Chapter 1

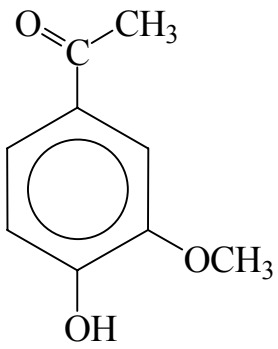
General Introduction

- 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)*.



Apocynin

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 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 (·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)*.

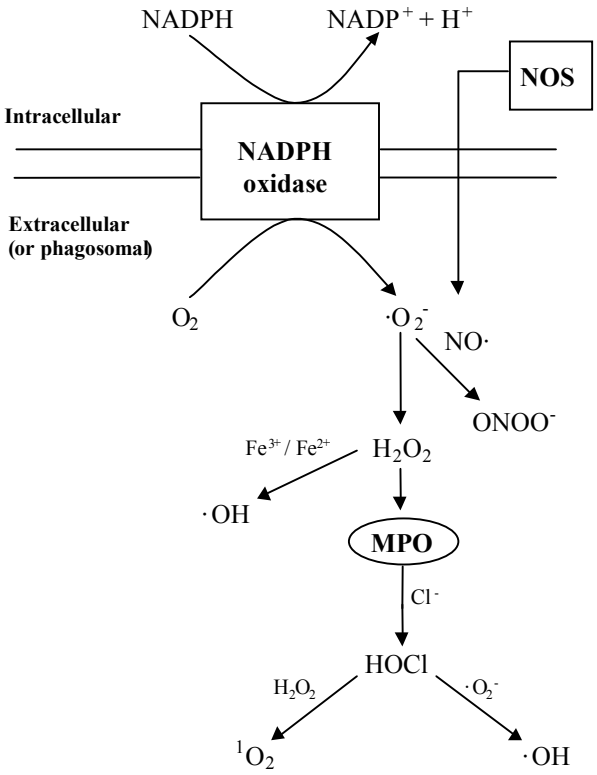


Figure 1. ***Reactive oxidant species production and reactions in stimulated neutrophils.*** *[NOS: nitric oxide synthase, MPO: myeloperoxidase; adapted from Hampton et al.(30)]*

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 flavocytochrome *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.

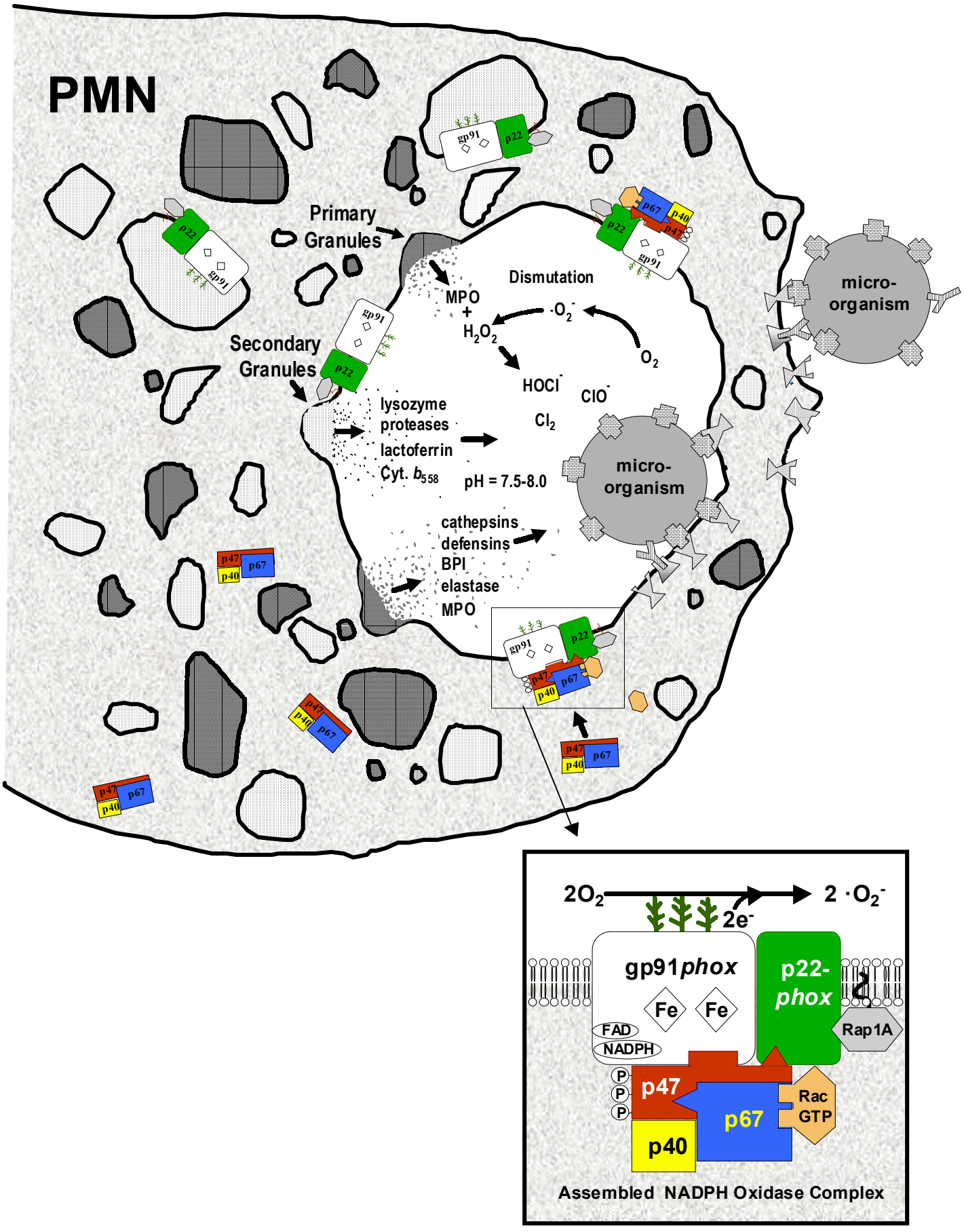


Figure 2. *NADPH oxidase assembly and subsequent ROS production by activated PMNs (picture by courtesy of Dr. F.R. DeLeo)*

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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