The Role of the Methoxyphenol Apocynin, a Vascular NADPH Oxidase Inhibitor, as a Chemopreventative Agent in the Potential Treatment of Cardiovascular Diseases

Jingjing Yu[1], Michel Weïwer[2], Robert J. Linhardt[1,2,3,*] and Jonathan S. Dordick[1,3,*]

[1]*Department of Biology,* [2]*Department of Chemistry and Chemical Biology* [3]*Department of Chemical and Biological Engineering, Center for Biotechnology & Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA*

Abstract: Oxidative stress has been linked to the origin and progression of cardiovascular diseases. Nicotinamide adenine dinucleotide phosphate, reduced form **(**NADPH) oxidase is a multi-component, NADPH-dependent enzyme that generates superoxide anion in the presence of molecular oxygen. The enzyme has been identified and characterized in all 3 vascular wall cell types and represents the major source of reactive oxygen species (ROS) production in the vascular wall. Inhibition of NADPH oxidase activation appears to suppress the sequence of cellular events that leads to a variety of cardiovascular diseases, including atherosclerosis. The naturally occurring methoxyphenol apocynin has been found to inhibit NADPH oxidase upon activation by peroxidases (e.g. soybean peroxidase, myeloperoxidase) or ROS under mild reaction conditions. Upon peroxidase-catalyzed activation, the apocynin oxidation products act to block the assembly and activation of NADPH oxidase. Although the mechanism of inhibition of NADPH oxidase remains largely unknown, apocynin's high effectiveness and low toxicity makes it a promising lead compound in the development of new therapeutic agents for cardiovascular diseases.

Keywords: Oxidative stress, reactive oxygen species, nicotinamide adenine dinucleotide phosphate, reduced form **(**NADPH) oxidase, apocynin, cardiovascular diseases.

1. INTRODUCTION

Polyphenols are common constituents of botanical extracts available in over-the-counter nutraceutical preparations and are found in different fruits and vegetables, olive oil, and beverages like red wine and tea. Consumption of polyphenols in the diet has been shown to reduce the likelihood of morbidity and mortality from coronary artery disease (CAD) [1]. The "*French Paradox*" (i.e. the low incidence of CAD despite diets high in lipids) is purportedly due to the regular drinking of red wine [2]. One way in which polyphenols are thought to act is through the inhibition of lipid peroxidation of low-density lipoprotein (LDL) [3]. Dietary supplements rich in polyphenols, such as black and green tea [4], olive oil [5], red wine [6], and licorice root extract [7], are associated with an increased resistance of plasma LDL oxidation. Indeed, consumption of tea extract, red wine or licorice extract by hyper-cholesterolemic apolipoprotein E (apoE)-deficient mice caused a significant reduction in atherosclerosis [7-9]. The antioxidant activity of polyphenols is clearly related to their chemical structure. For example, the two hydroxyl groups on the B phenol ring of the polyphenol glabridin are required for therapeutic activity that leads to a 50% reduction in the size of aortic lesions in apoE-deficient mice [10].

*Address correspondence to these authors at the Department of Biology, Department of Chemistry and Chemical Biology, Department of Chemical and Biological Engineering, Center for Biotechnology & Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA; Tel: 518-276-3404, 518-276-2899, Fax: 518-275-3405, 518-275-3405,
Emails: linhar@rpi.edu; dordick@rpi.edu

The *o*-methoxyphenol apocynin (4-hydroxy-3-methoxy-acetophenone or acetovanillone), is the major active constituent of plant extracts from *Picrorhiza kurroa*, which has been used as a traditional medicine in South Asia. Apocynin has been applied as a liver tonic, a cardiotonic, and in the treatment of jaundice and asthma [11, 12]. Conventional wisdom has held that this compound, as with other polyphenols, exerts its chemoprotective effects through antioxidative properties, e.g. direct free radical scavenging [13-16]. However, scavenging of radicals once formed is a relatively inefficient process, particularly given the high reactivity of ROS. A more compelling mechanism of the chemoprotective effect involves the inhibition of ROS generation by shutting down the catalytic activity of specific enzymes that generate reactive oxygen species (ROS) as byproducts of cell metabolism.

Recent investigations have suggested that apocynin's primary mode of action is through the inhibition of NADPH oxidase. Growing evidence for the involvement of NADPH oxidase in the vasculature as the source for ROS production suggests that this multi-component signaling enzyme may be an ideal target for the design of new vascular therapeutic agents [17]. Inhibition of NADPH oxidase activation will suppress the sequence of cellular events that leads to a variety of vascular diseases, such as atherosclerosis, wherein NADPH oxidase is activated and is the major source for the formation of ROS that modulate pathophysiological changes in vascular wall cells [18, 19]. Although the mechanism of inhibition by apocynin is not completely understood, the compound appears to be a prodrug that can be metabolized to the corresponding dimer or oligomers *via* the action of peroxidases [20]. Due to its high oral bioavailability, low

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toxicity and high efficacy *in vivo* the compound may serve as a promising lead as a therapeutic candidate.

2. OXIDATIVE STRESS AND ROS

2.1. Oxidative Stress

Oxygen metabolism, although essential for life, imposes a potential threat to cells because of the formation of ROS, such as superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), hypochlorous acid (HOCl) and a variety of other oxygenated products [21], all of which to varying extents can damage deoxyribonucleic acid (DNA), proteins, lipids, and carbohydrates. To avoid this damage, organisms have developed antioxidant defense systems that are is composed predominantly of 2 classes; ROS metabolizing enzymes and low-molecular-weight antioxidants (e.g. ascorbic acid and the tocopherols).

Oxidative stress can be defined as an imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates or repair the resulting damage. At high ROS levels, cellular reductants are consumed, resulting in a lower reducing capacity of the cells [22]. Severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis [23] and cell degradation [24, 25]. Oxidative stress has been linked to the origin and progression of chronic degenerative diseases (e.g., cancer, diabetes, atherosclerosis, as well as neurodegenerative diseases such as Alzheimer's and Parkinson's diseases) and it may also be important in aging [26]. Nevertheless, ROS production can be beneficial, for example, by the immune system to kill pathogens. Furthermore, moderate and controlled physiological concentrations of ROS are involved in signal transduction mechanisms important in cell growth, apoptosis and cell migration [27]. This suggests that normal cellular homeostasis is a result of a delicate balance between the rate of ROS formation and its elimination.

2.2. Production of ROS

The one-electron reduction of oxygen to $\bullet O_2^-$ is catalyzed by a variety of enzymes in the mitochondrial respiratory chain, cytochromes P450, xanthine oxidases (XOD), lipoxygenases, and NADPH oxidases. This process is known as respiratory or oxidative burst in phagocytes [30, 31]. Production of $\bullet O_2^-$ appears to occur within all aerobic cells, to an extent dependent on O_2 concentration. In mitochondria, 1-3% of electrons are thought to form $\bullet O_2^-$. During respiratory burst, the increase in O_2 uptake in neutrophils can be 10 to 20 times that of resting O_2 consumption [32].

Superoxide anion serves as the starting material for the production of a vast assortment of ROS, including oxidized halogens, free radicals, and singlet oxygen. Most of the oxygen consumed in this way will not be present as $\bullet O_2^-$. Under physiological conditions, superoxide dismutase (SOD) converts $\bullet O_2^-$ into the more stable H_2O_2. The importance of SOD in antioxidant defense is illustrated by a study in SOD_2-deficient mice that developed cardiomyopathy and neurodegeneration [33]. However, H_2O_2, which diffuses through cell membranes [34], is assumed to be involved in intracellular signaling pathways and at higher concentrations is toxic [35]. Thus, enzymatic defense against H_2O_2 provided by catalase and glutathione peroxidase is crucial.

Hydrogen peroxide is bactericidal only at high concentrations. Because of the limited membrane permeability of

Table 1. Common Oxidants and their Properties (Adapted from [28, 29])

Oxidant	Property
$\bullet O_2^-$, superoxide anion	One-electron reduced state of O_2, formed in many autoxidation reactions *via* the electron transport chain. Rather unreactive but can release Fe^{2+} from iron-sulphur proteins and ferritin. Undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis and is a precursor for metal-catalyzed •OH formation.
H_2O_2, hydrogen peroxide	Two-electron reduced state, formed by dismutation of $\bullet O_2^-$ or by direct reduction of O_2. Lipid soluble and thus able to diffuse across membranes.
•OH, hydroxyl radical	Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive and will easily attack most cellular components.
ROOH, organic hydroperoxide	Formed by radical reactions with cellular components such as lipids and nucleotides.
RO•, alkoxy and ROO•, peroxy radicals	Oxygen-entered organic radicals. Lipid forms participate in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.
HOCl, hypochlorous acid	Formed from H_2O_2 by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents, including thiols, methionine, and amino groups.
$OONO^-$, peroxynitrite	Formed in a rapid reaction between $\bullet O_2^-$ and NO•. Lipid soluble and similar in reactivity to HOCl. Protonation forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen dioxide.
Singlet oxygen	Generated in a photosensitized process by energy transfer from dye molecules such as rose bengal, methylene blue or porphyrins, or by chemical processes such as spontaneous decomposition of hydrogen trioxide in water or the reaction of hydrogen peroxide with hypochlorite.

H_2O_2, a variety of secondary oxidants have been proposed for the destructive capacity of phagocytes. In phagocytes, most of the H_2O_2 is consumed by myeloperoxidase (MPO), an enzyme released upon cellular stimulation [36-38]. 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 HOCl (Eq. 1) [39].

$$H_2O + Cl^- \xrightarrow{MPO} HOCl + \bullet OH \quad (1)$$

Hydroxyl radicals (•OH), formed by the iron catalyzed Fenton reaction (Eq. 2), are extremely reactive with most biological molecules but have a limited range of action [40].

$$H_2O + e^- + H^+ \xrightarrow{Fe_3^+/Fe_2^+} H_2O + \bullet OH \quad (2)$$

Finally, $\bullet O_2^-$ reacts readily with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a potential cytotoxic agent produced by inflammatory cells, with a second order rate constant of $6.7 \times 10^9\ M^{-1}s^{-1}$ [41, 42]. Although SOD stabilizes NO [41], its reaction is three times slower ($2.9 \times 10^9\ M^{-1}s^{-1}$) than that of $\bullet O_2^-$ and NO [43]. Given the rapid rate of the chemical reaction, there is probably some $\bullet O_2^-$ reacting with NO at any given time within the cells, suggesting a tenuous balance between these molecules that can be easily disturbed under pathological conditions [44]. Peroxynitrite is a potent, relatively stable, strong oxidant and nitrating agent with properties similar to those of hydroxyl radical [45-48] (Fig. (**1**)) [49].

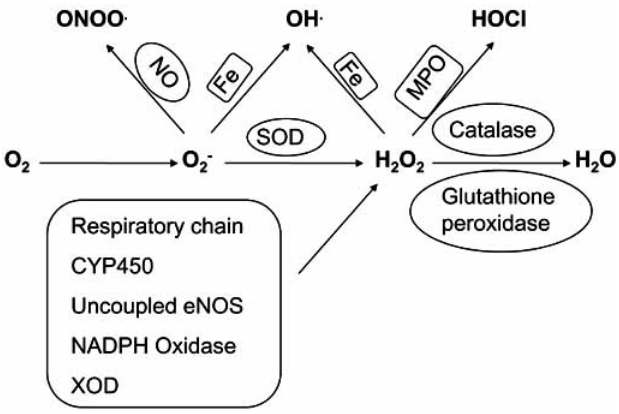

Fig. (1). ROS production and removal in biological system (modified from [49]).

2.3. Biology of ROS

In phagocytes, ROS production is used to kill invading microorganisms and pathogens. But recent studies involving vascular cells have demonstrated that ROS plays an integral role in regulating cell signaling pathways, often through the modulation of kinase and phosphatase activities or through gene transcription [50-52]. ROS have distinct functional effects on each vascular cell type and can play both physiological and pathophysiological roles. In smooth muscle cells and fibroblasts, ROS were found to promote cell proliferation and mediate cell migration [53]. In endothelial cells, ROS have been shown to induce signaling processes including apoptosis, expression of adhesion molecules, and angiogenesis [27]. All these processes are fundamental in the homeostasis of the vasculature, but oxidative stress followed by an over-stimulation of the pathways can lead to inflammation, hypertrophy, remodeling, and/or angiogenesis, which are hallmarks of many cardiovascular diseases, such as atherosclerosis, hypertension, diabetes, heart failure, and restenosis [54].

Increased production and release of ROS is considered to be the key event in the pathogenesis of endothelial dysfunction, which is caused by a decline in the bioavailability of NO [55]. This pathophysiological state is characterized by the impairment of the protective functions of NO leading to a loss of vasodilation, platelet aggregation, smooth muscle cell growth and migration, inflammation, and angiogenesis [44]. A loss of the NO bioavailability may be caused either by a decreased expression of endothelial Nitric Oxide Synthase (eNOS) [56], a lack of substrate or cofactors for eNOS [57], or accelerated NO scavenging by ROS, such as superoxide [58], lipid radicals [59], and hydroxyl radicals [60] (Fig. (**2**)) [44].

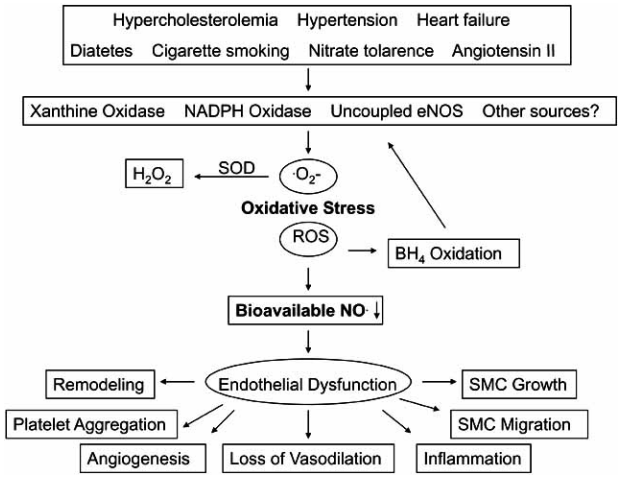

Fig. (2). Oxidative stress and endothelial dysfunction in cardiovascular diseases (modified from [44]).

3. NADPH OXIDASE

3.1. Phagocytic NADPH Oxidase Structure

Human NADPH oxidase is a complex enzyme that is composed of at least six protein components assembled at the cell membrane to catalyze the production of $\bullet O_2^-$ by the one-electron reduction of oxygen, using NADPH as the electron donor [61] (Eq. 3).

$$2\ O_2 + NADPH \rightarrow 2\ O_2^- + NADP^+ + H^+ \quad (3)$$

The structure and function of NADPH oxidases are well characterized in phagocytic cells (neutrophils, macrophages, and eosinophils). A functional phagocytic NADPH oxidase complex consists of the membrane-anchored flavocytochrome b_{558} (a heterodimer composed of $gp91^{phox}$ and $p22^{phox}$), cytosolic proteins $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$, and the low molecular-weight GTP-binding proteins, Rac 1 or 2 [50, 62-66].

Cytochrome b_{558} is anchored to the cell membrane by a series of hydrophobic transmembrane segments. Both a flavin and two heme redox centers are contained within the cytochrome heterodimer. The catalytic subunit gp91^{phox} (also termed Nox2 according to the new nomenclature [67]) is a highly glycosylated protein with a molecular weight of 65.3 kD, but running at 91 kD on an SDS-PAGE gel. It possesses six transmembrane α-helices and contains the binding sites for FAD and NADPH (Fig. (**3**)). The small subunit p22^{phox} associates with gp91^{phox} in a 1:1 complex and contributes to its maturation and stabilization. The C-terminal cytoplasmic portion of p22^{phox} has a proline-rich region (PRR) that contains a consensus PxxP around Pro^{156} (Fig. **3**). This motif is known to be a target of the Src homology 3 (SH3) domains of p47^{phox} [68, 69]. Studies show that p22^{phox} is phosphorylated in a phosphatidic acid-dependent manner at Thr^{132} or Thr^{147} upon activation, which is close to the region that interacts with p47^{phox} [70, 71] (Fig. (**3**)) [72].

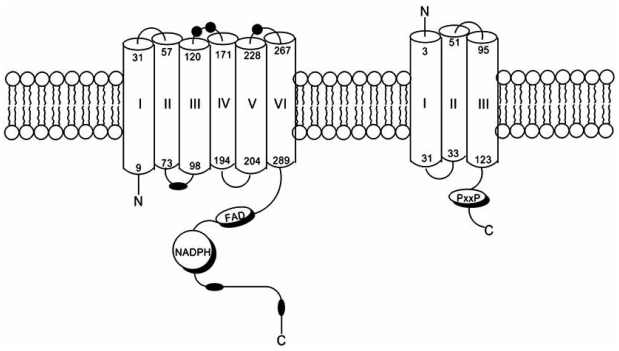

Fig. (3). Model of Flavocytochrome b558 [72]. The predicted transmembrane helices of gp91^{phox} and p22^{phox} are indicated. Glycosylation sites are indicated by dots and regions that are believed to interact with p47^{phox} in the active state in oval.

P47^{phox} contains a PX (phox homology) domain, two tandem SH3 domains (N-SH3 and C-SH3), a polybasic region/autoinhibitory region (PBR/AIR) that is rich in arginine and lysine residues and a PRR (Fig. (**4**)). The PX domain preferentially recognizes phosphatidylinositol (3,4)-bisphosphate and thereby contributes to membrane anchoring of p47^{phox} after activation-induced translocation [73]. In the resting state, p47^{phox} is in an autoinhibited form, where the PBR/AIR bundles the tandem SH3 domains and the linker between the tandem SH3 domains forming a closed structure [74] (Fig. (**4**)) [72].

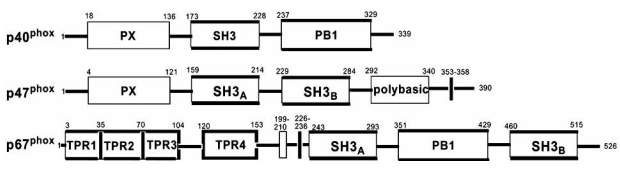

Fig. (4). Domain Structure of Cytosolic Subunits p40^{phox}, p47^{phox}, and p67^{phox} ([72]).

The positions of consensus PxxP motifs in p47^{phox} (amino acids 363–368) and p67^{phox} (amino acids 226–236) are indicated by thick bars. The locations of serine and threonine residues that become phosphorylated during activation are indicated by thin bars.

3.2. Assembly and Activation Mechanism of NADPH Oxidase

Tight regulation of NADPH oxidase activity is achieved by two mechanisms; the separation of the oxidase subunits into different subcellular locations during resting state (cytosolic and membrane-bound) and the modulation of reversible protein-protein and protein-lipid interactions [72]. In unstimulated, quiescent neutrophils, p47^{phox}, p67^{phox}, p40^{phox}, and Rac all reside in the cytoplasm. p47^{phox}, p67^{phox} and p40^{phox} may form a ternary complex with 1:1:1 stoichiometry [75]. However, it is possible that p47^{phox} exists separately from the p40^{phox}-p67^{phox} complex in resting cells, and that formation of the trimeric complex requires stimulation. This would then constitute the first step along the activation pathway [76] (Fig. (**5**)) [77].

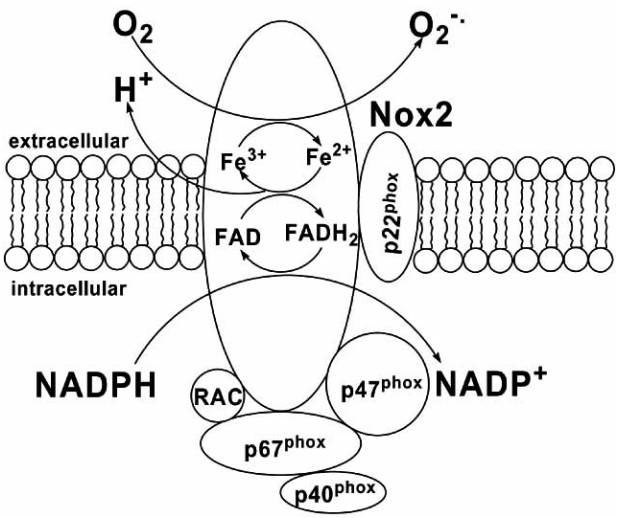

Fig. (5). Assembly of phagocytic NADPH oxidase (modified from [77]). FAD and $FADH_2$ are flavin adenine dinucleotide and flavin adenine dinucleotide, reduced form.

NADPH oxidase activation can be either receptor-mediated or receptor-independent. Typical receptor-dependent stimuli include opsonized zymosan (OPZ) [78], the bacterium-derived chemotactic tripeptide N-formyl-Met-Leu-Phe (fMLP) [79], complement components C5a, C3b and iC3b [80], and the lectin concanavalin A [81]. Long-chain unsaturated fatty acids and phorbol-12-myristate-13-acetate (PMA) are receptor-independent [82]. All these substances ultimately lead to p47^{phox} phosphorylation, Rac activation, and free fatty acid release that triggers NADPH oxidase-derived oxidative burst [83].

Upon cellular activation, some serine residues in the p47^{phox} PBR/AIR, including positions 303, 304, 315, 320, and 328, are phosphorylated by protein kinase C (PKC) or Akt [84, 85]. Once PBR/AIR binding to SH3 is released by phosphorylation, rearrangements of the SH3 domain may occur, forming an open structure that translocates and binds to the cytoplasmic PRR of membrane-bound p22^{phox} [74, 86, 87]. This results in a conformational change in the flavocytochrome b558 that allows electrons from NADPH at the cytosolic side of the membrane to be donated to molecular oxygen at the other side of the membrane, either at the out-

side of the cells or in the phagosomes containing ingested microorganisms that initiate $\bullet O_2^-$ generation. The docking of $p47^{phox}$ to cytochrome b558 also facilitates the interaction between $p67^{phox}$ and $gp91^{phox}$ that is required for the activation of the catalytic subunit [88]. The interaction between $p47^{phox}$ and $p22^{phox}$ is considered to be essential for NADPH oxidase activation [86, 89]. In addition, three interaction sites are reported to exist between $p47^{phox}$ and $gp91^{phox}$ [90]. Another critical step in the activation process is the translocation and binding of the activated GTPase Rac to cytochrome b558. Rac-GTP appears to anchor itself in the membrane through its prenylated tail, independent of $p47^{phox}$ [91].

Specific interactions through SH3 domains are required for the assembly and activation of the NADPH oxidase subunits [68, 69, 92] (Fig. **4**). SH3 domains are present in many signal transduction proteins and are known to mediate interactions *via* binding to proline-rich regions (PRRs) on target proteins. Both $p47^{phox}$ and $p67^{phox}$ contain two SH3 binding domains [16], while $p47^{phox}$ and $p22^{phox}$ each have a PRR. In the cytosol of resting cells, $p67^{phox}$ is complexed with $p47^{phox}$ through an interaction between the C-terminal SH3 domain of $p67^{phox}$ and the C-terminal PRR of $p47^{phox}$ [75]. Assembly of the cytosolic subunit complex with the membrane subunits absolutely requires binding between the N-terminal SH3 domain of $p47^{phox}$ to a PRR on the cytosolic C-terminus of $p22^{phox}$ (residues 151-160). A point mutation, $Pro_{156} \rightarrow Gln$, in the PRR of $p22^{phox}$ prevents $p47^{phox}$ binding [93]. In mapping the functional domains in the $p22^{phox}$ subunit using pentadecapeptide sequences derived by "peptide walking", $p47^{phox}$ has been shown to not only bind to the PRR of $p22^{phox}$, but also to a domain (residues 51-63) located on a loop exposed to the cytosol [94].

3.3. Vascular NADPH Oxidase

The observation that all 3 major vascular wall cell types (e.g., endothelial cells (EC), vascular smooth muscle cells (VSMC), and adventitial fibroblasts) possess NADPH oxidase has led to the descriptive term, vascular NADPH oxidase. Over the past few years, significant investigations have established that activation of vascular NADPH oxidase is the major determinant of the generation of ROS by the aforementioned cell types of the arterial wall, as well as the monocytes/macrophages and neutrophils that invade the arteries during atherosclerosis [16, 17, 95-97]. NADPH oxidase might initiate oxidative stress at early ages of vascular disease, and then trigger itself and other ROS sources leading to the progression of oxidative stress and endothelial dysfunction [98, 99]. NADPH oxidase together with other enzymes, e.g. XOD, lipoxygenases appears to act synergistically to augment ROS generation [100]. There has been growing interest in the vascular NADPH oxidase, largely because it is recognized that oxidative stress plays a critical role in the pathogenesis of vascular diseases.

A number of studies support the presence of a functional NADPH oxidase in EC, which is capable of producing ROS and similar to that of phagocytes [96, 101-107]. This oxidase was found to exist in a *preassembled* form, consisting of $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ [108]. The NADPH oxidase subunits expressed by EC are identical to that found in phagocytes. The messenger ribonucleic acid (mRNA) of EC $p47^{phox}$, $p67^{phox}$, $p22^{phox}$, and $gp91^{phox}$ have been sequenced and shown to share a near 100% homology with their corresponding neutrophil counterparts [96, 104]. Cytosolic $p47^{phox}$ and $p67^{phox}$ in EC are closely homologous to their phagocytic counterparts, however as stated above, in EC they may exist as a pre-formed complex prior to activation [108]. The close homology between the phagocytic and EC NADPH oxidase has facilitated mechanistic evaluation. Fibrobloasts, such as EC, express the flavocytochrome b_{558} subunits, $gp91^{phox}$ and $p22^{phox}$, as well as the cytosolic factors $p47^{phox}$ and $p67^{phox}$ [97]. In contrast, whereas VSMC contain $p47^{phox}$ and $p22^{phox}$ [17], the expression of $p67^{phox}$ and $gp91^{phox}$ has been difficult to demonstrate [109]. Interestingly, both $p67^{phox}$ and $gp91^{phox}$ are found expressed in VSMC of human resistance arteries [110-112].

Two homologues of $gp91^{phox}$, called Nox1 and Nox4, were recently identified in VSMC [111]. These homologues have been shown to function in place of $gp91^{phox}$. Nox1 is expressed in low amounts in VSMC where its activity and expression can be stimulated by mitogenic substances such as angiotensin II and platelet-derived growth factor (PDGF) [111]. In contrast to the relatively low level of Nox1 and $gp91^{phox}$, Nox4 is abundantly expressed in all vascular cells [91, 113, 114]. While the Nox homolog and the membrane-bound $p22^{phox}$ subunit are essential to maintain a stable unit capable of supporting electron transfer for superoxide generation [115], the role the cytosolic components play in the vascular NADPH oxidase remains unclear. This has important implications for the action and specificity of NADPH oxidase inhibitors. Evidence of a functional NADPH oxidase in VSMC, includes the transfection of VSMC with antisense to $p22^{phox}$ or Nox1, or the use of $p47^{phox}$ knockout cells, both of which result in a marked inhibition of NADPH-dependent $\bullet O_2^-$ generation by stimulated cells and a reduction of atherosclerotic lesions [109, 111, 116, 117].

Homologues of 2 cytosolic subunits, NoxO1 (homologue of $p47^{phox}$) and NoxA1 (homologue of $p67^{phox}$), have also been cloned from colon epithelial cells. Both subunits seem to be required for Nox1 activity [118]. In contrast, Nox4 activity seems to be independent of the known cytosolic subunits. Compared with its homologue $p47^{phox}$, NoxO1 does not have an autoinhibitory loop, which together with its pre-localization in the membrane, suggests an increased basal activity of NoxO1-based NADPH oxidase [119]. The precise role of NoxO1 and NoxA1 in the vasculature still remains to be determined. Recently, the expression of NoxA1 has been shown in VSMC of mouse carotid artery indicating that it replaces $p67^{phox}$ in the media of large vessels [91, 120]. The small GTPase Rac1 has been shown to be expressed in all vascular cells [121].

The exact assembly and activation of vascular NADPH oxidases is poorly understood. It is assumed that vascular $gp91^{phox}$-based NADPH oxidases follow a mechanism similar to that demonstrated for phagocytes. It has been suggested that $gp91^{phox}$ is responsive to agonists-stimulated ROS generation, such as tumor necrosis factor-α (TNF-α), and angiotensin II. In EC and fibroblast with $gp91^{phox\ -/-}$-mice these stimulators failed to promote ROS formation [53, 96, 122].

In contrast to phagocytic cells, the vascular NADPH oxidases produce constitutively low levels of ROS under basal conditions, 1-10% of the rate in leucocytes [121], while generating much higher levels in response to cellular perturbants, such as angiotensin II, thrombin, atherogenic LDL levels, hypertensive levels of shear stress, diabetic concentrations of glucose, growth factors like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and cytokines like tumor necrosis factor - α (TNF-α), and interleukin-1 (IL-1) [123]. These attributes are consistent with the integral role for vascular NADPH oxidase in cell signaling and activating events that lead to a variety of cardiovascular diseases. The observation that ROS generation occurs mainly intracellularly provides another hint for their role in mediating signal transduction.

The most studied stimulus of the vascular NADPH oxidase is angiotensin II, which increases the activity of the NADPH oxidase at 3 or more levels. First, there is rapid activation of c-Src and other kinases, leading to phosphorylation of $p47^{phox}$, which translocates to the membrane cytochrome complex [124]. A key role for $p47^{phox}$ in this process has been demonstrated by use of $p47^{phox-/-}$ mice. Isolated EC and VSMC from these knockout mice did not produce $\bullet O_2^-$ in response to angiotensin II [117, 125, 126]. In VSMC, EGF receptor transactivation is also involved, leading to sequential activation of phosphoinositide (PI) 3 kinase and the small G-protein Rac, all of which occurs within minutes of angiotensin AT1 receptor activation [127]. There is also some evidence for the involvement of Rho family guanosine triphosphate hydrolases (GTPases) in ROS generation in VSMC, and this is supported in suppression of NADPH oxidase by statins, which inhibit the synthesis of isoprenoids such as farnesylpyrophosphate and geranylgeranylpyrophosphate that are important posttranslational lipid attachments for intracellular signaling molecules such as the Rho GTPases [128]. A further level of action of angiotensin, and some other stimuli, is to increase the expression of NADPH oxidase subunits over hours to days [54]. All these events following stimulation with angiotensin serve to activate, promote and sustain electron flow through the cytochrome complex. In rat VSMC transfected with antisense Nox1 mRNA, ROS generation was inhibited in response to angiotensin II, but no change of basal ROS production was observed [111]. This indicates that Nox1 is essential for agonist-stimulated NADPH oxidase activity in VSMC. The importance of angiotensin II for activation of NADPH oxidase is underscored by studies showing that in oxidative stress, NADPH oxidase activation and some of the pathological features of hypertension and atherosclerosis are eliminated by angiotensin AT1 receptor antagonists [129, 130].

3.4. Pharmacology of NADPH Oxidases

The protective effects of NADPH oxidase inhibition on diseases such as atherosclerosis have been confirmed with an animal model of experimental atherosclerosis, e.g., the apoE-deficient mouse [131, 132]. However, the pharmacology of NADPH oxidase is still under intensive study. There is a long list of compounds/peptides that are known inhibitors of NADPH oxidase (Table **2**). However, most of these inhibitors have major impediments, in terms of bioavailability, efficacy, and toxicity for their therapeutic use. Moreover, most inhibitors appear to be either non-specific or their mechanism still remains unknown. For example, the proline-arginine rich peptide PR-39 blocks NADPH oxidase activity by binding to the SH3 domain of $p47^{phox}$ and, therefore, preventing its translocation to the cytochrome b558 [133-135]. However, non-specific effects of PR-39 have been observed because it also binds to SH3 domains of other proteins as well as interacting with membrane lipids [136, 137]. One of the more interesting classes of compounds with known NADPH oxidase inhibition activity is the phenolics. Due to their simplicity, abundance, and low toxicity, these com-

Table 2. Inhibitors of NADPH Oxidase [50, 53, 103, 138]

Category	Inhibitors
Cytosolic subunits translocation inhibitors	Catechols (3,4-dihydroxybenzaldehyde, caffeic acid, protocatechuic acid), *ortho*-methoxy-substituted catechols (apocynin, vanillin, and 4-nitroguaiacol), quinones (1,4-naphthoquinone), nitrosothiols (RSNO), nitric oxide, PR-39, gp91ds-tat, gliotoxin, 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)
Flavoprotein inhibitors	Diphenylene iodonium (DPI), quinacrine
Heme ligands	Imidazole, pyridine
Direct-acting thiol reagents	Disulfiram, penicillamine, phenylarsine oxide (PAO), gliotoxin
NADPH analogue	Cibacron blue
Redox active inhibitors	Quercetin, esculetin, DPI
Intracellular calcium antagonist	TMB-8
Calmodulin antagonists	W-7, Trifluoperazine
Rac inhibitors	Statins, Inhibitors of geranylgeranyltransferase (GGTI-286,GGTI-298), clostridia toxins
Unknown	Neopterin, plumbagin, S17834[6,8-diallyl 5,7-dihydroxy 2-(2-allyl 3-hydroxy 4-methoxyphenol)1-H benzo(b)pyran-4-one], VAS2870.

pounds may prove to be highly desirable as therapeutic candidates. One of the more active phenolics is apocynin, whose function is described below.

4. APOCYNIN

4.1. History and Structure

Apocynin (Fig. (**6**)) was first isolated from the roots of *Apocynum cannabinum* (Canadian hemp) in 1883. Although apocynin was first discovered in *A. cannabinum*, its occurrence is not restricted exclusively to the *Apocynaceae* family. In fact, it is a common compound in many plant species [139-143]. Furthermore, in the pulp and paper industry, apocynin is known as one of the degradation products of lignin [144, 145]. Canadian hemp was used as official remedies for dropsy and heart problems [146]. The constituents of Canadian hemp were re-investigated in 1908 by Finnemore, who developed a new procedure to isolate apocynin on a larger scale [147]. In 1971, isolation of apocynin from *Picrorhiza kurroa* Royle ex Bebth was reported [148]. *P. kurroa* is a small, perennial plant growing at high altitudes in the western Himalaya region, which has been used in traditional Ayurvedic medicine in South Asia, specifically in India and Sri Lanka. Apocynin was considered to be an important constituent contributing to the medicinal potential of this herb, although no specific details on the effectiveness of *P. kurroa* were known at that time. Extracts of this plant are currently available in over-the-counter preparations that are advertised as being useful for "watery fullness of cellular tissues-oedema; puffiness of eyelids and wrinkled lids; feet full and oedematous, pitting upon pressure; circulation sluggish; scanty urine; skin glistening; hemoptysis; menorrhagia, profuse, too often and too long continued; and full, relaxed uterus, with watery discharges [149]".

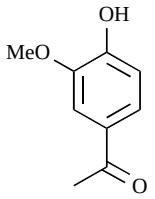

Fig. (6). Chemical structure of apocynin.

4.2. Chemistry

Apocynin possesses a faint vanilla odor and has a melting point of 115°C. It is slightly soluble in cold water, but freely soluble in hot water, alcohol, benzene, chloroform, and ether. Peroxidases can catalyze the oxidative polymerization of phenols under mild reaction conditions in the presence of the ROS H_2O_2 to produce a mixture of oligomeric species. Horseradish peroxidase (HRP) [150], soybean peroxidase (SBP) [151] and myeloperoxidase [20] have been used to produce oligophenol mixtures from a wide variety of phenols, including *o*-methoxyphenols such as apocynin.

Despite the emergence of apocynin and related compounds as potential prodrugs against the NADPH oxidase target, the products of peroxidase-catalyzed apocynin oxidation both *in vitro* and as metabolites of *in vivo* peroxidase action have not been fully characterized. In 2004, Antoniotti *et al.* studied SBP-catalyzed oxidation of apocynin and related *o*-methoxyphenols in the presence of H_2O_2 (Fig. (**7**)) [151]. A complex mixture of dimers to pentamers was obtained *via* both *ortho-ortho* and *ortho-meta* C-C coupling reactions. In some cases hydroxylated, demethylated, and quinone products were observed. The oxidative oligomerization was strongly influenced by the reaction pH. Slightly acidic conditions favored the formation of the dimer. The reaction at pH 7 resulted in a shift in the product spectrum to nearly equal fraction of trimers and tetramers, containing a large fraction of hydroxylated products. Products of the pH 8 reaction consisted of a major trimeric hydroxylated quinone in 40% yield. In addition to the solution-phase reactions, solid-phase apocynin oxidation has been performed with SBP on TentaGel® resins [151]. Using these conditions, a hydroxylated dimer attached to a hydroquinone (HQ) seed phenolic (HQ-apocynin-apocynin)-OH was selectively obtained after acid-cleavage. This dimer is similar to what was obtained in the solution phase reaction in the absence of HQ.

4.3. Mode of Action

Apocynin inhibits NADPH oxidase in neutrophils [11, 12] and non-phagocytic cells [104, 152]. This has been demonstrated in EC and neutrophils using immunoblots of cell membranes, where apocynin inhibits the translocation of cytosolic oxidase subunits (e.g. $p47^{phox}$ and $p67^{phox}$) to the membrane, thus preventing the assembly and activation of a functional NADPH oxidase complex [152, 153]. However, a detailed molecular and biochemical mechanism of apocynin's inhibitory activity remains unknown. Nevertheless, information is available on some of the activities of apocynin, which may shed clues onto the functional role of this phenolic in inhibiting NADPH oxidase.

In 1990, Simons *et al.* proposed that apocynin must be metabolically activated by means of a ROS or MPO-dependent mechanism in stimulated neutrophils [11]. A possible structure for this active metabolite – a quinone methide – was proposed in 1992 by Hart and Simons [154], but no scientific evidence was presented. Their hypothesis was later supported by Stolk *et al.* [12] who observed that apocynin requires conversion by peroxidases and ROS to exert its inhibitory effect. Oxygen uptake measurements revealed that in neutrophils there was a lag time of 2 to 3 min before the inhibitory effect of apocynin was observed and O_2 uptake was completely inhibited 7 min after addition serum-treated zymosan (STZ). The lag time appears to correspond to a sufficient conversion of apocynin to an active metabolite(s) catalyzed by peroxidase and ROS. This oxidative requirement was confirmed in neutrophils that lack MPO, where apocynin failed to inhibit NADPH oxidase activation [12, 154] and in MPO-deficient neutrophils in which the lag time was about 50% longer when compared with normal cells. Immunoblots of neutrophil membranes showed that translocation of the oxidase cytosolic components $p47^{phox}$ and $p67^{phox}$ to the membrane fraction at 7 min after STZ stimulation was markedly reduced when the neutrophils had been incubated with apocynin, but translocation was still normal after 2 min of stimulation. Recently, Müller *et al.* also reported that an apocynin-free fraction, which contained the major active metabolite, was obtained by *in vitro* activation of apocynin with human MPO and H_2O_2 and subsequent

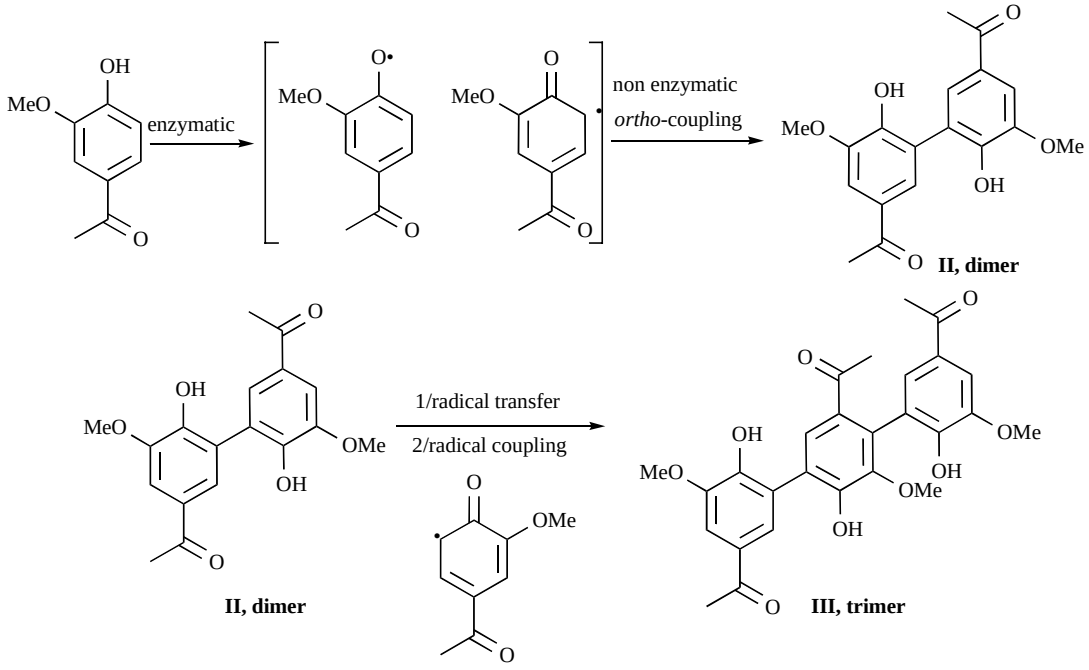

Fig. (7). Oxidative polymerization of apocynin [151].

chromatographic purification, but again no characterization of the structure of this metabolite was provided [155].

Similarly there is a lag time of 2 min before apocynin exerts an inhibitory effect in EC stimulated with arachidonic acid (AA) (Fig. (**8**)) [19]. Superoxide production was quantified by measuring the SOD-inhibitable reduction of cytochrome C. Immediately following this lag, superoxide production was nearly completely inhibited. An apocynin metabolite, diapocynin or the related quinone, oligoquinone, or oligophenol (an exact structure has not been identified) generated from apocynin through peroxidase catalysis, appears to act directly on NADPH oxidase to prevent enzyme complex assembly in the presence of AA, resulting in immediate inhibition. Johnson *et al*. recently demonstrated that apocynin dimer, obtained through a radical C-C *ortho-ortho* coupling of apocynin catalyzed by soybean peroxidase (SBP), is a more potent inhibitor than apocynin itself [156]. However, the precise chemical identification of the apocynin oxidation products has yet to be made.

Recently, Ximenes *et al.* reported the oxidative coupling of apocynin catalyzed by MPO [20]. They observed the corresponding dimer and a trimeric hydroxylated quinone that had previously been identified in the SBP-catalyzed reaction. Since apocynin impedes the migration of the cytosolic component $p47^{phox}$ to the membrane and this effect could be related to its conjugation with essential thiol groups (Cys^{378} residue on $p47^{phox}$), they studied the reactivity of apocynin and its MPO-catalyzed oxidation products with glutathione. They found that apocynin and its oxidation products do not react with glutathione. However, glutathione was efficiently oxidized by the apocynin radical during the MPO-catalyzed oxidation. This suggests that the reactivity of apocynin radical with thiol compounds may also be involved in the inhibitory effect on NADPH oxidase complex.

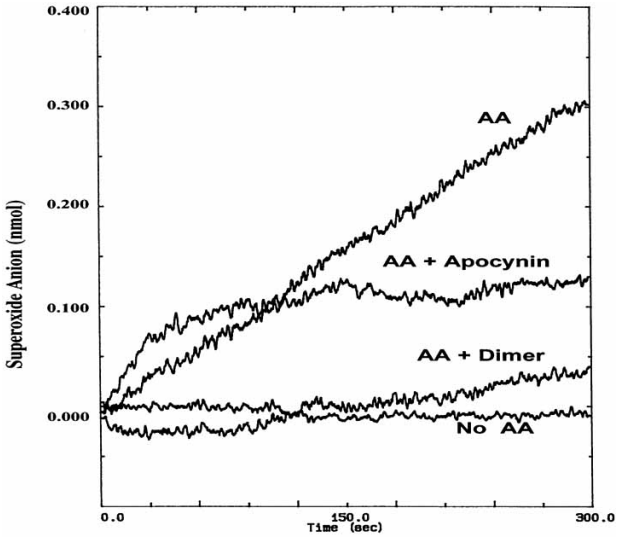

Fig. (8). Superoxide production by endothelial sonicates, in response to arachidonic Acid (AA), and in the presence and absence of 100 μM apocynin [19].

4.4. Cardiovascular-Related Biological Activities in Connection with NADPH Oxidase

The *in vitro* studies of apocynin provide a rationale for the more detailed study of its potential *in vivo* effects [157]. To that end, in the past decade apocynin has been extensively studied to help establish potential therapeutic applications.

4.4.1. Apocynin and Atherosclerosis

Atherosclerosis is one of the most common cardiovascular diseases in developed countries and another disease in

which ROS are thought to play an important role [158, 159]. Increased superoxide generation in vessels has been linked to the clinical risk factors for atherosclerosis and impaired endothelial NO function in patients with CAD [160]. Among the primary causes of atherosclerosis is a high serum level of LDL [161]. It has been suggested that LDL oxidation by cells of the arterial wall may be a key event in early atherosclerosis [159, 162]. In atherosclerosis pathogenesis, the initiating event is an injury to the blood vessel wall resulting in endothelial dysfunction. This disruption of the normal homeostatic regulatory function of the endothelium initiates a sequence of events including: (a) endothelial hyperpermeability; (b) inflammatory responses; and (c) VSMC proliferation, finally leading to plaque formation, plaque rupture, thrombosis, and tissue infarction. A causative relationship exists between these events and oxidative stress of the vascular wall [44].

Atherogenic levels of LDL have been shown to lead to a significant increase in NADPH oxidase dependent ROS production by the endothelium [163]. A significant role of NADPH oxidase was demonstrated in apo-lipoprotein E and $p47^{phox}$ double knockout mice ($ApoE^{-/-}/p47^{phox-/-}$) where a marked reduction of lesions was observed in the descending aorta [116]. Although the immediate product of NADPH oxidase ($\bullet O_2^-$) is not sufficiently reactive to induce LDL oxidation, it can be converted into other more reactive species as previously described, which are able to oxidize the lipoprotein directly and more efficiently, resulting in the formation and release of peroxidized fatty acids.

Many studies have focused on the role of apocynin on the pathogenesis of atherosclerosis. NADPH oxidase activation and concomitant ROS production has been reported to be required for macrophage-mediated oxidation of LDL which increases atherogenicity [158, 164]. An animal macrophage model showed that inhibition of the macrophage NADPH oxidase with apocynin (600 μM) inhibited macrophage-mediated oxidation of LDL by 89% compared with the control group [95]. Experiments with apocynin in endothelial cells showed similar results. Holland *et al.* reported that apocynin significantly impaired ROS production of EC NADPH oxidase stimulated by phospholipase A2 activator thrombin [102]. Apocynin also blocks increased ROS generation by EC in response to elevated LDL levels [18]. The likelihood that NADPH oxidase plays an integral role in the atherogenic process is supported by *in vitro* inhibitory studies using apocynin. At apocynin concentrations that inhibit NADPH oxidase the permeability of EC exposed to high LDL concentrations is reduced to levels seen in quiescent cells [18], EC-surface expression of cell adhesion molecules (e.g. E-selectin and vascular cell adhesion molecule-1 (VCAM-1)) and subsequent monocyte binding is greatly impaired [152], monocyte-mediated LDL oxidation is markedly reduced [54], and VSMC proliferation in response to growth factors is largely diminished [165].

The effect of NADPH oxidase inhibition on atherosclerosis has been tested in the hypercholesterolemic rabbit model by Holland *et al.* [19]. In these studies, New Zealand white male rabbits were fed a 1% cholesterol diet, and randomly divided into five groups with apocynin added to drinking water (0, 1, 10, 15, and 45 mg/kg/day). Following animal sacrifice at 3 months, analysis of the aortas from rabbits fed a 1% cholesterol diet in the absence of apocynin treatment showed typical diffuse atherosclerotic lesions covering 60% of the aortic surface area. By contrast, the aortas from hypercholesterolemic rabbits treated with apocynin (10-45 mg/kg/day) had markedly less atherosclerotic disease, covering less than 10% of the aortic surface. The total serum cholesterol levels of hypercholesterolemic rabbits with and without apocynin treatment were comparable, indicating that the protective effect of apocynin treatment was not dependent on lipid lowering. In summary, animal data is consistent with *in vitro* inhibitory studies indicating that inhibition of NADPH oxidase activation suppresses the sequence of cellular events leading to atherosclerosis. Apocynin may therefore be an interesting compound for potential use in the treatment of atherosclerosis.

4.4.2. Apocynin and Hypertension

Recent results suggest that oxidative stress plays an important role in the pathogenesis of renal damage in hypertension with different hypertension animal models. For example, in deoxycorticosterone acetate (DOCA)-salt hypertension rats, greater aortic NADPH oxidase activity and lower aortic Cu/Zn SOD activity were found, which could be responsible for the increased $\bullet O_2^-$ release and possibly contribute to increased blood pressure [166, 167]. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis demonstrated that DOCA-salt rats have significantly higher mRNA expression of $p22^{phox}$ [166]. In a spontaneously hypertensive rat model, $p47^{phox}$ expression was increased in the kidney, suggesting a role of ROS derived from NADPH oxidase in the development of high blood pressure [168]. Interestingly, administration with apocynin significantly decreased $\bullet O_2^-$ production in aortic rings. Moreover, long-term treatment of apocynin significantly decreased aortic $\bullet O_2^-$ production and systolic blood pressure [166]. Administration of apocynin to mice resulted in a similar effect [169]. Although the effect of apocynin on kidney function has not been fully investigated, it is a possible drug target that directly regulates NADPH oxidase activity.

4.5. Toxicity

Apocynin has a very good safety profile (LD_{50}: 9 g/kg upon oral administration in mice) [170] and side effects of apocynin have not been reported. Interestingly, apocynin does not interfere with the killing capacities of polymorphonuclear leukocytes (PMNs) [12]. Even when treated with apocynin for a 3-month period, rabbits do not show any signs of ill-health and other parameters compared to control-treated animals [19]. 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 uM [171]. This safety profile may be due to the relative inertness of the compound in the absence of metabolism. Moreover, such metabolism may be highly localized to regions in the vasculature with high concentrations of myeloperoxidase and H_2O_2. Such conditions are likely found under predominantly inflammatory conditions.

5. APOCYNIN AS A THERAPEUTIC TARGET FOR CARDIOVASCULAR DISEASES

The aforementioned studies provide evidence for a metabolic activation of apocynin; however, the exact mode of action of apocynin is still unclear. Currently, the extracts of *P. kurroa* are used as a complementary and alternative medicine. A representative sample extract of *P. kurroa* available over the internet is a 10 mg capsule. The suggested dosage in adults is one capsule daily. Each capsule is standardized to another constituent, Kutkin, at 5-10%. If the apocynin component of the extract is about 25% of Kutkin, that would be 0.125 to 0.25 mg apocynin per 10 mg capsule. But if a potentially therapeutic human dose was based on Phase I clinical trial of apocynin in humans by Leiden University Medical Center [172], at least 5-10 mg/day of apocynin would be needed. This would require about 20-40 extract capsules per day. Thus, a greater understanding of apocynin's mode of action would greatly benefit the development of an effective therapeutic dose for this nutraceutical product.

Animal and human studies have indicated a fundamental role of ROS in the pathogenesis of cardiovascular diseases, while NADPH oxidase has been shown to be the major source of ROS production in the vascular wall. In 1990, Simons *et al.* eventually established the pharmacological potential of apocynin through activity-guided isolation from the roots of *P. kurroa* [11, 173]. A variety of *in vivo* and *in vitro* studies have demonstrated apocynin's pharmacological value in cardiovascular diseases as an inhibitor of NADPH oxidase. Continued studies, together with elucidation of the precise mechanism of action of specific apocynin metabolites may provide a route to the design of novel therapeutic candidates for cardiovascular diseases.

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