show if these analogs have a similar mode of action as proposed for apocynin.

Strikingly, when luminol was used as enhancer molecule, this ROS-inhibiting effect was more or less independent of the stimulus used. 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 particular 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 ROS production *(25)*, whereas the site of action of lucigenin is the extracellular space, most probably since PMNs are practically impermeable to lucigenin *(26)*. Another aspect is that luminol-enhanced chemiluminescence is mainly dependent on the MPO-H_2O_2 system, whereas experiments with MPO-deficient PMNs have indicated that lucigenin-dependent chemiluminescence is independent of the MPO-H_2O_2 system *(27)* (Fig 3).

A more likely explanation for the lack of inhibitory activity of apocynin and analogs in PMA-induced, lucigenin-enhanced chemiluminescence could be the fact that apocynin supposedly needs metabolic conversion by the combined action of MPO and ROS to become activated *(5)*. In contrast to OPZ, stimulation of human neutrophils with PMA results in little or no MPO release *(28)* which may explain the absence of inhibitory activity of apocynin upon PMA stimulation. Furthermore, it is known that apocynin and analogs exhibit more or less H_2O_2-scavenging capacity *(8)*, 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 ROS production in this set up, which is in favor of the theory of metabolic activation and excludes a major role of H_2O_2- scavenging activity.

Besides being a potent inhibitor of ROS generation, apocynin also has other significant anti-inflammatory properties *(29-32)* 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 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 highly contribute to unravel the mechanisms underlying the anti-inflammatory activity of apocynin and to develop new apocynin-related anti-inflammatory drugs.

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Chapter

4

Apocynin inhibits peroxynitrite formation by murine macrophages

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- Voordat ik een fout maak, maak ik die fout niet -

(Johan Cruijff, 1997)

Abstract

Peroxynitrite ($ONOO^-$), the highly reactive coupling product of nitric oxide (NO) and superoxide anion ($\cdot O_2^-$), has been implicated in the pathogenesis of an increasing number of (inflammatory) diseases. At present, however, selective peroxynitrite antagonizing agents with therapeutic potential are not available. Therefore, apocynin (4′-hydroxy-3′-methoxy-acetophenone), a potent inhibitor of NADPH oxidase-dependent $\cdot O_2^-$ production was tested for its ability to interfere with peroxynitrite formation *in vitro*.

The murine macrophage cell-line J774A.1, stimulated with IFNγ/LPS, was used as a model system. Conversion of 123-dihydrorhodamine (123-DHR) to its oxidation product 123-rhodamine was used to measure peroxynitrite production.

Stimulated peroxynitrite formation could be completely inhibited by apocynin, by the superoxide scavenger TEMPO, as well as by the nitric oxide synthase inhibitor aminoguanidine. Apocynin and aminoguanidine specifically inhibited superoxide anion and nitric oxide formation, respectively, which was confirmed by measuring nitrite accumulation and lucigenin-enhanced chemiluminescence.

We conclude that upon stimulation, J774A.1 macrophages produce significant amounts of peroxynitrite, which is associated with nitric oxide production and NADPH oxidase-dependent superoxide anion formation. The NADPH oxidase inhibitor apocynin is a potent inhibitor of both superoxide anion and peroxynitrite formation by macrophages, which may be of future therapeutic significance in a wide range of inflammatory disorders.

Introduction

Peroxynitrite, a relatively stable intermediate, is formed by the diffusion-limited reaction of the free radicals nitric oxide (NO) and superoxide anion ($\cdot O_2^-$) *(1-3)*. Inflammatory cells such as neutrophils *(4)* and macrophages *(5)*, but also endothelial cells *(6)* can release $\cdot O_2^-$ and/or NO, potentially leading to peroxynitrite ($ONOO^-$) formation. Peroxynitrite is a highly reactive compound with various harmful effects on cells *(7)* and could therefore be an important microbicidal compound.

In contrast to the possible beneficial effects of peroxynitrite in host defense mechanisms, the anion may have deleterious effects on host tissues. A role for peroxynitrite has been hypothesized in a number of disorders. Examples are human asthma *(8)*, acute lung injury *(9)*, idiopathic pulmonary fibrosis *(10)*, inflammatory bowel disease *(11)*, and animal models for septic shock *(12)*. Inhibition of peroxynitrite formation, by inhibiting either NO or $\cdot O_2^-$ production, could be a useful tool to limit tissue damage in various circumstances.

Apocynin is a strong inhibitor of the reactive oxygen species (ROS)-generating enzyme NADPH oxidase in PMNs *(13)*. This NADPH oxidase complex is also present in macrophages *(14, 15)* and, since peroxynitrite is dependent on both NO and $\cdot O_2^-$, apocynin may also be an inhibitor of peroxynitrite formation as well. This may be of importance because, at present, specific iNOS inhibitors or peroxynitrite scavengers which can be used *in vivo* are not available. Apocynin has previously been demonstrated to be a powerful anti-inflammatory agent in rat models for arthritis *(16)*, colitis *(17)*, and ulcerative skin lesions *(18)* and in a rabbit model for atherosclerosis *(19)*.

In this study, apocynin was tested for its ability to inhibit peroxynitrite formation by murine macrophages. Therefore, the 123-DHR oxidation assay was validated as an index of peroxynitrite formation by immuno-stimulated J774A.1 macrophages, providing a tool to screen different compounds for their ability to prevent peroxynitrite formation. Evidence for the dependence of 123-DHR oxidation on both nitric oxide and superoxide was obtained by the use of inhibitors of nitric oxide synthase (aminoguanidine) and by the superoxide scavenger TEMPO (2,2,6,6-Tetramethylpiperidin). The specificity of aminoguanidine and apocynin was confirmed by measuring lucigenin-enhanced chemiluminescence and nitrite accumulation, respectively.

Materials & Methods

Materials

Apocynin was obtained from Carl Roth GmbH (Karlsruhe, Germany) and was further purified by recrystallization from water.

Aminoguanidine, LPS (E. coli 0111:B4), sulfanilamide, naphthyl-ethylene-diamide, lucigenin, PMA, and TEMPO (2,2,6,6-Tetramethylpiperidin) were obtained from Sigma (St. Louis, MO, USA). Murine recombinant IFNγ was obtained from Genetech Ltd. (USA), 123-DHR from Molecular Probes Europe (Leiden, The Netherlands), and $NaHCO_3$ and $NaNO_2$ from Merck (Darmstadt, Germany).

Murine Cell Culture

J774A.1 (ATCC, Manassas, VA, USA) macrophages were maintained in RPMI 1640 (supplemented with 10% fetal bovine serum, 10 mM Hepes, 4 mM glutamate, 2 mM pyruvate, 50 µg/mL gentamycin, Penicillin/Streptomycin and 100 µM β-mercapto-ethanol, all from Gibco-BRL, Paisley, Scotland) and cultured at 37 °C with 5% CO_2. Cells were passaged every 3 days.

Oxidation of 123-Dihydrorhodamine

Cells were incubated for 20 h with or without stimuli and/or inhibitors in the presence of 25 µM 123-dihydrorhodamine (123-DHR) in culture medium in 96-well microtiter plates. After incubation, 123-DHR conversion into 123-rhodamine was measured by fluorimetric analysis at excitation/emission wavelengths of 485 and 530 nm, respectively (Cytofluor 2350, B&L Systems, Maarssen, The Netherlands). Fluorescence due to auto-oxidation of 123-DHR was subtracted from the original measurements. Furthermore, oxidation of 123-DHR by the peroxynitrite donor SIN-1 (3-morpholinosydnonimine) was measured in the presence of 300 µM apocynin or 1.0 mM aminoguanidine in culture medium (2 h, 37 °C) as described above.

Griess assay

Nitrite concentrations were measured using the Griess reaction *(20)*. Briefly, 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamide in 5% phosphoric acid) was added to 100 µL of sample medium. After a 10-min incubation at room temperature, optical density was measured at 550 nm using a microplate reader (Bio-Rad, CA, USA). Calibration curves were obtained using $NaNO_2$ dissolved in incubation medium.

Measuring $\cdot O_2^-$ production

Macrophages, pre-stimulated overnight with IFNγ (50 U/mL) and LPS (10 µg/mL), were incubated in white 96-well (10^6 cells/well, 200 µL), flat-bottom microtiter plates, in the presence of lucigenin (400 µM) with and without inhibitors. Macrophages were additionally stimulated with PMA (phorbol-12-myristate-13-acetate, 10 nM). Chemiluminescence was monitored every 3 min for 0.5 s, during a period of 90 min using a Titertek luminoskan luminometer (TechGen International, Zellik, Belgium). Peak levels were used to quantitate chemiluminescence. Subsequently, the nitrite concentration in the medium was detected using the Griess assay. Experiments were performed in Hank's balanced salt solution (HBSS, Life Technologies, Paisley, Scotland) buffered at pH 7.35 with $NaHCO_3$.

Data analysis

All data were expressed as mean ± standard error of the mean (SEM). Data were statistically analyzed using ANOVA followed by post-hoc pair-wise comparison of the effects of different inhibitor concentrations compared to control levels. Results were considered statistically different at the $P < 0.05$ level.

Results

Oxidation of 123-DHR and nitrite accumulation

To determine the effect of peroxynitrite formation by stimulated murine macrophages, the oxidation of 123-DHR and nitrite accumulation was assessed. Upon combined stimulation with recombinant murine IFNγ (50 U/mL) and LPS (10 µg/mL) a significant (P<0.001) increase in 123-rhodamine formation and nitrite accumulation as compared with unstimulated macrophages was detectable in the 20-h supernatants (Fig. 1).

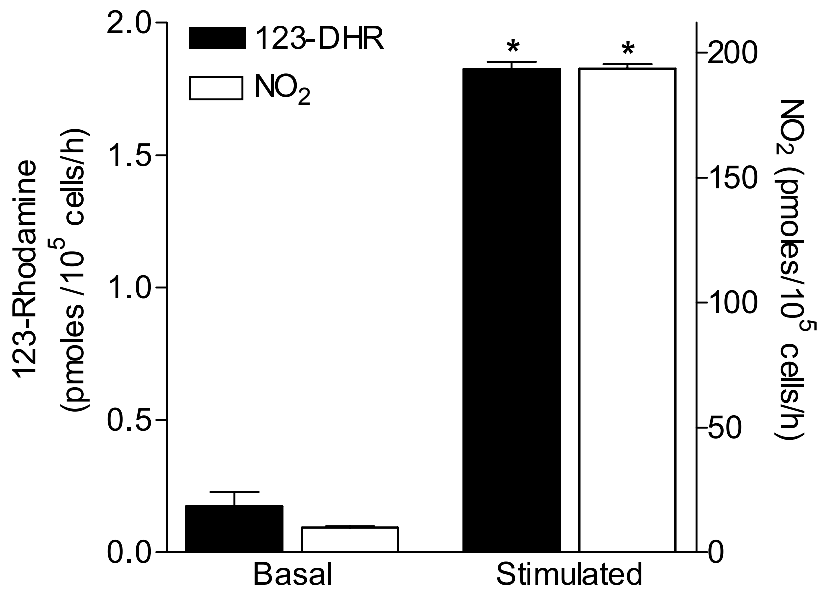

Figure 1. ***Effect of IFNγ (50 U/mL) and LPS (10 µg/mL) on 123-DHR oxidation (black bars) and nitrite accumulation (open bars) by J774A.1 macrophages compared with unstimulated cells (basal); (10^6 cells/mL incubated for 20 h).*** *Data represent mean ± SEM of six wells from three independent experiments. * Statistically significant difference (P<0.001) compared with controls.*

Effects of aminoguanidine, apocynin, and TEMPO on this stimulated 123-DHR oxidation and nitrite accumulation were determined after a 20-h incubation period of the cells in the presence of these compounds.

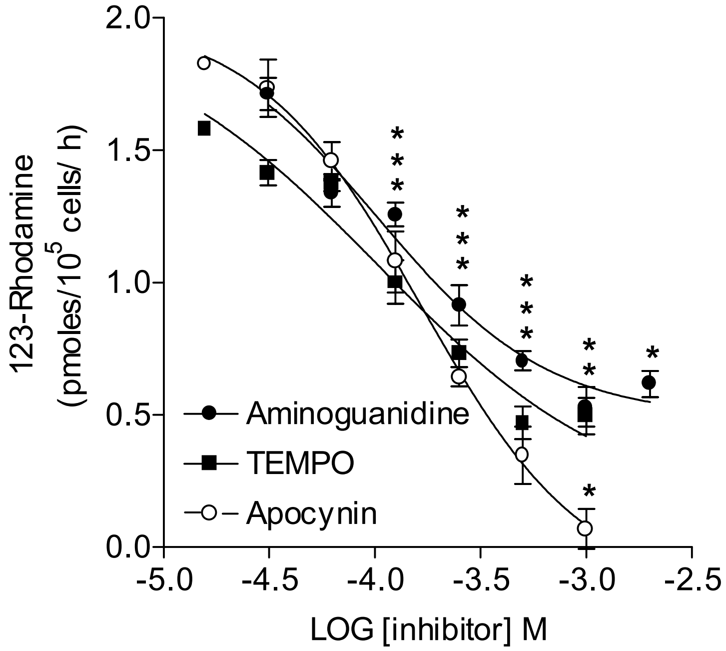

Figure 2. ***Effect of increasing dosages of aminoguanidine, TEMPO, and apocynin on IFNγ/ LPS stimulated 123-DHR oxidation by J774A.1 macrophages during 20 h of incubation.*** *Data represent mean ± SEM of six wells from three independent experiments. * Statistically significant difference (P<0.0001) compared with control incubations.*

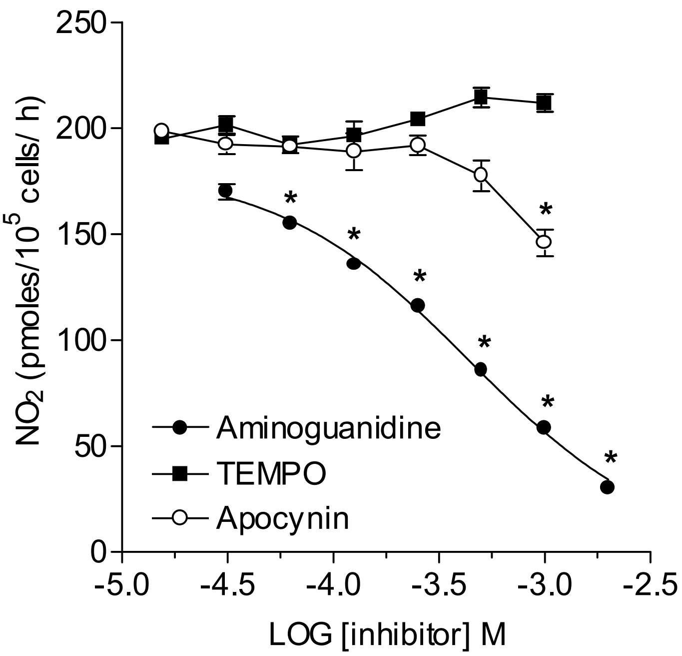

Figure 3. ***Effect of increasing dosages of aminoguanidine, TEMPO, and apocynin on IFNγ/ LPS stimulated nitrite accumulation by J774A.1 macrophages during 20 h of incubation.*** *Data represent mean ± SEM of six wells from three independent experiments * Statistically significant difference (P<0.0001) compared with control incubations.*

The nitric oxide synthase inhibitor aminoguanidine dose-dependently inhibited 123-rhodamine and nitrite accumulation with logEC50 values of -3.9 ± 0.06 M (125 μM) and –3.4 ± 0.03 M (398 μM), respectively (Figs. 2 & 3). Furthermore, the superoxide scavenger TEMPO (2,2,6,6-Tetramethylpiperidin) dose dependently inhibited 123-DHR oxidation [logEC50 –4.0 ± 0.1 M, (100 μM)] (Fig. 2), whereas nitrite accumulation was not affected by TEMPO (Fig. 3). The NADPH oxidase inhibitor apocynin completely inhibited 123-rhodamine accumulation [logEC50 –3.7 ± 0.03 M (199 μM)] (Fig. 2), whereas nitrite concentrations were practically unaffected (Fig. 3).

123-DHR oxidation by SIN-1

The peroxynitrite scavenging abilities of apocynin and aminoguanidine were investigated using the peroxynitrite donor SIN-1 (3-morpholinosydnonimine) which releases equimolar amounts of nitric oxide and superoxide anion. Neither apocynin (300 μM) nor aminoguanidine (1.0 mM) inhibited the SIN-1 mediated oxidation of 123-DHR (Fig. 4).

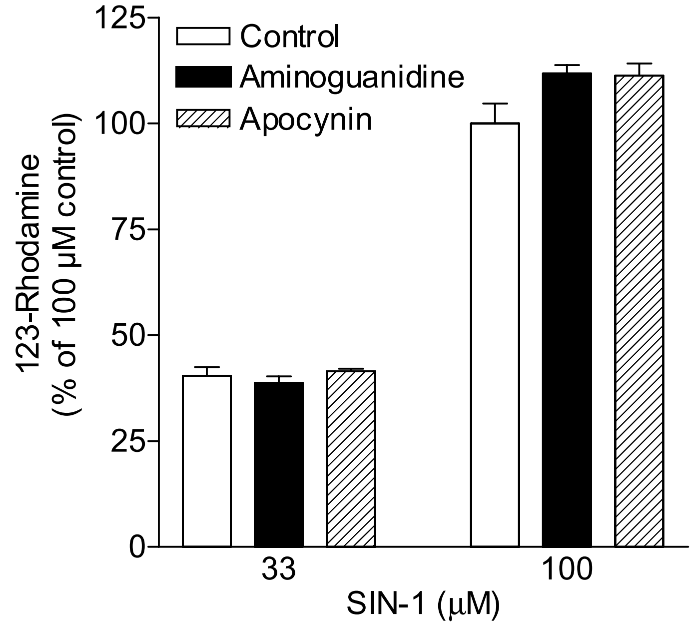

Figure 4. ***Effect of 1mM aminoguanidine (black bars) and 300 μM apocynin (hatched bars) on 123-DHR oxidation by the peroxynitrite donor SIN-1 (33 and 100 μM) in cell-culture medium compared with control incubations (open bars).*** *Data represent mean ± SEM of four replicate wells.*

Lucigenin-enhanced chemiluminescence

Lucigenin-enhanced chemiluminescence (LUC-CL) was measured in cells pre-stimulated with IFNγ and LPS overnight and additionally stimulated with PMA (10nM) just prior to chemiluminescence measurements. Apocynin dose-dependently inhibited LUC-CL (Fig. 5) with a logEC50 –3.5 ± 0.1 M (316 μM). Aminoguanidine,

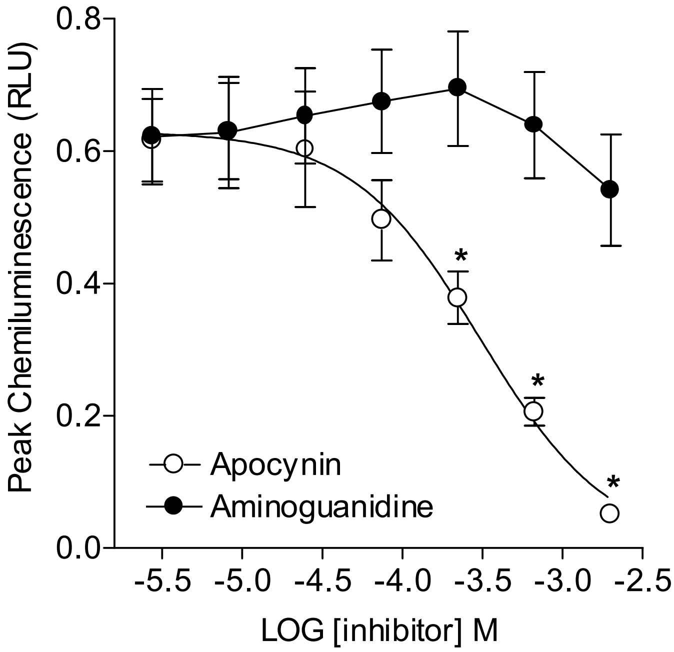

Figure 5. ***Effect of apocynin and aminoguanidine on peak lucigenin-enhanced chemiluminescence by J774A.1 macrophages pre-stimulated with IFNγ (50 U/mL) and LPS (10 μg/mL) for 20 h and additionally stimulated with PMA (10 nM) just prior to chemiluminescence measurements.*** *Data represent mean ± SEM of six replicate wells from two independent experiments. * Statistically significant difference (P<0.001) compared with controls.*

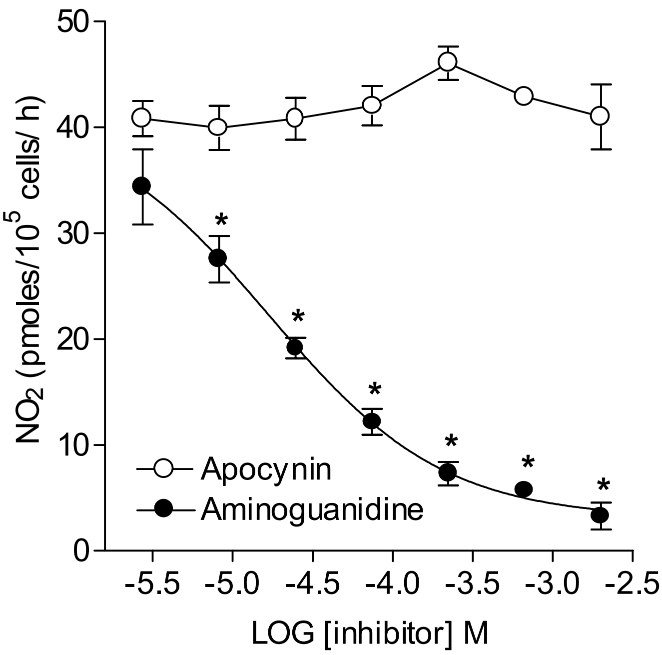

Figure 6. ***Effect of apocynin and aminoguanidine on nitrite accumulation after 90 minutes by J774A.1 macrophages pre-stimulated with IFNγ (50 U/mL) and LPS (10 μg/mL) for 20 h and additionally stimulated with PMA (10 nM).*** *Data represent mean ± SEM of six replicate wells from two independent experiments. * Statistically significant difference (P<0.001) compared with controls.*

however, did not affect LUC-CL (Fig. 5). After the 90-min chemiluminescence incubation, nitrite measurements revealed that aminoguanidine inhibited NO output with a logEC50 of 4.7 ± 0.1 (20 µM) during this short incubation period (Fig. 6). Apocynin did not affect nitrite accumulation in this system, not even at the highest concentration used (2 mM).

Discussion

It is important to stress that 123-DHR oxidation can only be attributed to peroxynitrite formation when shown to be dependent on both nitric oxide and superoxide anion. 123-DHR oxidation by stimulated human neutrophils, for example, is largely dependent on hydrogen peroxide formation and peroxidase activity *(21)* showing that 123-DHR is certainly not a specific probe for peroxynitrite. Inhibition of nitric oxide synthase by aminoguanidine revealed that the release of reactive oxygen species alone cannot explain 123-DHR oxidation measured in stimulated macrophages. Similarly, scavenging (TEMPO) or inhibition of superoxide formation (apocynin) showed that 123-DHR is insensitive to nitric oxide. Hence, 123-DHR oxidation by immuno-stimulated J774A.1 macrophages is mainly dependent on the simultaneous release of nitric oxide and superoxide anion and is therefore attributed to peroxynitrite considering the likelihood of the interaction between the two precursors *(22)*. Moreover, the present data suggest that NADPH oxidase is the main source of superoxide leading to peroxynitrite formation in J774A.1 macrophages since 123-DHR oxidation was completely inhibited by the NADPH oxidase inhibitor apocynin.

Neither apocynin nor aminoguanidine are scavengers of superoxide anion, nitric oxide, or peroxynitrite, since neither of the compounds inhibited SIN-1 mediated 123-DHR oxidation. SIN-1 is a donor of equimolar amounts of superoxide and nitric oxide and consequently of peroxynitrite *(23)*. Specificity of the inhibitors was further supported by lucigenin-enhanced chemiluminescence, which detects reactive oxygen species, but is insensitive to peroxynitrite and hypochlorous acid *(24)*. In this study, lucigenin-enhanced chemiluminescence could be completely blocked by apocynin, but was unaffected by aminoguanidine. The other way around, nitrite accumulation during these incubations was completely inhibited by aminoguanidine and was unaffected by apocynin.

The fact that apocynin and TEMPO did not enhance nitrite accumulation in the medium suggests that peroxynitrite mainly decays into nitrite during the incubations. The exact metabolic fate of peroxynitrite once formed in a biological environment, however, is not exactly clear at present. For example, several studies have shown an important role of carbon dioxide in peroxynitrite-mediated reactions *(25-27)*. Furthermore, the activities/concentrations of cellular antioxidant mechanisms are likely to play a pivotal role in downstream events initiated by peroxynitrite. Therefore, the reaction conditions for peroxynitrite and 123-DHR in the presence or inside cells may differ significantly from those in cell-free systems. For this reason, the calibration of the 123-DHR assay to exact concentrations of peroxynitrite as detected in cell-free systems may be questionable. Moreover, it is unknown whether peroxynitrite itself or downstream metabolites of the anion are responsible for the 123-DHR oxidation detected in immuno-stimulated macrophages.

Although the evidence for peroxynitrite formation *in vivo* is convincing, recent studies have suggested that other reactive nitrogen species maybe equally, or even more important mediators of oxidative tissue modifications during inflammatory processes *(28)*. The formation of the putative peroxynitrite footprint 3-nitrotyrosine, for example, has been demonstrated to be readily catalyzed by myeloperoxidase with hydrogen peroxide and nitrite as substrates *(29)*. Therefore, nitric oxide formation as such may not be an essential factor in reactive nitrogen species-mediated tissue alterations during inflammation. In contrast, superoxide and its downstream metabolites appear to play a pivotal role in all pathways leading to reactive nitrogen species described above. Consequently, limiting superoxide anion production by apocynin may prevent the formation of peroxynitrite as well as other reactive nitrogen species.

The present data clearly show that IFNγ and LPS-stimulated murine J774A.1 macrophages release high amounts of NO and superoxide anion, most probably leading to peroxynitrite formation. Moreover, stimulated 123-DHR oxidation by J774A.1 macrophages is dependent on both $\cdot O_2^-$ and NO, and therefore likely to be dependent on peroxynitrite or downstream metabolites of peroxynitrite.

This is the first time that the NADPH oxidase inhibitor apocynin is shown to be a potent inhibitor of 123-DHR oxidation by murine macrophages *in vitro*. Apocynin may therefore have potential therapeutic value as a tool to limit peroxynitrite formation in inflammatory conditions *in vivo*. Considering the lack of specific iNOS inhibitors that can be used therapeutically, apocynin could have additive value since it is not only a potent inhibitor of superoxide anion release, but also a powerful inhibitor of peroxynitrite formation.

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Chapter

5

Isolation, characterization and activity of diapocynin, an apocynin metabolite

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- Elk nadeel hebt zijn voordeel -

(Johan Cruijff, 1997)

Abstract

Apocynin is a potent inhibitor of NADPH-dependent ROS production in stimulated human PMNs. However, its mode of action has been subject of speculations. It has been assumed that apocynin is converted into a metabolite which accounts for its activity. A dimer of apocynin, diapocynin, was suggested to be this active compound. We established the formation of diapocynin upon incubation of apocynin with MPO and hydrogen peroxide. In addition, we showed the conversion of apocynin into diapocynin by OPZ-stimulated PMNs; the identity of diapocynin was unambiguously confirmed by NMR spectroscopy and mass spectrometry. Diapocynin was also chemically synthesized to obtain sufficient amounts for *in vitro* testing. Its activity on ROS-generation and oxygen consumption by stimulated PMNs, however, seems not consistent with the existing theories. Diapocynin was shown to be less active than apocynin, and, in contrast, appeared to be a potent scavenger of superoxide anions. We hypothesize that conversion of apocynin into diapocynin in activated PMNs results in an increased MPO activity which eventually accelerates the termination of the respiratory burst, in this way inhibiting production of ROS. Although not (very) active as exogenous agent, diapocynin as endogenous metabolite is most active.

Introduction

Reactive oxygen species (ROS) production by activated polymorphonuclear neutrophils (PMNs) plays an important role in the host defense against invading microorganisms. However, excessive production of ROS sometimes leads to tissue damage *(1,2)*. Therefore, inhibition of ROS production may be beneficial in certain inflammatory conditions *(3,4)*.

Apocynin is a potent and selective inhibitor of the NADPH oxidase-dependent production of ROS by stimulated human PMNs *(5)*. Apocynin was isolated by activity-guided isolation from *Picrorhiza kurroa (6),* and in the years following its discovery it has been used in many laboratories around the world. Although apocynin exhibits most promising activities in experimental animal models for colitis *(7)*, rheumatoid arthritis *(8)*, and atherosclerosis *(9)*, its exact mechanism of action has not been well defined. It has been proposed that conversion of apocynin into an active metabolite is a prerequisite for displaying inhibitory activity *(5, 10)*. This conversion is supposed to take place by the combined action of myeloperoxidase (MPO), an enzyme released from azurophilic granules by activated PMNs *(11)*, and the produced ROS. In literature, some structures have been reported for this 'active' apocynin metabolite. 't Hart *et al.* have suggested the formation of a quinone methide *(10)*, but so far no affirmative data have been published. In a United States Patent, Holland *et al*. have described the formation of an apocynin dimer, named diapocynin, upon treatment of apocynin with H_2O_2 and horse radish peroxidase (HRP) and claim this compound to be the active metabolite *(12)*. However, evidence concerning its identity as well as its actual formation in PMNs is lacking.

We decided to further investigate whether the formation of the postulated 'active' apocynin dimer actually occurs. Besides cell-free transformations using HRP as well as MPO, also *in vitro* conversion of apocynin by activated PMNs was studied. In addition, diapocynin was subjected to several *in vitro* assays to gain insight into its significance in the inhibition of NADPH oxidase-dependent ROS formation.

Materials & Methods

Reagents

Apocynin was purchased from Carl Roth GmbH (Karlsruhe, Germany). Before use, apocynin was purified by recrystallization from water. Hank's balanced salt solution (HBSS) was obtained from Life Technologies (Paisley, Scotland). MPO was a gift from Dr. J.P. Weiss, Department of Internal Medicine, University of Iowa College of Medicine, IA, USA. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless specified otherwise.

Derivatization of apocynin and GC-MS analysis

Apocynin was (trimethyl)silylated in ethyl acetate with MSTFA [N-methyl-N-(trimethylsilyl)trifluoroacetamide; Sigma/Aldrich, Zwijndrecht, The Netherlands] in a sealed vial at 70 °C for 40 min. The TMS derivative was analyzed using a Chrompack CP Sil 8 CB column (30 m; internal diameter 0.25 mm; film thickness 0.25 μm). After injection of the sample, the initial oven temperature of 100 °C was maintained for 1 min and then raised to 290 °C with a rate of 8 °C/min. The latter temperature was maintained for 5 min [Rt apocynin-TMS 12.20 min].

Apocynin: Spectral data and physical constants

Melting point: 111 °C

^{1}H NMR: For ^{1}H chemical shifts of apocynin in DMSO-d_6 and $CDCl_3$ (300 MHz) see Table 1. (NMR spectra were recorded using a Varian G-300 spectrometer).

^{13}C NMR (APT and HETCOR; DMSO-d_6; 75 MHz): δ 196.3 (C; C=O), 151.9 (C; C-3), 147.7 (C; C-4), 129.1 (C; C-1), 123.6 (CH; C-6), 115.1 (CH; C-5), 111.3 (CH; C-2), 55.8 (CH_3; OCH_3), 26.4 (CH_3)
^{13}C NMR-APT ($CDCl_3$; 75 MHz): δ 196.7 (C; C=O), 150.4 (C; C-3), 146.5 (C; C-4), 130.1 (C; C-1), 123.8 (CH; C-6), 113.7 (CH; C-5), 109.7 (CH; C-2), 55.9 (CH_3; OCH_3), 25.9 (CH_3)

EI-MS: (Relative abundances are given in parentheses)
[apocynin-TMS] *m/z*: 238 (51) $[M]^+$, 223 (94) $[M-CH_3]^+$, 208 (61) $[M-2CH_3]^+$, 193 (100), 165 (10) $[M-TMS]^+$, 149 (4) $[M-OTMS]^+$.

Synthesis of diapocynin

Diapocynin (5,5′-dehydrodiacetovanillone) was synthesized according to a method described by Elbs *et al.* for the synthesis of dehydrodivanillin *(13)*. To a solution of 1.0 g of apocynin (6 mMol) in 200 mL of hot water were added 75 mg of ferrous sulfate heptahydrate (0.3 mMol) and 810 mg of potassium persulfate (3 mMol). The reaction mixture was stirred on a boiling water bath for 30 min. After cooling, the precipitate was collected and dissolved in 4N sodium hydroxide. Subsequently, diapocynin was precipitated by addition of 4N hydrochloric acid, filtered off, washed and dried (yield: 60%).

Derivatization of diapocynin and GC-MS analysis

Diapocynin was silylated using a reaction mixture containing 10 mL of MSTFA, 20 mg of ammonium iodide, and 30 μL of ethanethiol. Derivatization was allowed to proceed in a sealed vial at 80 °C for 30 min. The TMS derivative was subjected to GC-MS analysis using a HP Ultra 1 column (18 m; internal diameter 0.2 mm; film thickness 0.11 μm). After injection of the sample, the initial oven temperature of 150 °C was raised to 250 °C with a rate of 10 °C/min [Rt diapocynin-$(TMS)_4$ 9.88 min].

Diapocynin: Spectral data and physical constants

Melting point: >260 °C (decomposition)

^{1}H NMR: see Table 1.

^{13}C NMR-APT (DMSO-d_6; 75 MHz): δ 196.4 (C=O), 149.3 (C; C-3), 147.6 (C; C-4), 128.1 (C; C-1),125.5 (CH; C-6), 124.6 (C; C-5), 109.8 (CH; C-2), 56.2 (OCH_3), 26.5 (CH_3)

IR spectroscopy (KBr), ν_{max} cm^{-1} (%T) 3315 (34.2; OH), 1666 (21.1; C=O), 1591 (24.3; aryl C=C), 1284 (14.5), 1203 (22.9), 1182 (23.7)

EI-MS: (Relative abundances are given in parentheses).
TMS-derivative *m/z* 618 (100) $[M]^{+}$, 603 (39), 587 (73), 529 (47), 441 (77) and 309 (34).

Enzymatic conversion of apocynin

To a solution of 2.0 mg of apocynin in 100 mL of water 100 μL HRP (0.1 mg/mL) or 100 μL MPO (0.01 mg/mL) and 24 μL of hydrogen peroxide (3%) were added under constant stirring. After 10 sec 1.5 mL of 0.1 M sodium thiosulfate was added. The mixture was stirred vigorously and after 2 min, the reaction was terminated by addition of 3 M sulfuric acid to pH < 3. The mixture was extracted three times with diethyl ether. Combined fractions were dried over anhydrous sodium sulfate and the solvent was evaporated.

The residue was subjected to thin layer chromatography (TLC) using precoated Si60 F_{254} silica plates (Merck, Darmstadt, Germany). Typically, 10-μL solutions were applied to the plate and dried. The plate was developed in a saturated chamber with a mixture of dichloromethane and methanol (9:1). Plates were examined under UV light at 254 nm and 366 nm.

Because enzymatic conversion as described above only yielded minute amounts of metabolite, the original concentration of apocynin was increased and the experiment was repeated several times. The residue obtained was subjected to column chromatography using silica gel 60 (column size 40 cm x 1.5 cm diameter) with dichloromethane/methanol (9:1) as eluent; fractions of 1 mL were collected. Fractions containing the reaction product were combined and the solvent was evaporated. The product was analyzed by TLC, GC-MS, ^{1}H NMR and IR-spectroscopy.

Metabolic conversion of apocynin by activated PMNs

PMNs ($1{\cdot}10^7$ cells/mL) were incubated with apocynin (20 μM) and stimulated with OPZ (200 μg/mL). After 7 min, 3 M sulfuric acid was added until pH < 3. The mixture was extracted three times with diethyl ether and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the residue was silylated and subjected to GC-MS analysis as described above for diapocynin.

Determining 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 buffer. Propidium iodide (PI; 1.5 mg) was dissolved in 10 mL of phosphate-buffered saline containing 2.5% quenching ink, 5% w/v EDTA, and 8 mg of bovine serum albumin. PMNs were labeled with the vital stain CFDA (10 μg/mL) at 20 °C for 15 min, washed, and resuspended in buffer to a concentration of 10^7 cells/mL. Amounts of 100 μL of this cell suspension were incubated with equal volumes of serially diluted sample at 37 °C for 15 min. Subsequently, the cells were washed and stained with 25 μL of PI/ink solution for the detection of cellular death. The percentage of dead cells was determined using a fluorescence microscope (Fluovert, Leitz, Wetzlar, Germany).

Measuring ROS production

Neutrophils were isolated from venous blood of healthy volunteers (Bloedbank Midden-Nederland, Utrecht, The Netherlands) as described by Verbrugh *et al.(14)*.

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 PMN suspension ($1{\cdot}10^7$ cells/mL) and 50 μL of luminol (120 μM) or lucigenin (400 μM) solutions were added. The neutrophils were triggered by adding 50 μL of opsonized zymosan A (OPZ; final concentration: 200 μg/mL) or PMA (final concentration 10 nM). Chemiluminescence was monitored every 2 min for 0.5 sec 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 HBSS buffered at pH 7.35 with $NaHCO_3$ and supplemented with 0.1% (w/v) gelatin to avoid cell aggregation (HBSS-gel). OPZ was obtained by incubation of washed commercial zymosan A with 1:10 diluted human pooled serum (HPS) 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 DMSO, stored at -20 °C, and diluted in HBSS to a final concentration of 40 nM immediately before use. In the experiments with azide, sodium azide (final concentration 1.0 mM) was added to the wells before the addition of OPZ.

Determining superoxide anion scavenging

In 96-well, white flat-bottom microtiter plates (Costar, Badhoevedorp, The Netherlands) test compounds were serially diluted in PBS (pH 7.4) to a final volume of 50 µL. Hypoxanthine (50 µL; final concentration 1 mM), lucigenin (50 µL; 0.1 mM), and either buffer or superoxide dismutase (SOD; 25 µL; 10 U/mL) were added. Superoxide anion ($\cdot O_2^-$) radical production was initiated by the addition of 25 µL of xanthine oxidase (10 mU/mL) and chemiluminescence was monitored every min for 0.5 sec during 15 min using a Fluoroskan *Ascent FL* luminometer (Labsystems, Breda, The Netherlands). Activity of the test compounds was calculated from the SOD-inhibitable part of the chemiluminescence signal. To exclude direct effects of test compounds on xanthine oxidase activity, uric acid formation was determined spectrophotometrically at 290 nm.

Oxygen consumption

Oxygen consumption was measured polarigraphically using an oxygen electrode (Radiometer, Copenhagen, Denmark) as described by Weening *et al. (15)*. In the reaction chamber, 300 µL of a PMN suspension ($5{,}4 \cdot 10^6$ cells/mL) was incubated with 325 µL of sample dilution (or buffer for control experiments) at 37 °C. Subsequently, 25 µL OPZ (25 mg/mL) was added and the rate of oxygen consumption was monitored for 15 min under constant gentle stirring.

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.

Results

Synthesis of diapocynin

Diapocynin was synthesized as described above. Its identity was confirmed by NMR spectroscopy and EI-MS. The ^1H NMR spectrum of diapocynin in $CDCl_3$ showed two doublets at δ 7.62 and 7.58 (J = 2.0 Hz; H-6 and H-2, respectively), and three singlets at δ 6.32 (4-OH), 4.01 (OCH_3), and 2.57 (CH_3). Chemical shifts, coupling constants, and assignments for apocynin and diapocynin in $CDCl_3$ and DMSO-d_6 are shown in table 1.

After derivatization with TMS, the mass spectrum (EI) of diapocynin showed a molecular ion $[M]^+$ at *m/z* 618 (also base peak) indicating its di-enol tautomer coupled to 4 TMS groups (Fig. 2). Other fragment ions were found at *m/z* 603 $[M-CH_3]^+$, 587 $[M-OCH_3]^+$, 529 $[M-OTMS]^+$, 441 and 309, the latter fragment representing an ion formed by cleavage of the silylated dimer between C5 and C5′. The IR spectrum of diapocynin is shown in Fig. 3.

Apocynin			**Diapocynin**		
$CDCl_3$					
H-2/6	7.53	2H, m	H-6	7.62	2H, d (*J* = 2.0 Hz)
			H-2	7.58	2H, d (*J* = 2.0 Hz)
H-5	6.94	1H, d (*J* = 8.5 Hz)			
4-OH	6.06	1H, s	4-OH	6.32	2H, s
OCH_3	3.95	3H, s	OCH_3	4.01	6H, s
CH_3	2.56	3H, s	CH_3	2.57	6H, s
DMSO-d_6					
H-6	7.50	1H, dd (*J* = 1.8, *J* = 8.2 Hz)	H-2/6	7.45	4H, m
H-2	7.44	1H, d (*J* = 1.8 Hz)			
H-5	6.86	1H, d (*J* = 8.2 Hz)			
4-OH	9.99	1H, s	4-OH	9.47	2H, s
OCH_3	3.82	3H, s	OCH_3	3.89	6H, s
CH_3	2.50	s*	CH_3	2.49	s*

* Signal obscured by residual protons in the solvent

Table 1. ***^1H NMR spectral data (ppm) of apocynin and diapocynin in $CDCl_3$ and DMSO-d_6 (300 MHz)***

Enzymatic conversion of apocynin

Apocynin was subjected to the protocol described by Holland *et al. (12)*, using HRP and H_2O_2 (Fig. 1).

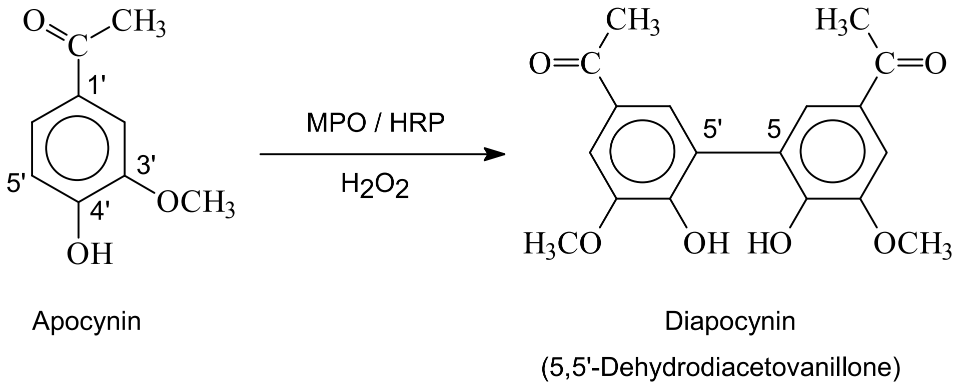

Figure 1. ***Peroxidase-mediated conversion of apocynin into diapocynin***

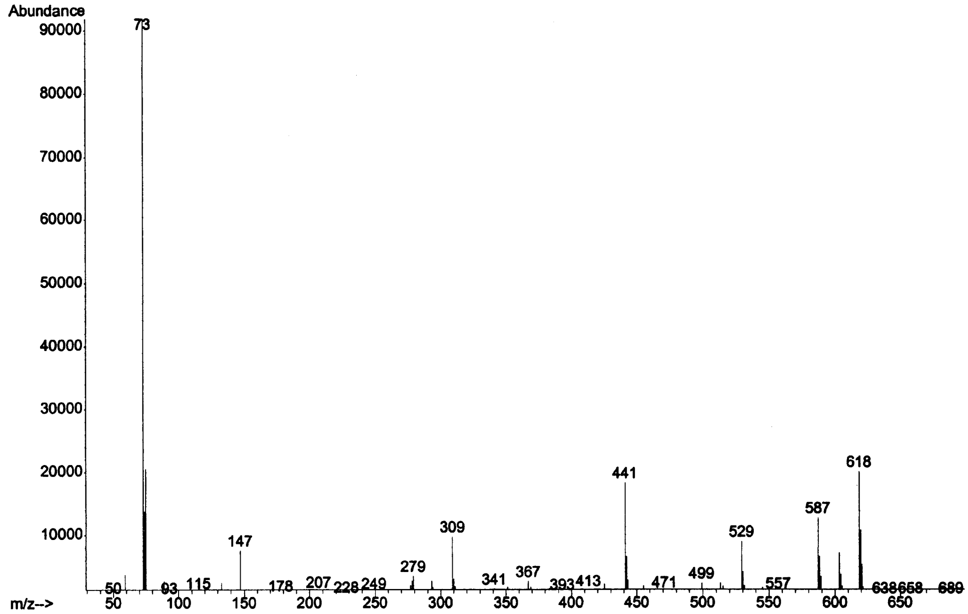

Figure 2. *Mass spectrum of diapocynin*

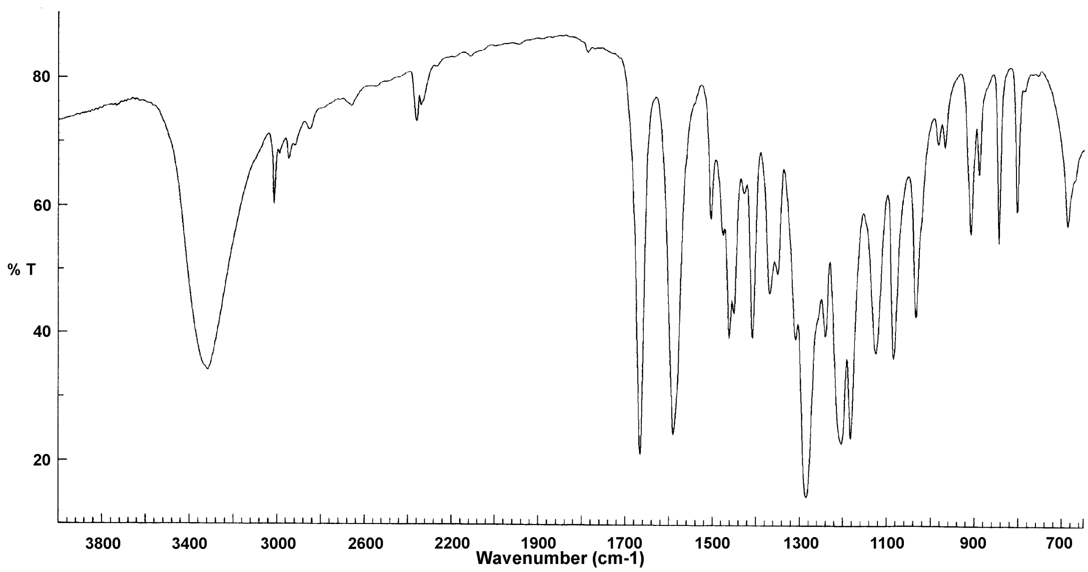

Figure 3. *IR spectrum of diapocynin*

TLC analysis of the resulting mixture showed two distinct spots, a major compound at Rf 0.62 representing apocynin and a minor (conversion) compound at Rf 0.46. The latter product was isolated by column chromatography. Using ^{1}H NMR, EI-MS and IR spectroscopy, the compound was shown to be 5,5′-dehydro-diacetovanillone (or diapocynin).

Using the above-mentioned protocol with MPO as enzyme, however, did not result in the formation of diapocynin (or any other product) in detectable amounts. Increasing MPO, H_2O_2 or apocynin concentrations also failed to yield any product. To determine whether the pH might play a role, the experiment was repeated using apocynin solutions with pH values ranging from 5.5 to 8.5 (intervals of 0.5). All reaction mixtures were analyzed using GC-MS.

Surprisingly, only at pH 8.0, MPO affected the conversion of apocynin into a detectable amount of diapocynin.

Metabolic conversion of apocynin by activated PMNs

To determine whether apocynin was actually converted into diapocynin by activated PMNs, cells were incubated with apocynin and triggered with OPZ. After 7 minutes, the process was terminated by the addition of sulfuric acid and the mixture was extracted with diethyl ether. After evaporation, the residue was silylated and subjected to GC-MS analysis.The EI-spectrum obtained was shown to be identical with that recorded for chemically synthesized diapocynin, proving the ability of activated PMNs to convert apocynin into diapocynin.

No diapocynin could be detected in control PMNs, apocynin-incubated PMNs without OPZ stimulation, and apocynin-incubated PMNs stimulated with PMA.

Determining cytotoxicity

To exclude any cytotoxic effects, we tested apocynin and diapocynin using the vital stain CFDA. CFDA-labeled PMNs were incubated with apocynin and diapocynin in concentrations up to 600 μM. Both compounds did not show any cytotoxic effects compared with control PMNs.

Cytotoxic effects of sodium azide were also determined. Sodium azide was not toxic in concentrations up to 2 mM.

Measuring ROS production

To investigate effects of diapocynin on the production of ROS by OPZ or PMA-stimulated human PMNs, chemiluminescence was measured using either luminol or lucigenin as enhancers. Activities of diapocynin were compared with those of apocynin. IC50 values of both compounds for different enhancers and stimuli are depicted in Fig. 4.

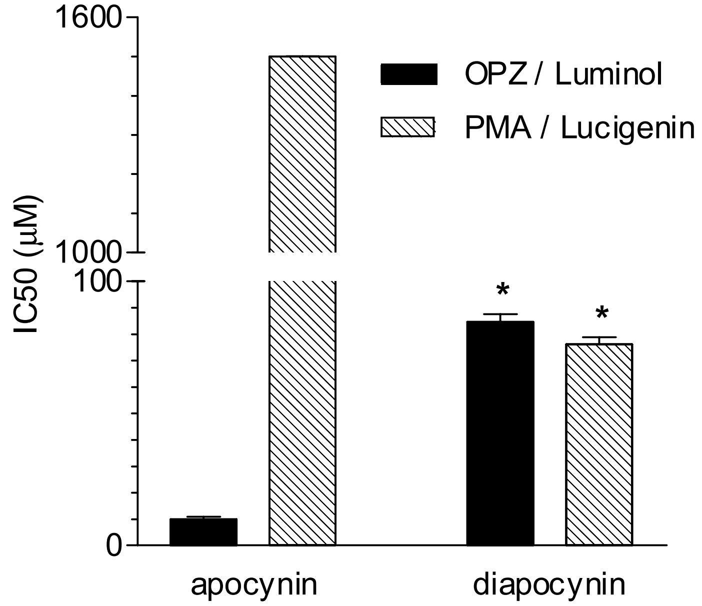

Figure 4. ***Inhibitory effects of apocynin and diapocynin on luminol and lucigenin-enhanced chemiluminescence responses of OPZ and PMA-stimulated human PMNs (n=8).*** *Values are depicted as mean IC50 values ± Standard Error of the Mean (SEM). * Significant difference compared with apocynin-treated PMNs ; P < 0.005.*

Upon stimulation with OPZ and luminol enhancement, the inhibitory activity of diapocynin was significantly lower than that of apocynin (IC50 values: 84 µM vs. 10 µM, respectively). Upon PMA stimulation and lucigenin enhancement, however, the inhibitory activity of diapocynin was significantly higher compared with apocynin (76 µM vs. 1500 µM, respectively; Fig. 4).

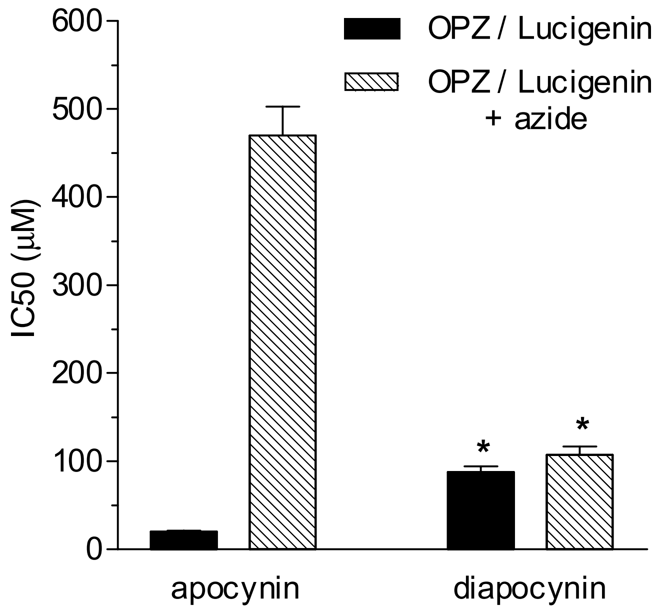

Figure 5. ***Effect of sodium azide on the inhibitory activity of apocynin and diapocynin measured as the lucigenin-enhanced chemiluminescence responses of OPZ-stimulated human PMNs (n=8).*** *Values are depicted as mean IC50 values ± Standard Error of the Mean (SEM). * Significant difference compared with apocynin-treated PMNs; P < 0.005.*

To investigate the role of MPO, apocynin and diapocynin were tested in the presence of the MPO inhibitor sodium azide using lucigenin as enhancer and OPZ as stimulus. Sodium azide was added to the cells before the addition of samples or stimulus. The concentration of azide used (1mM) did not show any cytotoxic effects. It was found that in the presence of azide the inhibitory activity of apocynin was completely diminished (20 µM vs. 470 µM respectively), whereas the activity of diapocynin was almost unaffected by azide (87 µM vs. 107 µM; Fig. 5).

Detection of superoxide anion scavenging

To discriminate between direct effects on NADPH oxidase activity and $\cdot O_2^-$-scavenging properties, $\cdot O_2^-$-scavenging abilities of apocynin and diapocynin were assessed using the hypoxanthine-xanthine oxidase system *(16)*. Lucigenin-enhanced chemiluminescence was measured in the presence of apocynin and diapocynin. Activity were calculated from the SOD-inhibitable part of the chemiluminescence signal. It was found that apocynin did not inhibit lucigenin-enhanced chemiluminescence (IC50 value: > 600 µM). However, diapocynin was shown to be a potent scavenger of superoxide anions (IC50: 9.8 ± 0.2 µM; Fig. 6). This effect could not be ascribed to direct inhibitory effects on xanthine oxidase, since the production of uric acid was not inhibited.

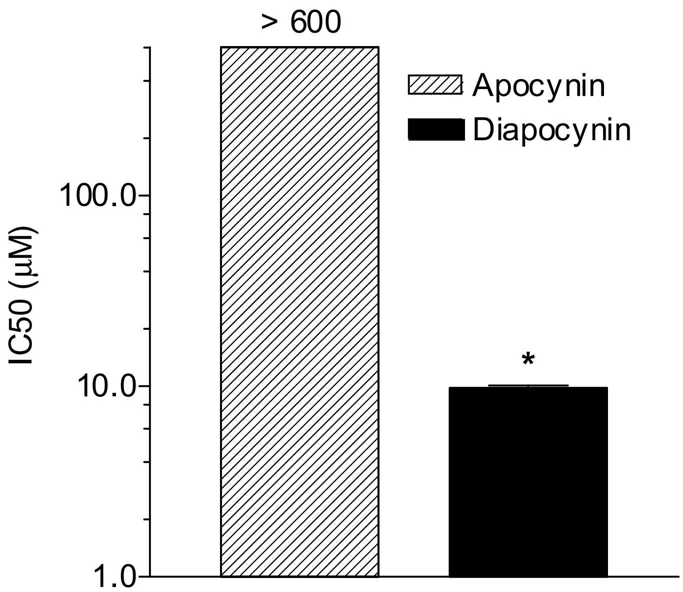

Figure 6. ***Effects of apocynin and diapocynin on the scavenging of superoxide anions (n=4).***
** Significant difference compared with apocynin; P < 0.01.*

Oxygen consumption

Upon activation, PMNs start to consume large amounts of oxygen accompanying ROS production *(17)*. To further evaluate the inhibitory effects of diapocynin on this NADPH oxidase-dependent process, its impact on oxygen consumption by activated human neutrophils was determined in comparison with apocynin.

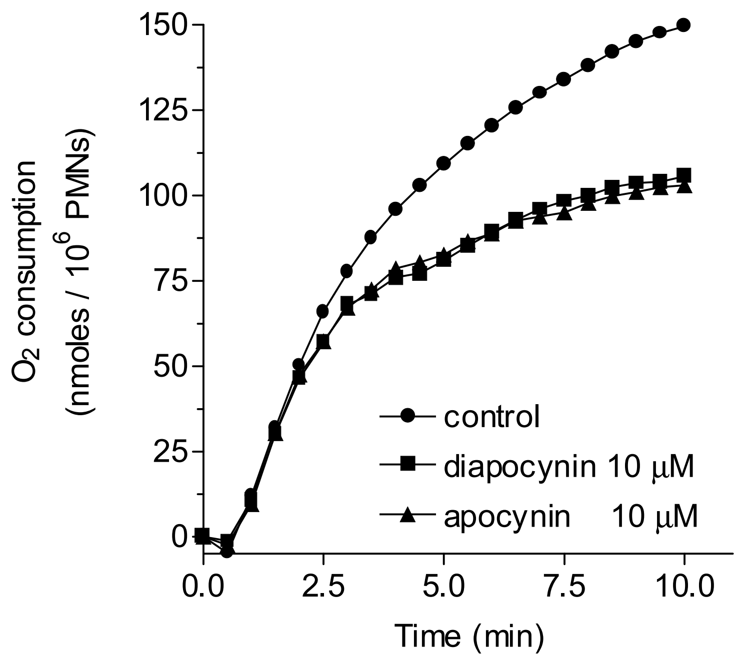

Figure 7. ***Effects of apocynin and diapocynin on oxygen consumption by OPZ-stimulated human PMNs.*** *Plots depict representative curves of three separate experiments.*

PMNs were incubated with apocynin or diapocynin and oxygen uptake upon OPZ stimulation was measured. Apocynin as well as diapocynin inhibited OPZ-induced oxygen uptake similarly (Vmax: 10.4 nmoles $O_2/10^6$ PMNs/min) compared with control cells (Vmax: 16 nmoles $O_2/10^6$ PMNs/min). No differences between both compounds could be observed.

Discussion

Although apocynin is generally known to be a potent inhibitor of phagocyte NADPH oxidase, its mechanism of action has not been well defined. It has been suggested that stimulated PMNs convert apocynin into an active metabolite by the action of MPO and ROS *(5, 10)*. Recently, Holland *et al.* claimed diapocynin, a dimer of apocynin, to be this active metabolite *(12)*. They described the formation of this compound, however, in a cell-free system using HRP, an enzyme not present in PMNs *(11)*. In addition, no spectroscopic evidence for the formation and the identity of diapocynin was provided.

In order to gain more insight into mechanisms underlying inhibitory activities of apocynin, we further investigated the formation and activities of diapocynin. Initially, we obtained diapocynin according to the protocol described by Holland *et al.* using HRP and H_2O_2, but the yield was rather low (approximately 10%). In order to obtain sufficient amounts for *in vitro* testing, diapocynin was synthesized by the procedure described by Elbs *et al.* using potassium persulfate, which led to a much higher yield of 60%. The identity of diapocynin (5,5′-dehydrodiacetovanillone) was confirmed by EI-MS, and ^{1}H NMR, ^{13}C NMR, and IR spectroscopy.

Now that HRP-mediated conversion of apocynin was established, we investigated the conversion of apocynin in the presence of MPO. Replacing HRP by MPO in the experimental set up described by Holland *et al.* did not result in detectable amounts of diapocynin or any other products. Further experimentation showed that MPO only converted apocynin into diapocynin at pH 8.0, illustrating the importance of pH for MPO activity. This finding seems to be consistent with the fact that phagosomal pH is under tight control and that in the first few minutes after phagosome formation a transient increase in pH to 7.8 - 8.0 is observed *(18, 19)*. MPO can reach levels of 1-2 mmol/L inside phagolysosomes *(18)* and it may well be possible that in the first minutes after phagolysosome formation, apocynin is converted into diapocynin.

Besides the MPO-mediated conversion described above, it was even more important to investigate diapocynin formation in PMNs. It was found that only in OPZ-stimulated PMNs apocynin was converted into diapocynin; in resting PMNs or those stimulated with PMA no diapocynin could be detected. So, dimerization of apocynin by stimulated human PMNs was demonstrated for the first time.

Although it was now established that activated PMNs convert apocynin into diapocynin, the activity of the latter compound still had to be proven. Therefore, diapocynin was tested in the chemiluminescence assay for its ability to inhibit NADPH oxidase activity in activated PMNs. For the supposed active metabolite, quite unexpectedly, diapocynin showed less inhibition of ROS production upon OPZ-stimulation and luminol-enhancement in comparison to apocynin (Fig. 4).

Upon PMA-stimulation and lucigenin-enhancement, the inhibitory activity of diapocynin was found to be similar to that in the OPZ/luminol system, whereas the activity of apocynin significantly decreased (Fig. 4). PMA-stimulated PMNs do not release MPO *(20)*, which prevents apocynin from exerting its activity *(21)*. Apparently, in this respect, the activity of diapocynin does not depend on MPO. Another indication came from experiments with sodium azide, an inhibitor of MPO activity *(22-24)*. It was observed that incubation of azide-treated PMNs with apocynin resulted in a 23-fold decrease in inhibitory activity upon OPZ-stimulation compared with control PMNs, whereas for diapocynin no significant difference was observed (Fig. 5).

To further investigate the activity of diapocynin, its activity on oxygen uptake by stimulated PMNs was examined. As previously described *(20)*, inhibition of oxygen consumption by apocynin showed a lag time of approximately 5 minutes. It was supposed that this time was needed for conversion of apocynin into its active metabolite. However, for diapocynin no differences in activity or inhibitory profile could be observed in comparison with apocynin.

Since the activity of diapocynin was shown to be independent of stimulus (OPZ or PMA) or enhancer (luminol or lucigenin), a common phenomenon observed for $\cdot O_2^-$ scavengers, we determined the scavenging properties of diapocynin. Diapocynin, in contrast to apocynin, appeared to be a potent $\cdot O_2^-$ scavenger (Fig. 6). The scavenging properties of diapocynin may in part explain its activity in the chemiluminescence experiments described above. However, its rather high IC50 value in the assay with OPZ and luminol, as well as its effect on oxygen consumption seem not consistent with an active metabolite as meant by Holland *et al*. *(12)*. For an active metabolite in this sense, it would be expected that diapocynin is at least as active as apocynin and shows a prompt inhibition of oxygen consumption without displaying any lag time.

To better explain our results, the 'active metabolite' theory may need to be extented. It has been reported that MPO plays a crucial role in the termination of the NADPH oxidase-dependent respiratory burst, since an increased MPO activity results in an accelerated termination of the respiratory burst *(25-27)*. It has also been shown that decreased levels of hydrogen peroxide (the substrate of MPO) result in increased MPO activity *(25)*. It is obvious, that scavenging of superoxide anions results in a decrease of hydrogen peroxide formation. So, besides effects due to direct scavenging of superoxide anions, the activity of diapocynin may be explained in terms of increasing MPO activity, resulting in an accellerated termination of the respiratory burst.

Furthermore, it is known from literature that lignins and lignin-related compounds not only scavenge $\cdot O_2^-$ *(28)*, but also potently stimulate the iodination of cells that contain MPO, such as PMNs and monocytes *(28-31)*. This increased iodination, which reflects increase of MPO activity *(32)*, was almost completely inhibited in the presence of the MPO inhibitors sodium azide and aminotriazole *(30,31)*. Importantly, only polymerized phenolic lignin-related structures were active, whereas degradation products of lignin had little or no effect *(30)*. In addition, diapocynin has been described to be a lignin model-compound *(33)*, whereas apocynin is considered to be a degradation product of lignin *(34, 35)*. In conlusion, we hypothesize that diapocynin increases MPO activity and thus accelerates the termination of the NADPH oxidase-dependent respiratory burst of stimulated human PMNs.

A final proposal for the mechanism of action of apocynin will be presented in the General Discussion (Chapter 7).

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Chapter 6

Effects of apocynin and analogs on the cytokine production by mononuclear cells

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- Je moet een vraag wel stellen zoals die gesteld hoort te worden -

(Johan Cruijff, 1993)

Abstract

Apocynin is a promising, plant-derived, nonsteroidal, anti-inflammatory compound that has been studied in different *in vitro* systems as well as in *in vivo* models for chronic inflammatory diseases. So far, apocynin is known as a specific inhibitor of NADPH-oxidase activity in stimulated neutrophils. This paper is dealing with possible other aspects of apocynin action, including inhibition of cytokine (IL-1, TNFα, IFNγ) production by cultured monocytes/macrophages and T cells, as well as inhibition of the proliferative response of T cells.

Our findings implicate that not only apocynin itself, but also combinations of apocynin with one or more of its major *in vivo* metabolites may be involved in its net *in vivo* effect. This putative synergistic activity will be subject of further *in vitro* studies.

Introduction

Apocynin has proven its potential value in the treatment of several experimental inflammatory diseases such as colitis *(1)*, atherosclerosis *(2)*, and rheumatoid arthritis *(3)*. It is a potent inhibitor of NADPH oxidase-dependent reactive oxygen species (ROS) production in activated human PMNs. Its putative mode of action involves metabolic conversion by MPO and ROS. Recently, Holland *et al.* proposed diapocynin to be the active metabolite of apocynin *(2)*. In Chapter 5, we described the isolation, characterization, and activity of diapocynin, and proposed a model for its mode of action. However, activities of other known metabolites of apocynin have not been studied yet.

The metabolization of apocynin *in vivo* is partially known, since Gjertsen *et al.* *(4)* and Daly *et al.* *(5)* reported that after a period of 20 hours following *i.p.* administration, 80% of apocynin was recovered unchanged in the urine of the animals, but that traces of apocynin had been converted into 3′,4′-dihydroxy-acetophenone or into 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol.

So far, apocynin is known as a potent and selective inhibitor of ROS production by activated PMNs *(6; & this thesis)*. Although PMNs play an important role in inflammatory diseases, they can not be accounted for all detrimental effects, since monocytic cells, T cells, and mononuclear cells-derived production of cytokines play a major role as well *(7-10)*.

To determine whether the metabolites mentioned above, may contribute to the activity of apocynin, these analogs were tested for their ability to inhibit ROS production by activated human PMNs. Furthermore, a pilot-study was performed on the activity of apocynin, diapocynin, 3′,4′-dihydroxy-acetophenone, and 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol to assess their effects on T-cell proliferation and their ability to inhibit production of interferon γ (IFNγ), tumor necrosis factor α (TNFα), interleukin 1 (IL-1), as well as IL-4 by T cells and monocytic cells.

Materials & Methods

Materials

Apocynin was obtained from Carl Roth GmbH (Karlsruhe, Germany) and was purified by recrystallization from water before use. Diapocynin was synthesized as previously described (Chapter 5). The apocynin analogs 3′,4′-dihydroxy-acetophenone and 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol were synthesized as described below. α-CD3/α-CD28 was obtained from the Central Laboratory of the Blood Transfusion Service (CLB, Amsterdam, The Netherlands). All other reagents were obtained from Sigma (St. Louis, MO, USA), unless stated otherwise.

Synthesis of 3′,4′-dihydroxy-acetophenone

3′,4′-Dihydroxy-acetophenone was prepared by *o*-demethylation of apocynin *(11)*. Protected from atmospheric moisture, 4.4 g (33 mMol) of anhydrous aluminium chloride (4.4 g,) was suspended in a solution of 5.0 g (30 mMol) of apocynin in 50 mL of dichloromethane while stirring. The mixture was cooled and 11.1 mL (132 mMol) of pyridine was added slowly (molar ratios of the reactants were pyridine: aluminium chloride: apocynin = 4.4:1.1:1). Subsequently, the resulting orange solution was refluxed at 45 °C for 24 h. After cooling to room temperature and addition of hydrochloric acid (15%) to pH<4, the aqueous phase was extracted exhaustively with diethyl ether. The diethyl ether extract was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The residue was found to contain a small amount of apocynin and was therefore purified by column chromatography over silica gel with chloroform/methanol (90:10) as eluent. Fractions containing 3′,4′-dihydroxy-acetophenone were combined and the solvent was evaporated. Yellow-orange crystals were obtained by recrystallization from chloroform (yield: 40%).

3′,4′-Dihydroxy-acetophenone: Spectral data and physical constants

Melting Point: 122-123 °C

^{1}H NMR (CD_3OD; 300 MHz): δ 2.48 (3H, s; CH_3), 6.81 (1H, dd, *J* = 7.7 Hz, *J* = 0.8 Hz; H-5), 7.42 (2H, m; H-2/6)
^{13}C NMR (CD_3OD; 75 MHz): δ 199.7 (C=O), 152.3 (C-3), 146.4 (C-4), 130.8 (C-1), 123.5 (C-6), 116.1 (C-5/2), 115.8 (C-5/2), 26.2 (CH_3)

EI-MS *m/z*: 152 $[M]^+$, 137 (base peak) $[M\text{-}CH_3]^+$, 109 $[M\text{-}CH_3CO]^+$

IR spectroscopy (KBr), ν max cm^{-1} (%T): 3375 (55.4; OH), 1664 (57.6; C=O), 1591 (55.2; aryl C=C), 1525 (68.5; aryl C=C), 1446 (69.1; aryl C=C), 1367 (69.1), 1294 (59.7), 1230 (62.7), 1124 (68.2)

Synthesis of 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol

1-(4′-hydroxy-3′-methoxyphenyl)-ethanol was obtained by reduction of apocynin with sodium boron hydride. Sodium boron hydride (113 mg; 3 mMol) was suspended in 10 mL of 1,2-dimethoxyethane (DME). The suspension was stirred and a solution of 500 mg (3 mMol) of apocynin in 3 mL of DME was added slowly. Methanol (1.2 mL; 30 mMol) was added after 6 h, and after another 16 h the reaction was terminated by addition of 1N hydrochloric acid to pH< 4. Subsequently, the mixture was extracted exhaustively with diethyl ether. The combined ether layers were dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography over silica gel using chloroform/methanol (90:10) as eluent. Fractions containing 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol were combined and the solvent was evaporated; a crystalline product was obtained which purity was proven by GC-MS (yield: 50%).

Derivatization of 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol and GC-MS analysis

1-(4′-hydroxy-3′-methoxyphenyl)-ethanol was (trimethyl)silylated in ethyl acetate with MSTFA [N-methyl-N-(trimethylsilyl)trifluoroacetamide; Sigma/ Aldrich, Zwijndrecht, The Netherlands] in a sealed vial at 70 °C for 40 min. The TMS derivative(s) was(ere) analyzed using a Chrompack CP Sil 8 CB column (30 m; internal diameter 0.25 mm; film thickness 0.25 μm). After injection of the sample the initial oven temperature of 100 °C was maintained for 1 min and then raised to 290 °C with a rate of 8 °C/min. The latter temperature was maintained for 5 min [Rt 11.70 min].

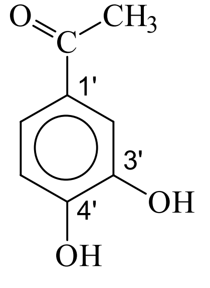
1-(4′-hydroxy-3′-methoxyphenyl)-ethanol: Spectral data and physical constants

Melting Point: 107 °C

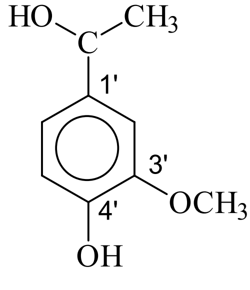
^{1}H NMR (CD_3OD; 300 MHz) δ: 6.95 (1H, d, *J* = 2.1 Hz; H-2′), 6.77 (1H, dd, *J* = 8.1 Hz, *J* = 2.1 Hz; H-6′), 6.73 (1H, d, *J* = 8.1 Hz; H-5′), 4.73 (1H, q, *J* = 6.3 Hz; H-1), 3.85 (3H, s; OCH_3), 1.40 (3H, d, *J* = 6.3 Hz; CH_3)
^{1}H NMR (DMSO-d_6; 300 MHz) δ: 9.14 (1H, s; 4′-OH), 7.29 (1H, s; H-2′), 7.10 (2H, m; H-5′/6′), 5.00 (1H, q, *J* = 6.3 Hz; H-1), 4.15 (3H, s; OCH_3), 3.74 (1H, d, *J* = 6.6 Hz; 1-OH), 1.68 (3H, d, *J* = 6.3 Hz; CH_3)

EI-MS: (Relative abundances are given in parentheses).
[1-(4′-hydroxy-3′-methoxyphenyl)-ethanol-$(TMS)_2$] *m/z*: 312 (14) $[M]^+$, 297 (100) $[M-CH_3]^+$, 267 (8), 223 (14) $[M-OTMS]^+$.

IR spectroscopy (KBr), ν max cm^{-1} (%T): 3446 (52.4; OH), 3128 (59.2; OH), 2968 (58.3), 1603 (63.1), 1527 (57.9), 1468 (67.1), 1385 (64.3), 1284 (54.6),1263 (56.7), 1217 (62.3), 1159 (59.2)



3',4'-dihydroxy-acetophenone



1-(4'-hydroxy-3'-methoxyphenyl)-ethanol

Measuring ROS production

Neutrophils were isolated from venous blood of healthy volunteers (Bloedbank Midden-Nederland, Utrecht, The Netherlands) as described by Verbrugh *et al.(12)*.

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 PMN suspension ($1 \cdot 10^7$ cells/mL) and 50 µL of a luminol solution (120 µM) was added. The neutrophils were triggered by adding 50 µL of opsonized zymosan A (OPZ; final concentration 200 µg/mL). Chemiluminescence was monitored every 2 min for 0.5 sec 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 HBSS buffered at pH 7.35 with $NaHCO_3$ and supplemented with 0.1% (w/v) gelatin to avoid cell-aggregation (HBSS-gel). OPZ was obtained by incubation of washed commercial zymosan A with 1:10 diluted human pooled serum (HPS) at 37 °C for 30 min. After washing, the opsonized product was resuspended in HBSS (final concentration: 0.8 mg/mL).

Mononuclear-cell cultures

Peripheral blood mononuclear cells (MNCs) were isolated from healthy volunteers according to standard procedures. Blood was diluted 1:1 with Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Paisley, Scotland) containing 0.81 mM SO_4^{2-}, supplemented with glutamine (2 mM), penicillin (100 IU/mL) and streptomycin sulphate (100 µg/mL; DMEM$^+$). MNCs were isolated by density centrifugation using Ficoll-Paque (Pharmacia, Uppsala, Sweden). The viability of the cells, checked by trypan blue exclusion, always exceeded 95%. Subsequently, MNCs were cultured ($0.5 \cdot 10^6$ cells/mL) in 96-well plates (in the presence or absence of compounds) in DMEM$^+$ supplemented with 10% human male AB$^+$ serum (Red Cross Blood Transfusion Center, Utrecht, The Netherlands).

Experimental design

To mimic the conditions in the rheumatoid joint *(13, 14)*, MNCs were stimulated with the bacterial antigen *E. coli* lipopolysaccharide (LPS 0111:B4; 10 ng/mL). The production of TNFα, and IL-1 (in the presence or absence of compounds) was determined in the 4-day culture supernatants of the cells.

T cells were stimulated with αCD3/αCD28 monoclonal antibodies (diluted 1:500.000). During the last 48 h of the cell culture, the cells were additionally stimulated with ionomycin/PMA (1.0 µg/mL and 50 µg/mL, respectively). The cytokines IFNγ and IL-4, as estimates of T-helper 1 (T_H1) or T_H2 cell activity, respectively, were measured in the supernatants.

The effect of the compounds on T-cell proliferation was assessed in quadruplo per donor by [^{3}H]-thymidine incorporation during the last 18 h of culture according to standard procedures.

Measuring cytokines

Supernatants of PMNC cultures were harvested, separated from cellular material by centrifugation (5 min, 450xg), frozen in liquid nitrogen, and stored at –20 °C for no longer than 3 months. All cytokines were determined by ELISA (Medgenix, Flerus, Belgium). The detection limits were 30, 10, 10, and 10 pg/mL for IL-1, TNFα, IFNγ, and IL-4, respectively.

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.

Results

Inhibition of ROS production

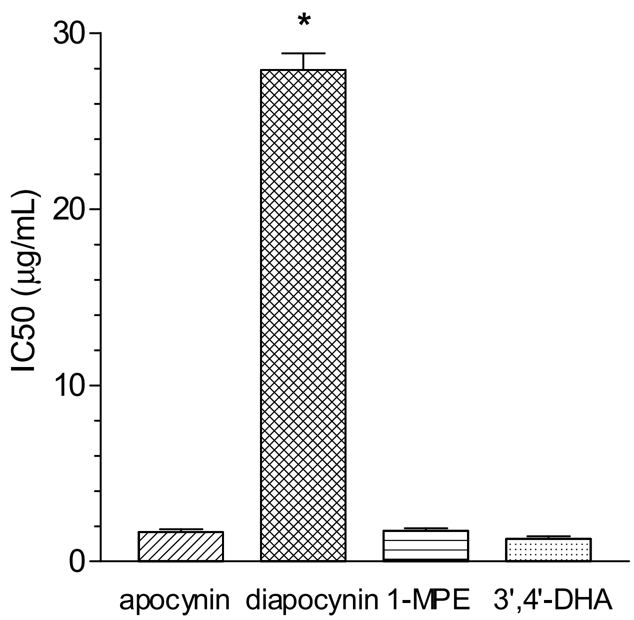


Figure 1. ***Inhibitory effects of apocynin, diapocynin, 1-(4'-hydroxy-3'-methoxyphenyl)-ethanol (1-MPE), and 3',4'-dihydroxy-acetophenone (3',4'-DHA) on the luminol-enhanced chemiluminescence response of OPZ-stimulated human PMNs (n=6).*** *Values are depicted as mean IC50 values ± Standard Errors of the Mean (SEM). * Significant difference compared with apocynin-incubated PMNs; P<0.001.*

To investigate the effects of the apocynin metabolites 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol and 3′,4′-dihydroxy-acetophenone on the production of ROS by OPZ-stimulated human PMNs, chemiluminescence was measured using luminol as enhancer. IC50 values were compared with IC50 values of apocynin and diapocynin, which were determined previously (Chapters 3 & 5, respectively).

The IC50 values of 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol and 3′,4′-dihydroxy-acetophenone (10.5 μM and 8.5 μM, respectively) were not significantly different from that of apocynin (10 μM). Diapocynin was significantly less active (IC50: 84 μM), as previously reported in Chapter 5.

IL-1 and TNFα production by MNCs

To investigate the effects of the apocynin and apocynin metabolites on cytokine release by MNCs, levels of cytokines in the supernatants of the cell cultures were determined.

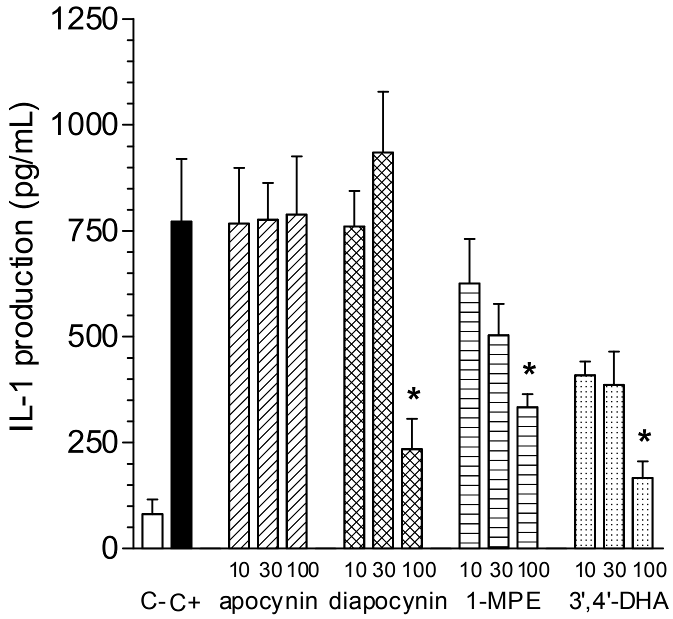


Figure 2. ***Effects of apocynin, diapocynin, 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol (1-MPE), and 3′,4′-dihydroxy-acetophenone (3′,4′-DHA) on the production of IL-1 by LPS-stimulated MNCs (n=3).*** *Values are depicted as mean values ± Standard Errors of the Mean (SEM). Concentrations of the compounds are depicted as μg/mL. C- and C+ represent control cells without and with stimulation, respectively. * Significant difference compared with stimulated controls; P<0.05.*

Although IL-1 production was not inhibited by the three doses of apocynin tested, diapocynin, 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol (1-MPE), and 3′,4′-dihydroxy-acetophenone (3′,4′-DHA) showed significant inhibition, albeit at the highest concentration tested.

As shown in figure 3, apocynin, diapocynin, and 3′,4′-DHA, showed dose-dependent inhibition of TNFα production; significant results were only observed at the highest concentration used. 1-MPE was found to be not active.

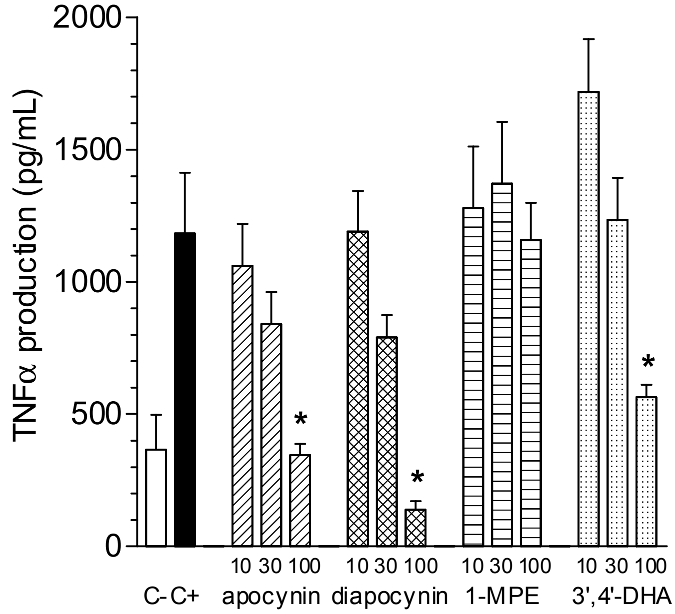


Figure 3. ***Effects of apocynin, diapocynin, 1-MPE, and 3′,4′-DHA on TNFα production by LPS-stimulated MNCs (n=3).*** *Values are depicted as mean values ± SEM. * Significant difference compared with stimulated controls; P<0.05.*

T- cell derived cytokines

The T_H1 and T_H2 cell cytokines IFNγ and IL-4, respectively, were determined in culture supernatants of αCD3/αCD28 and ionomycin/PMA-stimulated MNCs.

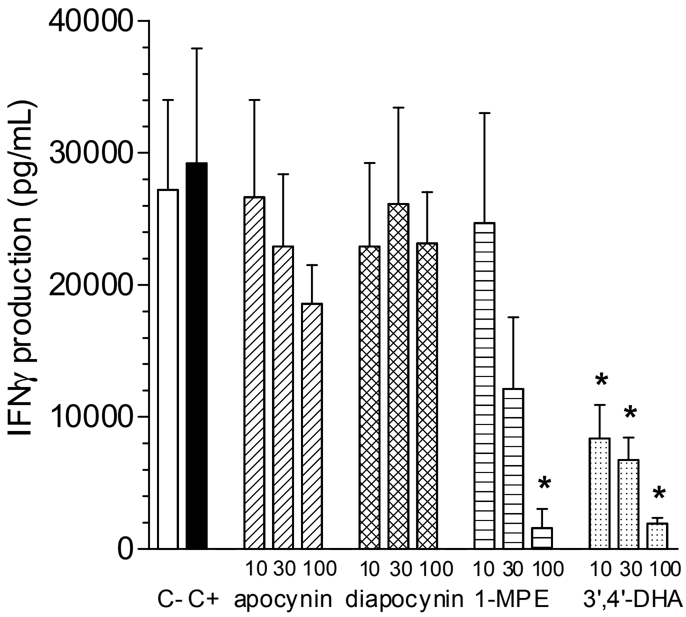


Figure 4. ***Effects of apocynin, diapocynin, 1-MPE, and 3′,4′-DHA on IFNγ production by αCD3/αCD28 and ionomycin/PMA-stimulated MNCs (n=3).*** *Values are depicted as mean values ± SEM. * Significant difference compared with stimulated controls; P<0.05.*

Unexpectedly, unstimulated cells showed increased levels of IFNγ production. Compared with the stimulated controls, no significant inhibitory effects on IFNγ production were observed for apocynin and diapocynin-incubated MNCs (Fig. 4). 3′,4′-DHA, however, showed significant, dose-dependent inhibition, whereas 1-MPE was only inhibitory at the highest concentration.

For IL-4, all values obtained were below the detection limit of the assay (data not shown).

T-cell proliferation

T-cell proliferation, as measured by [^{3}H]-thymidine incorporation, was determined for all compounds.

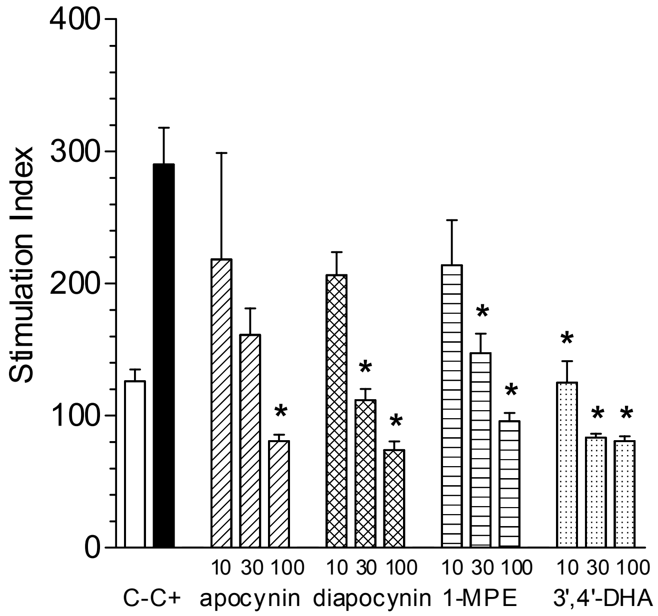


Figure 5. ***Effects of apocynin, diapocynin, 1-MPE, and 3′,4′-DHA on T-cell proliferation (n=3).*** *Values are depicted as SI (stimulation index) and represent mean values ± SEM. * Significant difference compared with stimulated controls; P<0.05.*

All compounds showed dose-dependent inhibition of T-cell proliferation. Significant inhibition of apocynin was only obtained at a level of 100 μg/mL; diapocynin and 1-MPE showed significant inhibition at 30 and 100 μg/mL, whereas 3′,4′-DHA was significantly active at all concentrations. Inhibitory effects could not be attributed to cytotoxicity as tested by trypan blue exclusion.

Discussion

The nonsteroidal anti-inflammatory drug apocynin is a promising lead-compound in the treatment of several experimental inflammatory conditions, including colitis ulcerosa and rheumatoid arthritis in rats *(1, 2)* and atherosclerosis in rabbits *(3)*. Although different inflammatory diseases may involve different sets of inflammatory cells, including mast cells, neutrophils (PMNs), eosinophils, monocytes, T cells, and even B lymphocytes (as antigen-presenting cells), the effects of apocynin studied so far have been mostly restricted to the inhibition of NADPH-dependent reactive oxygen species (ROS) production by neutrophils. Neutrophils are considered to be involved in the onset *(15, 16)*, rather than in later phases of inflammatory processes wherein monocytes and T cells play a major role *(17, 18)*. Therefore, in order to get an impression of the effects of apocynin later on in a monocyte- and/or T-cell-mediated inflammatory reaction, our attention in this paper was focussed on the action of apocynin on the mononuclear blood cells of healthy donors, with special reference to T-cell and monocyte-mediated effects *(17, 18)*.

Thus far, the *in vitro* effects of apocynin on monocyte- and/or T-cell-mediated reactions have not been studied thoroughly, whereas the effects of the apocynin metabolites on these processes have not been studied at all. Therefore, not only apocynin, but also its major *in vivo* metabolites diapocynin (the putative active principle in the inhibition of PMN-dependent ROS production), 3′,4′-dihydroxy-acetophenone, and 1-(4′-hydroxy-3′-methoxy-phenyl)-ethanol were included in this experimental study as well.

In contrast to what is known about mononuclear cells (MNCs) from patients with rheumatoid arthritis *(19)*, the production of the monocyte-derived, pro-inflammatory cytokine IL-1 by mononuclear cells from healthy donors was not affected by apocynin, at least in the concentrations tested (Fig. 2). In contrast, all three apocynin metabolites inhibited IL-1 production by monocytes from healthy subjects, albeit mostly only at the highest concentration tested.

Regarding TNFα production, the results obtained for apocynin were consistent with those from Mattsson *et al.*, who described a moderate inhibition of TNFα production by adherent mononuclear cells *in vitro (20)*. Diapocynin and 3′,4′-dihydroxy-acetophenone sorted similar effects as apocynin, whereas 1-(4′-hydroxy-3′-methoxy-phenyl)-ethanol was totally devoid of inhibitory effect. Since all IL-4 levels remained below the detection limit of the assay, conclusions with regard to possible inhibitory effects of apocynin and apocynin metabolites on T_H2 cells could not be drawn.

In contrast to previous observations on mononuclear cells from rheumatoid arthritis (RA) patients *(19)*, apocynin-incubated T cells were not inhibited in their IFNγ response. Although our unstimulated cells from healthy donors showed already high levels of IFNγ production, the highest concentration of 1-(4′-hydroxy-3′-methoxy-phenyl)-ethanol and all three doses of 3′,4′-dihydroxy-acetophenone tested showed significant inhibitory effects (Fig. 4).

Results obtained with the T-cell proliferation assay were the most consistent: all three apocynin metabolites tested, inhibited the proliferative T-cell response, at least at the two highest dose levels (Fig. 5).

In conclusion, it can be stated that in this pilot-study, apocynin, diapocynin, and the other metabolites only are able to inhibit monocyte cytokine production and T-cell proliferation at concentrations which are significantly higher than the IC50 values obtained in the ROS inhibition assay. Considering the dosage of apocynin and its metabolism in the *in vivo* situation *(4)*, it may seem unlikely that the *in vitro* effects reported here could contribute to the reported *in vivo* effects.

However, it should be noted that also the *in vivo* effects of apocynin in the experimental rat RA model *(19)* were in fact much stronger than could be expected on basis of established *in vitro* activity. Possible explanations for this inconsistency between the *in vivo* and *in vitro* observations may be: (i) apocynin or one of its metabolites are concentrated in the major inflammatory cell, (ii) apocynin and one or more of its metabolites act synergistically, (iii) combinations of the latter two possibilities or (iv) apocynin or its metabolites affect production (or activity) of other important pro-inflammatory cytokines. Since the second possibility can easily be studied *in vitro* as well as *in vivo*, near-future studies will be focused on the putative synergistic effects between apocynin and its metabolites.

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Chapter 7

General Discussion

- Je gaat het pas zien als je het doorhebt -

(Johan Cruijff, 1994)

In recent years, there has been a gradual shift in immunological focus away from the specific antigen recognition mechanisms of adaptive immunity, back to primary host defense mechanisms. It has become clear that innate immune responses exhibit a degree of specificity and are more complex than was ever supposed *(1)*. Studies on innate immunity have led to the discovery of common molecular mechanisms used for host defense in mammals, insects, and even plants *(2, 3)*. Innate or natural immunity is the ability to respond immediately to an infectious challenge, regardless of previous exposure of the host to the invading agent. Elements of the innate immune system include phagocytic cells, *i.e.* polymorphonuclear leukocytes (PMNs) and mononuclear phagocytes like macrophages, and the complement cascade of circulating soluble pre-enzymic proteins. PMNs and macrophages play a key role in the first line of defense against invading microorganisms, and conditions in which these cells do not function properly can cause serious problems *(4-6)*. Besides their beneficial properties, PMNs may also have adverse effects, since the production of reactive oxygen species (ROS) by activated PMNs may exacerbate the inflammatory response and may give rise to tissue injury *(7-9)*. Inflammation and reactions of the innate immune system can often be controlled by the use of steroids, but sometimes adverse effects of these drugs make the cure worse than the disease *(10-12)*. Therefore, advanced research is focused on the discovery of potent non-steroidal anti-inflammatory drugs (NSAIDs).

In a search for such anti-inflammatory compounds, we obtained apocynin by activity-guided isolation in 1989. Although apocynin as a molecule had been described before *(13-15)*, we discovered its potent inhibitory activity on NADPH oxidase-dependent ROS production by stimulated human PMNs *(16)*. In following experiments, the activity of apocynin appeared to be dependent on myeloperoxidase (MPO), an enzyme present in the azurophilic granules of PMNs and released upon activation *(17)*. A more detailed mode of action has been subject of speculations, however. It has been proposed that apocynin has to be converted into an active metabolite by the combined action of MPO and ROS *(18-20)*. Several structures have been suggested for this metabolite *(18, 21)*, but no convincing evidence has been provided. The studies described in this thesis contribute to the understanding of the mode of action of apocynin.

In Chapter 2, we demonstrated that in activated PMNs, apocynin inhibits extracellular as well as intracellular production of superoxide anions and other ROS. Since it was established that apocynin does not act as a scavenger of superoxide anions, this may point at a direct effect of this compound on the neutrophil NADPH oxidase.

The proposed conversion of apocynin into an active metabolite by the combined action of MPO and ROS was supported by experiments concerning the increased oxygen uptake by activated PMNs which preceeds ROS production. The inhibition of oxygen consumption by apocynin showed a lag time of approximately 5 min, which is probably the time needed for its conversion into the active substance. Moreover, when PMNs had been incubated with apocynin in the presence of OPZ, upon a second stimulation with OPZ, no lag time was observed indicating the presence of an active apocynin metabolite inside the PMN.

Previously, it was assumed that apocynin inhibited NADPH-dependent ROS production by interfering with translocation of cytosolic components necessary for the assembly of the NADPH oxidase complex *(20)*. Based on our results, however, no discrimination between inhibition of assembly or acceleration of dissociation of the NADPH oxidase complex could be made.

In Chapter 3, structure activity relationship studies (SAR) are described; these studies were performed to determine whether changes in the molecular structure of apocynin would lead to the discovery of more potent inhibitors of NADPH oxidase activity. SAR studies may also provide more information with regard to the active site(s) of the molecule. Experiments with apocynin analogs showed that the introduction of an additional methoxy-group at position C-5 leads to an increase in inhibitory activity. Future experiments have to show if the analogs investigated have a similar mode of action as proposed for apocynin.

In recent years, more and more attention has been paid to the role of reactive nitrogen species (RNS) in inflammatory diseases. Nitric oxide and its derivative peroxynitrite play an important role in inflammation of the respiratory tract *(22, 23)*. Apocynin was capable of inhibiting peroxynitrite formation by murine macrophages (Chapter 4). This finding may explain the observations of Muijsers *et al.* who reported that *i.p.* as well as orally administrated apocynin significantly inhibited allergen-induced hyperresponsiveness in mice *(24)*.

Since in literature diapocynin has been proposed to be the active metabolite of apocynin *(21)*, we focussed our attention on this dimer. First of all we proved its actual existence: its formation from apocynin in a cell-free system with MPO and hydrogen peroxide, as well as in OPZ-stimulated PMNs (Chapter 5). The identity of diapocynin was confirmed by NMR spectroscopy and MS. Diapocynin was also chemically synthesized for *in vitro* testing. The dimer appeared to be less active than apocynin in inhibiting ROS production by stimulated PMNs. In contrast, it showed similar inhibition of oxygen consumption, and appeared to be a potent scavenger of superoxide anions. These findings strongly indicated that the existing theories had to be revisited. A new model for the mode of action of apocynin is proposed below.

Finally, effects of apocynin and some metabolites [*i.e.* diapocynin, 3′,4′-dihydroxyacetophenone, and 1-(4′-hydroxy-3′-methoxyphenyl)-ethanol] on cytokine production by mononuclear cells and T cell proliferation are described in Chapter 6. Studies on mechanisms underlying inhibition of cytokine production and T cell proliferation by apocynin may contribute to the understanding of its long-term effects in inflammatory conditions.

Proposed mechanism of action of apocynin

The fact that apocynin is converted into diapocynin by activated PMNs (Chapter 5), makes this dimer a likely candidate for the suggested active metabolite.

The activity of diapocynin, and thereby the mode of action of apocynin, may be explained as follows.

It has been reported that polymerized phenolic lignin-related compounds significantly increase iodination inside PMNs *(25, 26)*. This iodination reflects the MPO activity *(27)*. It should also be noted here that diapocynin has been described as a lignin model-compound *(28)*. Furthermore, it is known that increased activity of MPO leads to an accelerated termination of the NADPH oxidase-dependent respiratory burst *(29)*. The involvement of MPO was further demonstrated by Klebanoff *et al.*, who showed that respiratory burst activity was increased in MPO-deficient PMNs *(30-32)* as well as in PMNs treated with the MPO inhibitor sodium azide *(29, 33)*.

Therefore, we hypothesize that in the phagolysosome, by combined action of MPO and ROS, diapocynin is formed which increases MPO activity, and thereby accelerates the termination of the respiratory burst resulting in decreased NADPH oxidase activity.

Moreover, we discovered that diapocynin is a potent scavenger of superoxide anions (Chapter 5). Since it has been shown that decreased levels of hydrogen peroxide result in an accelerated termination of the respiratory burst *(29)*, scavenging of superoxide anions by diapocynin, and thereby a decrease of hydrogen peroxide, is significant in this respect.

In understanding the activity of apocynin, the topology of events is really crucial. There is a significant difference between extracellular events and those taking place inside the phagolysosome *(34, 35)*.

Our observation that diapocynin displayed a lower activity than apocynin in the chemiluminescence assay upon OPZ-stimulation and luminol-enhancement (Chapter 5) may be explained by the fact that incubation of PMNs with (exogenous) diapocynin represents another condition than diapocynin being formed (endogenously) from apocynin inside the phago(lyso)some. The log P value of diapocynin, representing lipophilicity, calculated according to Hansch and Leo *(36)*, appeared to be significantly higher than that of apocynin (1.40 and 0.89, respectively). Consequently, (exogenous) diapocynin will readily enter the cell membrane of the PMN and stay there without migration to the cytosol. Also the difference in pH between the extracellular space and the phago(lyso)some may be quite important. Inside the phagolysosome, the pH can increase to 8 during the first minutes of phagocytosis *(34, 35)*. Calculated pK_a-values of diapocynin and apocynin were 8.4 and 8.9, respectively *(36)*. This may mean that within the phagolysosome, diapocynin is more likely to occur in a charged (deprotonated) form than apocynin. In this way, endogenously formed diapocynin will not easily enter the cell membrane, thus allowing the substance to exert its activity within the phagolysosome. A schematic representation of our model is depicted in Figure 1.

However, there may be another mechanism involved in the activity of apocynin. The formation of diapocynin within the phagolysosome is most likely to proceed via an intermediate radical. A similar phenomenon has been reported by

McCormick *et al.*, who demonstrated the formation of a tyrosyl radical in the conversion of L-tyrosine into its 3,3′-dimer upon incubation with H_2O_2 and MPO *(37)*. In our case, we could also confirm the existence of a relatively stable radical derived from apocynin in the presence of H_2O_2 and MPO by experiments using electron spin resonance (ESR) spectroscopy (unpublished results). Reactive apocynin radicals will not pass the cell membrane, but may interact with the flavocytochrome *b* at the intraphagosomal side causing conformational changes in this protein-complex which may eventually lead to dissociation of the NADPH oxidase complex (Fig. 1). However, this theory needs further confirmation; therefore, the effects of apocynin radicals on NADPH oxidase activity will be subject of our future studies.

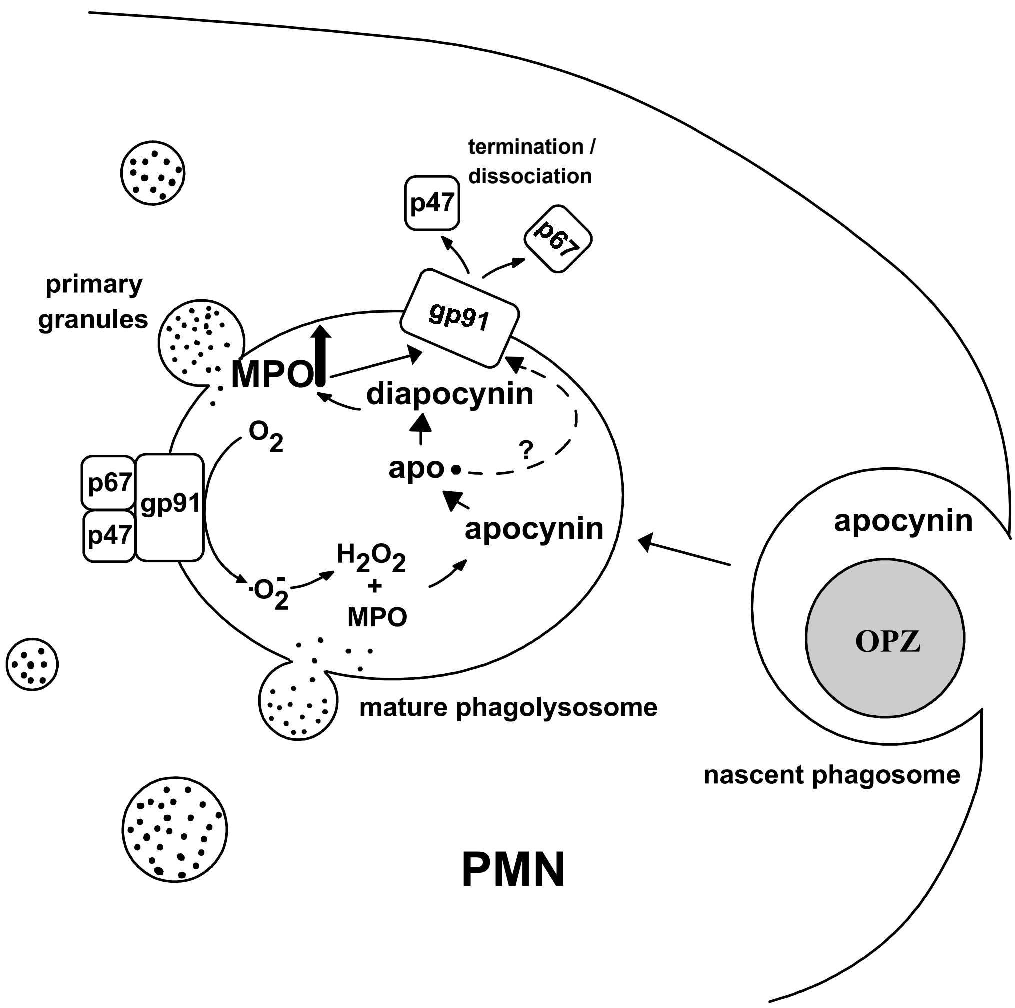

Figure 1. ***Supposed mechanism of action of apocynin.*** *(apo • represents the apocynin radical, MPO* 🡅 *represents increase of MPO activity).*

The possibility of diapocynin being the active metabolite also makes the theory of Stolk *et al.* less likely. These authors suggested that apocynin (or better its active metabolite) inhibits the assembly of NADPH oxidase by interfering with the translocation of the cytosolic proteins $p47^{phox}$ and $p67^{phox}$ *(20)*. The release of MPO and ROS, and thereby the conversion of apocynin into diapocynin, does only occur in the phagolysosome. However, translocation of the cytosolic factors $p47^{phox}$ and $p67^{phox}$ to the membrane-bound flavocytochrome *b* is a process that takes place in the cytosol of PMNs. So, diapocynin will only be able to inhibit the assembly of NADPH oxidase when it passes the cell membrane and enters the cytosol. Considering its log P and pK_a-values, this seems unlikely. We suggest that the activity of apocynin reflects rather the accelerated dissociation of the NADPH oxidase complex than inhibition of its assembly.

Possible role of apocynin in the plant

Being a phytogenic compound, the activities of apocynin described so far raise the question what role it may play in the plant. It is well known that many plants produce certain chemicals upon wounding or threatening events like attacks of insects or microorganisms *(38-40)*. These secondary metabolites contribute to the defense of the plant. For example, salicylic acid is a potent bactericidal and fungicidal agent *(41, 42)*. Its derivatives have been used effectively for many years to treat inflammations in humans *(43, 44)*. But also in the plant itself salicylic acid acts as a protective compound; it plays a critical role in the activation of plant defense responses after pathogen attack by inducing systemic resistance to infection *(45, 46)*. Experiments have shown that salicylic acid is required for signal transduction at local levels in infected plants, and that its mode of action may include inhibition of catalase activity which results in increased levels of hydrogen peroxide *(47)*.

So, it may well be possible that anti-inflammatory properties of apocynin also play a role in plant defense. Although apocynin is described to occur in many plants, not much is known about its role in these species. It has been reported that in several plants, fungal infections may give rise to the synthesis of fungitoxic compounds (phytoalexins) (*48-51)*. In *Amelanchier ovalis* and *Photinia davidiana*, infection was accompanied by increasing amounts of antifungal phenolics, most of which appeared to be released from bound (glycosidic) forms. One of these phenolics was identified as apocynin *(52)*. This indicates that in some plant species the increased production of apocynin may correlate with fungal infection.

In contrast, it has also been described that small phenolic compounds like apocynin, 4′-hydroxyacetophenone, and acetosyringone in particular, are able to induce the virulence (*vir*) genes of *Agrobacterium tumefaciens (53-55)*. This Gram-negative soil bacterium causes crown gall tumors after infecting the wound sites of most dicotyledonous plants *(56, 57)*. In this case, apocynin is not beneficial to the host, but may serve as a signal-molecule instead, which is recognized by receptors of

A. tumefaciens and thereby used by the bacterium to invade the plant and induce infection.

An approach to obtain more information about the role of apocynin in plants (*e.g. Picrorhiza kurroa*) may be their infection with different pathogens and subsequent analysis of apocynin levels in comparison with healthy controls.

Most interestingly, plants also possess an NADPH oxidase, which is quite similar to that of mammals *(58-60)*. So, future experiments focused on the interaction of apocynin with this herbal enzyme complex may also contribute to a better understanding of its mechanism of action and its potential anti-inflammatory properties.

Future Perspectives

Considering the anti-inflammatory activities of apocynin, we may conclude this compound definitely deserves further study. Besides providing data to further prove its mechanism of action, apocynin may be an interesting model-compound in the development of new potent NSAIDs.

Since ROS play an important role in many inflammatory diseases, there may be quite some pathological conditions in which apocynin may prove its beneficial activity. In particular in the treatment of colitis and atherosclerosis, apocynin may serve as the basis for the development of useful drugs. In this respect, beneficial effects of apocynin have been demonstrated in animal models *(21, 61)*. As a consequence, apocynin should further be tested in clinical trials, in particular since this compound is virtually devoid of side effects *(21, 62)*.

Apocynin may also become of interest in the relatively new and rapidly increasing field of vanilloid receptors. The discovery of vanilloid receptors *(63-67)* has led to extensive research on vanilloid-sensitive neurons *(68, 69)*. Compounds related to capsaicin, *(E)*-*N*-[(4-hydroxy-3-methoxyphenyl)-methyl]-8-methyl-6-nonenamide, collectively referred to as vanilloids, interact at a specific recognition site (vanilloid receptor), almost exclusively expressed by primary sensory neurons involved in nociception *(70, 71)* and neurogenic inflammation *(72, 73)*. Desensitization to endogenous vanilloids is a promising therapeutic approach to alleviate neuropathic pain and pathological conditions in which neuropeptides released from primary sensory neurons play a major role (*e.g.* vasomotor rhinitis). The use of capsaicin, however, is limited by its irritancy *(72)*, and therefore the synthesis of novel vanilloids with an improved pungency/desensitization ratio is an on-going objective. Interestingly, it has also been reported that acute capsaicin administration in rats protects against the ulcerative action of trinitrobenzene sulfonic acid (TNBS), most likely via the release of protective neuropeptides from capsaicin-sensitive nerve endings *(74)*. Since apocynin shares the vanillyl-like motif with capsaicin, its possible interaction with vanilloid receptors certainly deserves attention. Investigations in this respect may contribute to the development of new potent vanilloid-like drugs.

Final conclusion

Apocynin is a tiny, but mighty molecule. Its proposed mode of action in stimulated human PMNs involves endogenous formation of a dimer (diapocynin) inside the phagolysosome, which leads to increase of MPO activity and eventually to an accelerated termination of the NADPH oxidase-dependent respiratory burst.

Our investigations also indicate that molecules with so-called 'a-specific scavenger properties' may be of greater significance than thus far assumed.

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List of abbreviations

CFDA	5-Carboxy fluorescein diacetate
CGD	Chronic granulomatous disease
DCF	Dichlorofluorescein
3′,4′-DHA	3′,4′-Dihydroxy-acetophenone
123-DHR	123-Dihydrorhodamine
DME	1,2-Dimethoxyethane
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EI-MS	Electron ionization-mass spectrometry
ELISA	Enzyme-linked immunosorbant assay
ESR	Electron spin resonance
GC-MS	Gas chromatography-mass spectrometry
HBSS	Hank's balanced salt solution
HPS	Human pooled serum
HRP	Horse radish peroxidase
IBD	Inflammatory bowel disease
IC50	Concentration giving rise to 50% inhibition
IFN	Interferon
IL	Interleukin
IR	Infrared
LPS	Lipopolysaccharide
1-MPE	1-(4′-Hydroxy-3′-methoxyphenyl)-ethanol
MNC	Mononuclear cell
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
NADPH	Nicotinamide adenine dinucleotide phosphate
NHS	Normal human serum
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
OPZ	Opsonized zymosan
PBMNC	Peripheral blood mononuclear cell
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear neutrophil
RA	Rheumatoid arthritis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SIN-1	3-Morpholinosydnonimine
SOD	Superoxide dismutase
TEMPO	2,2,6,6-Tetramethylpiperidin
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TNBS	2,4,6-Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor

Nederlandse Samenvatting

- Als ik zou willen dat je het begreep,
legde ik het wel beter uit -

(Johan Cruijff, 1992)

Polymorfonucleaire neutrofielen (PMNs) zijn witte bloedcellen die behoren tot de granulocyten. Zij danken hun naam aan hun typische kern die vaak uit meerdere lobben bestaat. PMNs komen in grote aantallen voor in het bloed van gezonde mensen en in het geval van een ontsteking kan het aantal cellen nog drastisch toenemen. PMNs zijn beweeglijke cellen en kunnen de bloedbaan gemakkelijk verlaten door tussen de endotheelcellen, die het bloedvat bekleden, heen te dringen Hierdoor kunnen ze zich in de omringende weefsels begeven. PMNs zijn relatief kortlevende cellen met een halfwaardetijd van 6-7 uur in het bloed en 1-4 dagen in weefsels buiten de bloedbaan. PMNs vormen een eerste verdedigingslinie tegen binnengedrongen micro-organismen. Bij ontstekingen worden PMNs in grote aantallen aangetrokken naar de plek van infectie, waar ze de veroorzakers van de infectie (bv. een bacterie) onschadelijk kunnen maken door die te omstulpen en vervolgens op te nemen, een proces dat fagocytose wordt genoemd. Naast fagocyteren kunnen PMNs ook allerlei enzymen en reactieve zuurstofmetabolieten (reactive oxygen species = ROS) afgeven die de bacterie kunnen beschadigen en eventueel doden. PMNs bezitten receptoren die binnengedrongen bacteriën kunnen herkennen en binden waarna de cel geactiveerd wordt. Wanneer PMNs geactiveerd worden, nemen ze aanzienlijke hoeveelheden zuurstof op uit hun omgeving, een proces dat bekend staat als de “oxidatieve burst”. Dit proces is onafhankelijk van de normale cellulaire ademhaling en kan zelfs met cyanide niet geremd worden. De opgenomen zuurstof wordt vervolgens omgezet in ROS zoals superoxide anion ($\cdot O_2^-$), waterstofperoxide (H_2O_2) en het hydroxyl radicaal ($\cdot OH$). Na reactie van H_2O_2 met het eveneens door de geactiveerde PMNs vrijgezette enzym myeloperoxidase (MPO), kan het uiterst reactieve hypochloriet (HOCl) worden gevormd.

Het enzymcomplex dat verantwoordelijk is voor de omzetting van zuurstof in deze zuurstofradicalen is het NADPH-oxidase. Het NADPH-oxidase is een complex enzym dat bestaat uit diverse eiwitbouwstenen. In ongestimuleerde cellen is het NADPH-oxidase niet volledig geassembleerd en dus inactief. Het bestaat uit een membraan-gebonden gedeelte en enkele essentiële bouwstenen, waaronder $p47^{phox}$ en $p67^{phox}$, die in het cytosol zijn gelokaliseerd. Wanneer de cellen geactiveerd raken, migreren deze cytosolische eiwitten naar de celmembraan toe en binden zich aan het membraan-gebonden gedeelte. Na de assemblage is het actieve NADPH-oxidase in staat om de opgenomen zuurstof om te zetten in reactieve zuurstofmetabolieten. Zoals reeds vermeld zijn deze zuurstofmetabolieten (ROS) erg belangrijk bij de afweer tegen bv. bacteriën, maar in sommige gevallen kan de productie van deze zeer reactieve verbindingen leiden tot beschadiging van het omliggende weefsel. Deze beschadigingen kunnen een nieuwe ontsteking tot gevolg hebben. Dit betekent dat PMNs zowel gunstige als schadelijke effecten kunnen hebben bij de verdediging van het lichaam tegen infecties. Daarom kan het bij ontstekingen gunstig zijn om de productie van deze ROS te remmen.

Veel onderzoek is gericht op het vinden van verbindingen die heel specifiek de productie van ROS door geactiveerde PMNs remmen. Soortgelijk onderzoek leverde aan het eind van de jaren tachtig de stof apocynin op. Apocynin werd op geleide van activiteit geïsoleerd uit de plant *Picrorhiza kurroa*. Deze medicinale plant groeit hoog in de Himalaya en wordt in India en Nepal al eeuwenlang gebruikt bij de behandeling van ontstekingen. Apocynin bleek een potente remmer van de productie van ROS door geactiveerde PMNs. Bovendien bleek apocynin niet toxisch te zijn en geen bijwerkingen te hebben, en liet het de fagocyterende capaciteiten van de cellen intact. Dit betekent dat apocynin een ideaal geneesmiddel zou kunnen zijn bij de behandeling van ziektes waarbij de productie van ROS een rol speelt, omdat het alleen de ROS-productie remt maar de cellen nog steeds in staat stelt om bacteriën op te ruimen door middel van fagocytose en deze in het fagolysosoom te vernietigen met enzymen. Verder onderzoek aan apocynin leverde meer interessante toepassingen van dit molecuul op. In de behandeling van darmontsteking (colitis) en gewrichtsontsteking (artritis) in proefdiermodellen bleek apocynin een veelbelovend experimenteel medicijn te zijn.

Alhoewel verschillende onderzoeken werden uitgevoerd om de activiteit van apocynin te verklaren, werd er nooit een eenduidig model opgesteld dat overtuigend bewijs opleverde voor het werkingsmechanisme van apocynin. Uit experimenten was gebleken dat apocynin alleen actief was wanneer PMNs gestimuleerd werden met geöpsoniseerd zymosan (OPZ), dat een experimentele benadering is van een bacterie, en niet met de chemische stimulus PMA (een phorbol ester). In tegenstelling tot stimulatie met PMA, heeft stimulatie met OPZ tot gevolg dat PMNs MPO vrijzetten. Ook bleek dat apocynin niet actief was als bij stimulatie van PMNs met OPZ de MPO-remmer azide werd toegevoegd. Toen vervolgens bleek dat apocynin niet actief was in alveolaire macrofagen (die geen MPO bezitten), werd al snel de conclusie getrokken dat de activiteit van apocynin MPO-afhankelijk zou zijn. Het idee was nu dat apocynin onder invloed van een combinatie van MPO en de geproduceerde reactieve zuurstofmetaboliet H_2O_2, het substraat van MPO, omgezet zou worden in een actieve metaboliet die verantwoordelijk zou zijn voor de uiteindelijke activiteit. Uit verdere experimenten werd de conclusie getrokken dat apocynin de assemblage van het NADPH oxidase zou remmen door de translocatie van de cytosolische bouwstenen $p47^{phox}$ en $p67^{phox}$ naar het membraan-gebonden gedeelte van het oxidase-complex te remmen.

In dit proefschrift is het werkingsmechanisme van apocynin nader onderzocht. In Hoofdstuk 2 worden experimenten beschreven die de theorie bevestigen dat MPO inderdaad noodzakelijk is voor de activiteit van apocynin. Hoewel apocynin inderdaad een tijdsafhankelijke afname te zien geeft van de cytosolische bouwstenen in het gevormde NADPH-oxidase-complex, is dit geen bewijs dat apocynin feitelijk de assemblage van het NADPH-oxidase-complex remt. Het zou namelijk ook mogelijk kunnen zijn dat apocynin de afbraak van het reeds gevormde oxidase-complex versnelt. Door deze versnelde afbraak zouden de cytosolische eiwitten, nadat het complex eerst is opgebouwd, weer los kunnen laten van het membraangebonden deel. Dit heeft een niet meer verder functionerend NADPH-oxidase-complex tot gevolg en daardoor dus geen ROS-productie meer.

Om te bestuderen of een specifiek gedeelte van het apocynin-molecuul verantwoordelijk zou kunnen zijn voor de activiteit, werden verschillende analogen van apocynin getest, die alle slechts op één plek van het molecuul verschillen van apocynin (Hoofdstuk 3). Wanneer de activiteit van deze stoffen op de ROS-productie wordt vergeleken met die van apocynin, zou misschien iets kunnen worden gezegd over het werkingsmechanisme van apocynin. Uit de experimenten bleek dat zodra er een extra methoxy groep (–OCH_3) op positie C-5 werd geïntroduceerd, de remmende activiteit op de ROS-productie duidelijk toenam. Zo'n onderzoek naar de relaties tussen structuur en activiteit kan erg belangrijk zijn voor de ontwikkeling van een eventueel medicijn.

Naast PMNs kunnen mononucleaire cellen (MNCs) ook een belangrijke rol spelen bij ontstekingen. Vooral macrofagen spelen een grote rol bij het opruimen van bacteriën en restmateriaal. Macrofagen produceren voornamelijk reactieve stikstofmetabolieten zoals stikstofmonoxide (NO) en in mindere mate ook ROS. Uit de reactie van het gevormde NO en het tevens geproduceerde superoxide anion kan het zeer reactieve peroxynitriet ($ONOO^-$) worden gevormd. Deze reactieve stikstofverbindingen (reactive nitrogen species = RNS) zijn vooral belangrijk bij ontstekingen van de luchtwegen, zoals astma, waar ze voor een verslechtering van de situatie kunnen zorgen. Uit de literatuur was reeds gebleken dat apocynin in een cavia-model voor astma zorgde voor een afname in de vernauwing van de luchtpijp. Om te onderzoeken of dit gunstige effect van apocynin te danken zou kunnen zijn aan remming van stikstofmetabolieten, werd apocynin getest op geactiveerde macrofagen en werd het effect op de productie van peroxynitriet gemeten. Zoals blijkt uit de resultaten die beschreven worden in Hoofdstuk 4, remt apocynin duidelijk de productie van peroxynitriet. Dit zou de eerder gevonden activiteit van apocynin kunnen verklaren en zou tevens kunnen betekenen dat apocynin een potentieel medicijn zou kunnen zijn in de behandeling van astma.

In 1999 werd beschreven dat apocynin door het enzym horseradish peroxidase (HRP) en H_2O_2 omgezet werd in diapocynin. Dit molecuul, bestaande uit twee gekoppelde apocynin-moleculen, zou als actieve metaboliet verantwoordelijk zijn voor de uiteindelijke activiteit van apocynin. HRP, net als MPO een peroxidase, komt van nature niet voor in PMNs. Experimentele gegevens over diapocynin werden in de literatuur niet vermeld. In het in Hoofdstuk 5 beschreven onderzoek wordt de vorming, de identiteit, en de uiteindelijke activiteit van diapocynin verder onderzocht. Het beschreven protocol met HRP werd niet alleen succesvol herhaald, maar ook de vorming van diapocynin door MPO en H_2O_2 werd voor het eerst aangetoond. De identiteit van diapocynin werd gecontroleerd, bevestigd en gedocumenteerd door verschillende detectiemethoden te gebruiken. Nu het mogelijk bleek te zijn om diapocynin via enzymatische omzetting in de reageerbuis te maken, restte nog de vraag of diapocynin ook binnenin PMNs gevormd zou worden. Uit experimenten waarin PMNs in de aanwezigheid van apocynin werden gestimuleerd met OPZ bleek dat dit inderdaad het geval was. De enige voorwaarde was echter dat de de oplossing waarin het proces plaatsvond een pH van 8 moest hebben. Deze pH blijkt een fysiologische pH te zijn in de eerste minuten na stimulatie van PMNs met OPZ. Na stimulatie met PMA werd geen diapocynin gevonden, wat opnieuw de rol van MPO bevestigde. Uiteindelijk werd de activiteit van diapocynin op de ROS-productie gemeten en vergeleken met die van apocynin. Hieruit bleek dat de actieve metaboliet diapocynin niet een directe remmer van ROS-productie was zoals apocynin, maar dat het de reeds gevormde ROS wegvangt: een proces genaamd scavenging. De vraag was nu of deze activiteit van de actieve metaboliet van apocynin de uiteindelijke activiteit van apocynin zou kunnen verklaren. Uit bestudering van de literatuur bleek dat verschillende stoffen die qua structuur op diapocynin lijken, de activiteit van MPO binnenin de PMN kunnen verhogen, wat een versnelde beëindiging van de oxidatieve burst tot gevolg heeft. Hieruit mag wellicht worden geconcludeerd dat ook diapocynin, als actieve metaboliet van apocynin, de ROS-productie remt door het beëindigen van de NADPH-oxidase- activiteit. Of scavenging van superoxide anionen door diapocynin hierbij ook nog een rol speelt zal uit verder onderzoek moeten blijken.

Door hun grote mobiliteit zijn PMNs vaak de eerste cellen die bij ontstekingen ter plaatse zijn. In ontstekingsreacties spelen geactiveerde PMNs en de geproduceerde ROS meestal alleen een rol in de acute fase van de infectie. De lange termijn-effecten van ontstekingen worden voornamelijk gestuurd door mononucleaire cellen, zoals T-cellen die na stimulatie verschillende cytokinen kunnen produceren. Deze cytokinen zijn belangrijke onstekingsmediatoren en spelen vooral een rol bij meer chronische ontstekingen zoals reumatische artritis (RA). Apocynin is getest in een RA-model in ratten en blijkt de bij RA veelvuldig voorkomende zwellingen van de gewrichten te verminderen. In Hoofdstuk 6 worden experimenten beschreven met betrekking tot de effecten van apocynin, diapocynin en enkele andere apocynin-metabolieten op de productie van verschillende cytokinen die een rol spelen bij RA. Hieruit bleek dat apocynin en de metabolieten de productie van verschillende cytokinen door gestimuleerde mononucleaire cellen remmen. Het feit dat de remming plaatsvond bij concentraties die veel hoger lagen dan bij remming van ROS-productie door PMNs, zou kunnen betekenen dat apocynin en metabolieten van apocynin in deze cellen elkaars activiteit versterken. Dit fenomeen staat bekend als synergisme. Deze activiteit van apocynin op mononucleaire- en T-cellen zou misschien de verklaring kunnen zijn van de lange termijn-effecten van apocynin bij de behandeling van RA.

Concluderend kan gesteld worden dat apocynin een erg interessante verbinding is die zeker verdere studie verdient. Dit kleine molecuul is een potente remmer van de ROS-productie door geactiveerde PMNs. Deze activiteit wordt veroorzaakt doordat apocynin in de geactiveerde cellen wordt omgezet in een actieve metaboliet. Deze actieve metaboliet zorgt, in tegenstelling tot wat in de literatuur wordt beweerd, voor een versnelde beëindiging van de oxidatieve burst en daardoor voor een afname van de ROS-productie. Het feit dat apocynin alleen wordt omgezet onder invloed van het enzym MPO, betekent dat het een specifieke remmer van het NADPH-oxidase is, want niet gestimuleerde cellen zetten geen MPO vrij. Het belang van verder onderzoek naar apocynin wordt verder onderschreven door de interessante resultaten die met deze stof werden verkregen in verschillende diermodellen voor chronische ontstekingen. Mogelijk kan verder onderzoek naar apocynin een veilig en werkzaam medicijn opleveren voor de behandeling van ontstekingen waarbij geactiveerde PMNs een rol spelen.

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Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 25 augustus 1966 in Kampen. Zijn VWO diploma behaalde hij in mei 1986 aan het Johannes Calvijn Lyceum te Kampen. In hetzelfde jaar begon hij aan de studie Biologie aan de Rijksuniversiteit Utrecht. In augustus 1988 brak hij deze studie af en meldde zich aan bij de Hogeschool Utrecht, waar hij de Hogere Laboratorium Opleiding volgde. Tijdens deze opleiding verrichte hij een stage van 9 maanden bij de afdeling Farmacologie van het RIVM onder begeleiding van Dr. M.A.M. Gouw en Dr. J. Wemer. Deze studie werd in januari 1992 succesvol afgerond door het behalen van het diploma.

Van januari 1992 tot augustus 1993 was hij werkzaam als zoölogisch analist bij de vakgroep Farmacotherapie van de Faculteit Farmacie, Universiteit Utrecht onder supervisie van Dr. J.P.M. Dam en Prof. dr. A.J. Porsius. Van augustus tot november van hetzelfde jaar was hij kortstondig werkzaam als analist bij de vakgroep Klinische Farmacie, sectie Biofarmacie, onder supervisie van Dr. C. Oussoren en Prof. Dr. G. Storm.

Na een korte periode als vrijwilliger bij de vakgroep Farmacologie, werd in juni 1994 begonnen met de aanstelling als analist bij de vakgroep Farmacognosie (Faculteit Farmacie, Universteit Utrecht) onder supervisie van Dr. C.J. Beukelman en Prof. dr. R.P. labadie.

Op 1 april 1998 werd deze functie omgezet in een AIO positie bij de vakgroep Medicinal Chemistry, waarin het in dit proefschrift beschreven onderzoek werd verricht onder begeleiding van Dr. C.J. Beukelman, Dr. A.J.J. van den Berg, Prof. Dr. H. van Dijk en Prof. dr. R.P. Labadie. Een deel van het onderzoek werd in deze periode uitgevoerd op de University of Iowa College of Medicine (Department of Internal Medicine, Division of Infectious Diseases) in Iowa City, Iowa, U.S.A. onder supervisie van Prof. dr. W.M. Nauseef en Dr. F.R. DeLeo.