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Trimer hydroxylated quinone (IIIHyQ) derived from apocynin targets cysteine residues of p47^{phox} preventing the activation of human vascular NADPH oxidase

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Abstract

Enzymatic derived oligophenols from apocynin can be effective inhibitors of human vascular NADPH oxidase. An isolated IIIHyQ has been shown to inhibit endothelial NADPH oxidase with an IC₅₀ ~30 nM. *In vitro* studies demonstrated that IIIHyQ is capable on disrupting the interaction between p47^{phox} and p22^{phox}, thereby blocking the activation of the Nox2 isoform. Herein, we report the role of key cysteine residues in p47^{phox} as targets for the IIIHyQ. Incubation of p47^{phox} with IIIHyQ results in a decrease of ~80% of the protein free cysteine residues; similar results were observed using 1,2- and 1,4-naphthoquinones, while apocynin was unreactive. Mutants of p47^{phox}, where each Cys was individually replaced by Ala (at residues 111, 196 and 378) and Gly (at residue 98), were generated to evaluate their individual importance in IIIHyQ-mediated inhibition of p47^{phox} interaction with p22^{phox}. Specific Michael addition on Cys₁₉₆, within the N-SH3 domain, by the IIIHyQ is critical for disrupting the p47^{phox}-p22^{phox} interaction. When a C196A mutation was tested, the IIIHyQ was unable to disrupt the p47^{phox}-p22^{phox} interaction. However, the IIIHyQ was effective at disrupting this interaction with the other mutants, displaying IC₅₀ values (4.9, 21.0, and 2.3 μM for the C111A, C378A, and C98G mutants, respectively) comparable to that of wild type p47^{phox}.

Keywords

Human vascular NADPH oxidase; Enzyme inhibition; Apocynin derived oligophenols; p47^{phox}; p22^{phox}

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INTRODUCTION

Reactive oxygen species (ROS) regulate cellular signaling, affecting several aspects of cellular function, such as proliferation, migration, gene expression, and cell death. Several enzymes are expressed in the vasculature and contribute to ROS generation (e.g., endothelial nitric oxide synthases, cytochrome P450s, xanthine oxidase, and NADPH oxidases). Among this group of enzymes, NADPH oxidases catalyze superoxide ($O_2^{\bullet-}$) synthesis that triggers formation of ROS (e.g., hydrogen peroxide and hydroxyl radical), species that play a critical role in the development of oxidative stress-related cardiovascular diseases, including ischemia, restenosis, stroke, and arteriosclerosis [1–4]. At least three isoforms of NADPH oxidase are expressed in vascular endothelial cells (VEC), Nox1, Nox2 and Nox4 [5,6], with Nox2 playing a critical role in ROS generation [7]. For example, Nox2 is upregulated ~8-fold and its production of $O_2^{\bullet-}$ increases 2–3 fold under induced oxidative stress [7]. Nox 2 has 100% homology with the NADPH oxidase found in neutrophils, an enzyme involved in pathogen neutralization. Similar to the neutrophil enzyme, Nox2 undergoes a complex assembly of membrane- and cytosol-associated subunits to generate the activated enzyme. As depicted in Fig. 1A, translocation of cytosolic subunits (p47^{phox}, p67^{phox}, p40^{phox} and Rac) occurs through binding to the membrane p22^{phox} and gp91^{phox} subunits [8–11]. Phosphorylation of serine residues in p47^{phox} results in conversion of inactive p47^{phox} into an active form of p47^{phox} and allows interaction of p47^{phox} Src homology 3 (SH3) domain with proline rich regions (PRR) of p22^{phox} (Figure 1B); this interaction is essential for activation of the Nox2 isoform [12,13].

Selective inhibitors for NADPH oxidase have been developed, some of which block the interaction of cytosolic subunits with membrane proteins (e.g., the flavonoid derivative S17834, 6,8-diallyl-5,7-dihydroxy-2-(2-allyl-3-hydroxy-4-methoxyphenyl)-1-*H*-benzo-[*b*]-pyran-4-one, and peptides such as the antibiotic PR-39) [14–16]; *in vivo* studies have shown that this flavonoid derivative reduced aortic superoxide anion levels by 40% and aortic atherosclerotic lesions by 60% in apolipoprotein E-deficient mice [15]. In particular, polyphenols have gained significant attention because of their ability to bind proline rich proteins [17,18]. Apocynin is a well-studied inhibitor of NADPH oxidase. Despite the growing number of studies with this phenolic compound, there remain questions of its precise role in NADPH oxidase inhibition. Some studies have revealed that apocynin is not a direct inhibitor of NADPH oxidase [19, 20], while other studies suggest that apocynin acts as a simple antioxidant [19, 21]. However, metabolism *in vivo* is expected to convert apocynin into reactive molecules, including oligophenols and their quinone analogs [22]. The latter has been observed *in vivo*. Thallas-Bonker and coworkers found that apocynin attenuates cytosolic superoxide generation in induced diabetic rats [23]. However, it is possible that the observed effect on diabetic rats was due the conversion of apocynin into active metabolites. Indeed, Johnson et al. [20] and Kanegae et al. [24] found a dimer derived from apocynin to be an effective inhibitor, but its precise mode of action has not been determined, although oxidized oligophenolic compounds have been shown to bind to thiol groups and contribute to NADPH oxidase inhibition [25].

In our previous work, we reported a broad complex mixture of derived oligophenols (up to heptamers) obtained from the peroxidase-catalyzed oxidation of apocynin and consisting of demethylated, hydroxylated, and quinone forms [26]. We isolated and fully characterized a trimer hydroxylated quinone (IIIHyQ), which showed strong inhibitory activity ($IC_{50} = 30$ nM) against endothelial cell-based NADPH oxidase. The IIIHyQ was able to disrupt the *in vitro* interaction between a His-tagged p47^{phox} (His-p47^{phox}) and a PRR peptide biotin-p22^{phox} ($IC_{50} = 1.60$ μ M) [26]. A linear correlation existed between the inhibitory activity against EC-NADPH oxidase and the ability to disrupt the interaction between biotin-p22^{phox} and His-p47^{phox}, suggesting that apocynin derived oligophenols are capable of preventing

p47^{phox}-p22^{phox} interaction *in vivo*. We now hypothesize that IIIHyQ may bind to critical cysteine residues of p47^{phox}, and in particular Cys¹⁹⁶ located in the N-SH3 domain (Figure 1C) via Michel adducts. Indeed, similar adducts are well known to occur between benzoquinone, naphthoquinone, anthraquinone or dopamine quinone with Cys residues in proteins [27,28].

In the current work, we have focused on gaining a more complete understanding of the mechanism of NADPH oxidase inhibited by apocynin-derived IIIHyQ. We evaluated the effect of IIIHyQ on the interaction of biotin-p22^{phox} and His-p47^{phox}. Four p47^{phox} mutants were expressed and purified, each containing a simple Cys replacement, thus sequentially eliminating potential targets for quinone binding. This approach resulted in the identification of Cys₁₉₆ as a critical target for IIIHyQ leading and potentially preventing p47^{phox}-p22^{phox} interaction. We also evaluated the toxicity of the IIIHyQ compared with apocynin using PC-12 cells. This set of results establishes apocynin derived oligophenols, and particularly metabolism-generated quinones, as candidates for potential therapeutic applications.

MATERIALS AND METHODS

Materials

Apocynin, soybean peroxidase (SBP), solvents, phosphate buffered saline (PBS) tablets, H₂O₂, thiazolyl blue tetrazolium bromide, Tween 20, 3,3',5,5'-tetramethyl-benzidine (TMB), sodium caseinate, fetal bovine serum, heparin, and endothelial growth supplement were purchased from Sigma-Aldrich (St. Louis, MO). Endothelial cells, PC-12 cells and F12K medium were purchased from ATCC (Manassas, VA). *E. coli* BL21 (DE3), *E. coli* Top 10 competent cells, isopropyl β-D-1-thiogalactopyranoside (IPTG), Lucifer Yellow Iodoacetamide and Ni-affinity column (Probond system) were purchased from Invitrogen (Carlsbad, CA). Primers were obtained from Integrated DNA Technologies (Coralville, IA). Antibodies were purchased from Upstate Biotechnology (Waltham, MA). High-affinity streptavidin-coated-96 well plates were purchased from Pierce. LC-MS analyses were performed on a Shimadzu LCMS-2010A. Samples for LC-MS were separated in an Agilent Zorbax 300SB-C₁₈ column (5 μm, 2.1 × 150 mm). Silica gel 230–400 mesh was purchased from Natland International Corporation (Morrisville, NC). Thin layer chromatography (TLC) plates were purchased from Merck (Whitehouse Station, NJ). Microplate reader analyses were performed in a Perkin-Elmer, HTS 7000, Bio Assay Reader.

Enzymatic production of IIIHyQ from apocynin

IIIHyQ was synthesized via SBP-catalyzed oxidation of apocynin as described previously [26, 29]. Briefly, apocynin (6 mmol) was dissolved in 5 mL of dimethylformamide (DMF) and transferred to 490 mL phosphate buffer (50 mM, pH 7). SBP (5 mL of a 1 mg/mL solution) was added and the reaction was initiated by using a syringe pump to introduce H₂O₂ (30% w/v) at 0.1 mL/min for 12 min to afford 12 mmol H₂O₂. Finally, the reaction was stopped after 2 h. Soluble and precipitated phases were separated by centrifugation and ethyl acetate was added to the supernatant to extract organic compounds. The extracted supernatant fraction was dried and stored at –20°C under argon. Dried powder (290 mg) was dissolved in chloroform and loaded onto a silica gel column (15 g) and eluted with a gradient of petroleum ether:ethyl acetate (2:1 to 0:1). Unreacted apocynin was recovered in the early fractions (210 mg, R_f 0.62 with petroleum ether:ethyl acetate, 1:1) and further elution with pure ethyl acetate furnished the IIIHyQ as a white powder (14 mg, R_f 0.34 with petroleum ether:ethyl acetate, 1:1). TLC, NMR and High Resolution Mass Spectrometry (HRMS) analyses were performed as previously reported [26].

Site directed mutagenesis

Four mutants of His-p47^{phox} were obtained by site directed mutagenesis using the original plasmid (pET-28a (+), 5369 bp) used for production of recombinant His-p47^{phox} wild type, C98G C111A, C196A, and C378A. Primer design was performed following the guidelines of the QuickChange® Lightning Site-Directed Mutagenesis Kit from Stratagene (Santa Clara, CA); primers (reverse, R, and forward, F) for each mutant are: C98GF (GGCACACTTACCGAGTACGGCTCCACGCTCATGAGCCTGC), C98GR (GCAGGCTCATGAGCGTGGAGCCGTACTCGGTAAGTGTGCC); C111AF (CACCAAGATCTCCCGAGCTCCCCACCTCCTCGACTT), C111AR (AAGTCGAGGAGGTGGGGAGCTCGGGAGATCTTGGTG); C196AF (GAGCGGTTGGTGGTTCGCTCAGATGAAAGCAAAGC), C196AR (GCTTTGCTTTTCATCTGAGCGAACCACCAACCGCTC); C378AF (CCTCATCTGAACCGCGCTAGCGAGAGCACCAAGC), C378AR (GCTTGGTGCTCTCGCTAGCGCGGTTTCAGGATGAGG). The calculated melting temperature for each primer was 78°C and the cycling parameters used for PCR are described in the instruction manual of the mutagenesis kit from Stratagene. After PCR reactions, DNA was inserted into *E. coli* Top 10 cells and plasmid purified samples were sent for sequencing (MCLab, San Francisco, CA) to confirm the correct mutations (see Supporting Information for primer design).

Production and purification of His-p47^{phox} and biotin-p22

A proline-rich p22^{phox} peptide N'-151PPSNPPRPPAEARK165-C', which was biotinylated at the N-terminus and amidated at the C-terminus was obtained from Genemed Synthesis Inc. (South San Francisco, CA). A biotin group was attached through a 4-residue spacer consisting of SGSG. The purity of the peptide was 99.99%. Endothelial cell (EC)-derived p47^{phox} (wild type) DNA (6-His tagged) was obtained from U. of Albany and Stratton VA Medical Center and confirmed by DNA sequence analysis (U. of Maine). His-p47^{phox} proteins (wild type and mutants) were expressed in BL21 (DE3) cells for 9 h using (IPTG 0.5 mM) at 35°C. The protein was purified using a Ni-affinity column (ProBond System) and the purity (80%) was calculated with the Image J software (NIH).

Biotin-p22 and His-p47^{phox} interaction

Interaction of His-p47^{phox} (mutants and wild type) with the biotin-p22^{phox} peptide was assessed using ELISA, as previously described [26, 30]. Briefly, experiments were performed in high-affinity streptavidin-coated-96 well plates. To block non-specific binding sites, each well was re-blocked with 300 µL of PBS supplemented with 0.1% (v/v) Tween 20 and 1% sodium caseinate. To each well, 100 µL of biotin-p22^{phox} (2 µM) peptide solution were added and incubated at room temperature for 1 h. After washing each well four-times with 300 µL PBS-Tween 20 solution, 100 µL of 0.30 µM His-p47^{phox} (in PBS-Tween solution containing 1% sodium caseinate) and IIIHyQ (0 – 1000 µM) were added to each well and incubated at room temperature for 1 h. Unbound IIIHyQ was removed by washing four times with 300 µL/well PBS-Tween 20 solution. The amounts of bound His-p47^{phox} were quantified by adding 100 µL/well of polyclonal goat anti-p47^{phox} (diluted 1:2000 in PBS-Tween 20 solution containing 1% sodium caseinate) and incubating at room temperature for 1 h. Each well was washed four times with 300 µL PBS-Tween 20 solution and incubated with 100 µL/well of HRP-conjugated rabbit anti-goat IgG secondary antibody (diluted 1:10000 in PBS-Tween solution containing 1% sodium caseinate) at room temperature for 1 h. The plate was finally washed four times with 300 µL/well of PBS-Tween solution and two additional washes with 300 µL/well of PBS. Detection of peroxidase activity was performed with a ready-to-use TMB liquid substrate by adding 200 µL/well and incubating at room temperature for 30 min. The reaction was stopped with 100 µL/well of 0.5 M H₂SO₄ solution and the absorbance was read at 450 nm in the microplate.

reader. All experiments were performed in triplicate and results were quantified from a standard curve of the interaction between biotin-p22^{phox} (2 μ M) and His-p47^{phox} (0 to 0.5 μ M). As positive controls, similar experiments were carried out using 1,2-naphthoquinone and 1,4-naphthoquinone and phenylarsine oxide (PAO) was used as a negative control.

Quantification of cysteines

Reactivity of cysteines with quinones was determined using the fluorescent dye Lucifer yellow iodoacetamide (following the protocol from Invitrogen). After purification in a Ni-column, wild type His-p47^{phox} buffer was exchanged with 10 mM phosphate buffer (pH 7.0) using an Amicon ultracentrifuge filtration device (5 kDa MWCO). Wild type His-p47^{phox} (50 μ M) was then incubated with the IIIHyQ, using 1,2- and 1,4- naphthoquinone as positive controls, and apocynin as negative control (each compound at a final concentration of 500 μ M). Lucifer yellow iodoacetamide (10 mM) was added and the reaction allowed proceeding at 4°C overnight in dark. To separate excess dye and protein conjugate, desalting spin columns (Zeba Spin Desalting Columns, 7k MWCO, 0.5 mL) were used three times until complete elimination of background fluorescence. After removal of the dye, protein concentration was determined by bicinchoninic acid (BCA) protein assay. Finally, fluorescence spectra were taken at an excitation wavelength of 426 nm, showing a maximum of emission intensity ~530 nm.

Intracellular production of superoxide via DHE staining

Qualitative determination of intracellular superoxide was performed via dihydroethidium (DHE) fluorescence. DHE permeates endothelial cells, and in the presence of superoxide and other ROS is oxidized to the red fluorescent 2-hydroxyethidium and ethidium, respectively, which are trapped with DNA resulting in bright red fluorescence. Although DHE can react with other ROS [31–33], the following method describes the generation of O₂^{•-} after the selective activation of vascular NADPH oxidase, which is the progenitor of other ROS (e.g., hydrogen peroxide and hydroxyl radical). Endothelial cells were incubated overnight in black, clear-bottom 96-well cell binding surface plates in DMEM in the absence of phenol red. DMEM was removed and the cells were washed twice with PBS and then fresh DMEM (90 μ L) was added. EC were incubated for 30 min and 37°C with phorbol myristate acetate (PMA, 1 μ M) to activate NADPH oxidase. After consecutive washes with PBS and addition of fresh DMEM (90 μ L), EC were incubated with 1 mM IIIHyQ, apocynin, and phenylarsine oxide (PAO) [34] for 2 h at 37°C. DMEM was removed and cells washed two times with PBS, and fresh DMEM was then added. Finally, the cells were incubated with DHE (3 μ M) for 30 min and then NADPH (100 μ M) to generate O₂^{•-}; the experiment was performed in the dark. Cell images were captured after 30 min with a Zeiss LSM 510 laser scanning confocal microscope at excitation and emission wavelengths of 520 and 610 nm, respectively.

Superoxide anion radical (O₂^{•-}) scavenging capacity assay

The O₂^{•-} scavenging capacity of IIIHyQ was measured by competition with a molecular probe, nitroblue tetrazolium (NBT), following synthesis of O₂^{•-} by the hypoxanthine-xanthine oxidase (HPX-XOD) system. Briefly, 200 μ L of NBT solution (0.34 mM in PBS), 500 μ L HPX (2 mM in PBS), and 100 μ L of IIIHyQ prepared at the desired concentration (or the solvent only control) were vortex mixed for 5 s. Then, 200 μ L of XOD (0.56 U/mL in PBS) were added and vortex mixed for 30 s. Samples were taken every 10 min to measure the absorbance at 560 nm. NBT has a yellow color that upon reduction by O₂^{•-} forms the blue formazan.

Cell toxicity assay

Toxicity of IIIHyQ and apocynin were determined by MTT assay using rat adrenal medullar cells (PC12). Cells were cultured in modified DMEM supplemented with 5% fetal bovine serum, 10% horse serum, and 1% penicillin-streptomycin. The cell suspension (110,000 cells/mL) was separated into aliquots of 90 μ L in a 96-well microtiter plate (CellBIND, Corning, Lowell, MA) and allowed to adhere for 24 h. Afterward, 10 μ L of IIIHyQ and apocynin solutions were added (to cover a range of concentrations from 0 to 1 mM, in 1% DMSO) to the microtiter plate, and the cells were further incubated for 48 h at 37 °C. The medium was then removed, and the cells were washed with PBS. Next, DMEM (200 μ L) and thiazolyl blue tetrazolium bromide (50 μ L of 2.5 mg/ml) were added to each well for 3 h at 37 °C. Finally, these solutions were removed, 250 μ L of DMSO was added, the plate was shaken (~ 75 rpm) for 20 min, and the absorbance was measured at 562 nm. The cytotoxicity values were normalized to the PBS (1% DMSO) control without IIIHyQ and apocynin.

RESULTS AND DISCUSSION

Interaction of the IIIHyQ with p47^{phox}

The mechanism of NADPH oxidase inhibition by apocynin-derived enzymatic oxidation products remains unclear. In phagocytes, myeloperoxidase is expected to convert apocynin into active metabolites [24,35,36]. Along these lines, we generated a library of potential inhibitors NADPH oxidase, resulting in the identification of a IIIHyQ as a strong inhibitor of the human EC-NADPH oxidase via blocking the interaction between p47^{phox} and p22^{phox} subunits. We hypothesize that the IIIHyQ may bind to critical Cys residues on p47^{phox} preventing its translocation to the membrane. p47^{phox} contains four Cys residues at positions 98, 111, 196, and 378. Cys₁₉₆ is located within the N-SH3 domain, which binds the PRR of p22^{phox} (Figure 1B).

To elucidate the role of specific Cys residues, and particularly the Cys₁₉₆ of p47^{phox} on interaction with p22^{phox} in the presence of IIIHyQ, four mutants (C98G-p47^{phox}, C111A-p47^{phox}, C196A-p47^{phox}, and C378A-p47^{phox}) of recombinant p47^{phox} were generated. Dose response analysis of IIIHyQ on C98Gp47^{phox}, and C378Ap47^{phox} enzyme-linked immunosorbant assay (Figure 2A) shows that the apocynin-derivative disrupts the interaction with biotin-p22^{phox} giving comparable IC₅₀ values (4.9, and 2.3 μ M respectively; Figure 2B, and 2E) to the value previously reported using the wild type p47^{phox} (1.60 μ M) [26], while IC₅₀ value using C111Ap47^{phox} (21.0 μ M) was an order of magnitude higher (Figure 2C). However, when Cys₁₉₆ was replaced by Ala, the IIIHyQ was unable to block the interaction over the entire dose range (Figure 2D). This was interesting, as Cys₁₉₆ is the only cysteine located within the SH3 domain, and particularly within the N-terminal SH3 domain that binds the PRR of p22^{phox}. Cys₁₉₆, therefore, appears to be a critical target to prevent protein-protein interactions necessary for NADPH oxidase activation. Several quinones (for example 1,2- and 1,4- naphthoquinones) are known to form Michael adducts with cysteines residues [28]. As controls, 1,2-naphthoquinones and 1,4-naphthoquinone were evaluated for their effects on the interaction of His-p47^{phox} with biotin-p22^{phox}. Using wild-type His-p47^{phox}, both quinones were able to prevent this protein-protein association with comparable IC₅₀ values (0.2 and 0.7 μ M respectively; Figures 3A and 3B). However, when the mutant C196Ap47^{phox} was used, neither naphthoquinone had any effect (Figures 3C and 3D).

The reactivity of the IIIHyQ on p47^{phox} Cys residues was determined using the fluorescent dye Lucifer Yellow iodoacetamide (Figure 4A). The 1,2- and 1,4-naphthoquinones were used as positive controls, and apocynin was used as a negative control. The fluorescence spectra (Figure 4B) show a significant decrease (~80%) of total Cys-thiol groups after the

incubation with the IIIHyQ, as well as the naphthoquinones, while incubation with apocynin did not result in appreciable reduction in the fraction of free Cys residues. This result suggests that IIIHyQ is not selective for Cys₁₉₆, which is consistent with the results of Park and co-workers using the fluorescent probe N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethyleneamine [37]. The latter and the reduction of ~80% of total cysteines indicate that the IIIHyQ is not specific for Cys₁₉₆ but its modification seems to be critical to prevent the interaction with p22^{phox}.

Effect of the IIIHyQ on NADPH oxidase following enzyme activation

NADPH oxidase inhibition can be a result of at least two mechanisms. One mechanism involves interference of enzyme activation, e.g., by preventing translocation of cytosolic subunits to the cell membrane. A second mechanism involves the inhibition of the already assembled and activated enzyme. Along these lines, it is important to note that few attempts have been made to evaluate inhibition when the NADPH oxidase is already activated and the target sites for the inhibitors may be restricted. For example, PAO is ineffective as an inhibitor once NADPH oxidase is activated [38]. We proceeded to investigate whether the IIIHyQ was an effective inhibitor following NADPH oxidase activation. To that end, we activated the enzyme within EC with PMA and followed intracellular O₂•⁻ formation by DHE, a cell-permeable dye that reacts with O₂•⁻ to generate 2-hydroxyethidium [31, 33]. The production of O₂•⁻ was then examined upon addition of IIIHyQ. As shown in Figure 5A, the level of O₂•⁻ decreased in the presence of IIIHyQ in relation to apocynin and PAO, although the inhibition was not as striking as addition of IIIHyQ prior to NADPH activation. Nevertheless, it is interesting that IIIHyQ can achieve some degree of inhibition once enzyme assembly had occurred. One explanation is that IIIHyQ may be able to bind to Cys₁₉₆ even after the p47^{Phox} – p21^{Phox} association takes place. If this occurs, then once IIIHyQ binds to Cys₁₉₆, the two enzyme subunits dissociate, thereby eliminating enzyme activity. Another possibility is that other protein-inhibitor interactions take place. For example, it has been demonstrated that polyphenols have affinity to physically interact with PRR of proteins and induce conformational changes [17,18]. Interactions between N-SH3 domain of p47^{phox} and PRR of p22^{phox} occur through Van der Waals and hydrogen bond interactions [9,39]. Similar interactions may occur with polyphenols that can disrupt the association of p47^{Phox} – p21^{Phox}. Importantly, IIIHyQ does not scavenge. Similar interactions may occur with polyphenols that can disrupt some of those interactions resulting in a reversible effect on NADPH oxidase. As a control, we determined that the IIIHyQ does not scavenge O₂•⁻ and the reduction of O₂•⁻ levels is due a loss of enzymatic activity (Figure 5B)

Toxicity of apocynin and IIIHyQ

Our results suggest that IIIHyQ does not target a specific cysteine, and thus may be indiscriminately reactive toward other cysteines residues on other proteins within cells, thereby affecting critical pathways that may damage or kill cells. We therefore evaluated the cytotoxicity of IIIHyQ via the standard MTT assay using PC12 cells. As shown in Figure 6, the dose response plots of apocynin and IIIHyQ were similar and only 20% loss in viability occurred at a concentration of 1 mM. This value is far higher than the IC₅₀ values obtained during the NADPH oxidase activity assay (30 nM) and the biotin-p22^{phox}-p47^{phox} (1.60 μM) [26], indicating a potentially wide therapeutic window. These cytotoxicity results are similar to those with the stilbene resveratrol and its peroxidase generated oxidation products [40].

CONCLUSIONS

We have studied the mechanism of inhibition of the enzymatically derived IIIHyQ from apocynin, with specific attention given to the interaction of p22^{phox} and p47^{phox}. Cysteine residues of p47^{phox} appear to be the primary target for the quinone, with Cys₁₉₆ in the N-SH3 domain playing a critical role as a site where inhibition. Specifically, the IIIHyQ is ineffective when Cys₁₉₆ is replaced by alanine. The IIIHyQ also reacts with other Cys residues in p47^{phox}; however, inhibition is eliminated only when a likely Michael addition to the Cys₁₉₆ occurs. Similar results were obtained with the structurally simpler 1,2- and 1,4-naphthoquinones. Despite the relatively low selectivity of the IIIHyQ on Cys residues, the compound showed minimal cytotoxicity *in vitro*. Enzymatic oligomerization of apocynin represents an attractive alternative to generate potential inhibitors of NADPH oxidase. Characterization of IIIHyQ and its mechanism of action provide additional insight into the structural features for potent inhibitors of vascular NADPH oxidase, which may prove valuable in the search for effective therapeutic interventions to cardiovascular diseases related to oxidative stress [34].

Highlights

- Inhibition of human vascular NADPH oxidase by a IIIHyQ derived from apocynin
- IIIHyQ blocks the p22^{phox}-p47^{phox} interaction preventing the activation of NADPH oxidase
- IIIHyQ targets cysteine residues of p47^{phox} as a potential mechanism of inhibition
- Cys196 within the N-SH3 domain plays a critical role as a target of the IIIHyQ
- Toxicity of the IIIHyQ is low and comparable with the apocynin toxicity

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Figure 1.

(A) Active conformation of human vascular NADPH oxidase (Nox2 isoform); Interaction between NADPH oxidase subunits p22^{phox} and p47^{phox}. (B) N-SH3 domain of p47^{phox} interacts with the proline rich region (PRR) of p22^{phox}. (C) Cysteine at the 196 position of p47^{phox} is the only cysteine located within the N-SH3 domain and is considered a critical target for quinones to prevent the interaction with p22^{phox}.

Figure 2.

Interaction between biotin-p22^{phox} and recombinant His-p47^{phox} (wild type and mutants) in presence of IIIHyQ. (A) Scheme of the ELISA, (B) Dose response using C98Gp47^{phox} (● IC₅₀ = 4.9 μM), (C) C111Ap47^{phox} (● IC₅₀ = 21.0 μM), (D) C196Ap47^{phox} (● IC₅₀ = not determined), and (E) C378Ap47^{phox} (● IC₅₀ = 2.3 μM).

Figure 3.

ELISA controls with quinone structures. Effect of 1,2- and 1,4- naphthoquinones on the interaction between biotin-p22^{phox} and recombinant His-p47^{phox}. (A) biotin-p22^{phox} – wild type His-p47^{phox} interaction with 1,2-napthoquinone (■ IC₅₀ = 0.2 μM), (B) biotin-p22^{phox} – wild type His-p47^{phox} interaction with 1,4-napthoquinone (■ IC₅₀ = 0.7 μM), (C) biotin-p22^{phox} – C196Ap47^{phox} interaction with 1,2-napthoquinone, and (D) biotin-p22^{phox} – C196Ap47^{phox} interaction with 1,4-napthoquinone (IC₅₀ values of C and D were not determined).

Figure 4. Quantification of cysteines in recombinant His-p47^{phox} incubated with apocynin, IIIHyQ, 1,2-naphthoquinone, and 1,4-naphthoquinone. (A) Chemical representation of the Michael adduct between IIIHyQ and Cys. (B) Fluorescent spectra of Lucifer iodoacetamide complexed with cysteines of wild type p47^{phox}.

Figure 5.

(A) Intracellular $O_2^{\bullet-}$ detection by DHE staining in endothelial cells. Inhibitory activity of apocynin, IIIHyQ, and PAO on previously activated NADPH oxidase. (B) $O_2^{\bullet-}$ scavenging capacity of IIIHyQ (0–1 mM); ■ IIIHyQ = 0.0 μ M, ● IIIHyQ = 0.01 μ M, ▲ IIIHyQ = 100.0 μ M, ▼ IIIHyQ = 1000.0 μ M

Figure 6.
MTT assay. Cytotoxicity of apocynin and IIIHyQ on PC-12 cells