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Original article

Activities of apocynin in cytotoxicity assays of potential pathological relevance

Shaun P. Wong[a], Vai Hong Fong[a,b], Amandio Vieira[a,*]

[a] Nutrition and Metabolism Research Laboratory, Biomedical Physiology BPK, Simon Fraser University, Burnaby, BC, Canada
[b] Department of Neurology, Far Eastern Memorial Hospital, Taiwan

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ABSTRACT

Low levels of some reactive oxygen and nitrogen species (ROS, RNS) are of physiological importance; but high levels result in oxidative stress and can perturb many cell functions including signal transduction and transport, and contribute to aging and chronic diseases. Apocynin (1-(4-hydroxy-3-methoxyphenyl) ethanone) is a phytochemical with reported antioxidant activities in some experimental models of human disease. The major objectives of the current study were to test the antioxidant capacity of apocynin in a hemin-peroxide assay, and test its capacity to moderate pro-oxidant-dependent inhibition of a cell function—endocytic transport. Apocynin, tested at concentrations up to 20 μM, did not exhibit statistically significant antioxidant activity (94.3 ± 7.8% relative to controls, $p > 0.05$) in the oxidation assay. When tested against the inhibition of endocytic transport by hydrogen peroxide, apocynin treatment did not significantly rescue such inhibition in the cell types tested ($p > 0.05$, relative to peroxide alone). When cells were treated with a cytotoxic protein aggregate that increased both ROS and RNS, apocynin treatment only inhibited production of the latter (30.0 ± 3.6% inhibition relative to controls without apocynin, $p < 0.05$). The results provide evidence that apocynin, unlike other phytochemicals such as curcumin, does not exhibit antioxidant activity in the heme-peroxide assay. The results also provide the first evidence that apocynin does not rescue hydrogen peroxide-mediated inhibition of endocytic transport, nor prevent hydrogen peroxide production by a cytotoxic protein aggregate. In the latter toxicity assay, however, apocynin could moderate oxidative stress by decreasing cellular levels of RNS.

1. Introduction

Plant extracts and purified phytochemicals are often tested for potential pharmacological activities involving in vitro biochemical reactions such as oxidation assays. As part of ongoing work to identify phytochemicals with therapeutic potential against human disease, and analyze the molecular and cellular mechanisms of action, we are developing assays to screen phytochemicals (as well as phytochemical combinations, phytoextracts, and their metabolites) for antioxidant activities, modulation of cell signaling, influence upon epigenetic programs, and modulation of endocytic transport. In the current study, we employ some of these assays to analyze the activities of a phytochemical, apocynin (acetovanillone), and further examine the relation between oxidative stress and endocytic transport.

Apocynin is a methoxy substituted catechol first prepared from *Apocynum cannabinum* (L., dogbane) and subsequently isolated from *Picrorhiza kurroa* (Royle ex Benth., alpine plant of the Himalayas). *Picrorhiza* species contain a variety of bioactive phytochemicals including apocynin [1–3], and have been used as part of traditional medicine in Asia. For purified apocynin, a range of potential therapeutic activities have also been reported: e.g., antioxidant, anti-inflammatory, protection of cerebrovascular integrity [4–9]; and some of these actions are attributed to inhibition of NADPH oxidase activities (see Section 4).

Based on reports from our laboratory and others that apocynin can have antioxidant activity in some experimental systems [6–9], and that pro-oxidants can inhibit a cellular transport function, endocytosis [10–13], we tested apocynin for possible rescue of pro-oxidant-mediated inhibition of such transport. This phytochemical, however, did not exhibit a statistically significant moderation of pro-oxidant mediated endocytic transport inhibition. Apocynin also did not exhibit a statistically significant antioxidant activity ($p > 0.05$) in a hydrogen peroxide-hemin-based oxidation assay. Further analyses in cell toxicity assays provided evidence that

* Corresponding author. Fax: +1 778 7824251.
E-mail address: avvieira@sfu.ca (A. Vieira).

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apocynin can moderate production of reactive nitrogen species (RNS), but not production of the reactive oxygen species (ROS) hydrogen peroxide.

2. Materials and methods

2.1. Reagents and cell line

All reagents used were of analytical grade and purchased from Sigma–Aldrich, unless noted otherwise in the sections below. Misfolded, aggregated TTR (aTTR) was prepared by a low pH treatment as previously reported [14,15], and analyzed by turbidity and thioflavin binding according to standard procedures [15,16]. The human epidermoid carcinoma cell line (A431) was obtained from American Type Culture Collection (ATCC). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, from Invitrogen). The medium was supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). Cells were maintained in humidified 5% CO_2 incubator at 37 °C.

2.2. Animals and primary cells

Murine primary liver cells were prepared from male BALB/c mice, ages 7–14 months, as described below. The procedure was based on a previously reported EDTA-sucrose method [17]. After perfusion of the circulation with physiological saline, the gall bladder was removed, and the liver rinsed first with PBS then with EDTA-sucrose (ES) solution (250 mM sucrose and 4 mM EDTA). In a plastic dish containing 5 ml of ES, the liver was chopped and torn into the smallest possible pieces, and placed for 5 min on ice. The tissue suspension was then passed five times through a 5-ml syringe (no needle), five times through a 1-ml pipette tip cut ~5 mm from end to create wider opening. The suspension was collected into tubes, and dish further rinsed with 0.5–1.0 ml of ES, followed by a brief centrifugation of the tubes at low g-force (2 s, $50 \times g$) to pellet tissue debris. The supernatant was carefully transferred to new tubes which were subsequently centrifuged, $50 \times g$ for 3 min to pellet the cells. Each liver cell pellet was resuspended in 1 ml of complete cell culture medium (DMEM-FBS) and re-centrifuged, $100 \times g$ for 1 min to pellet cells. The cell pellet was then resuspended in about 2 volumes of PBS-BSA-SFM (PBS, pH 7.4, containing 1% w/v bovine serum albumin mixed with equal volume of SFM), and kept at room temperature for ~20 min before being used for experiments. Cells were then re-centrifuged ($100 \times g$, 1 min) and resuspended in the appropriate solutions for subsequent experiments. Primary cells were prepared from different animals for each of the separate experiments (Table 1 and Fig. 2).

2.3. Hemin-based oxidation assay

Oxidation assays, performed in the presence or absence of apocynin, included hemin, hydrogen peroxide, and TMPD (*N*,*N*,*N*′,*N*′-Tetramethyl-1-4-phenylenediamine dihydrochloride) as the ultimate redox indicator, cf. [18,19]. The final concentrations in the assay were as follows: apocynin (up to 20 μM, or no apocynin in the control reactions), 1 μM hemin, 1 mM hydrogen peroxide, and 100 μM TMPD. The assays were performed on 96-well plates and absorbance (600 nm) was measured at different time intervals up to 10 min using a multi-well plate reader.

Table 1
Inhibition of endocytosis by hydrogen peroxide (0.5 mM) and effects of apocynin (20 μM) on the inhibition. Results are shown as a percentage of untreated controls ± standard deviation of mean (n = 4–6 for each treatment). Apocynin did not result in a statistically significant increase ($p > 0.05$) in endocytic transport of the indicated ligands, transferrin (Tf) and transthyretin (TTR).

Cell type	Ligand	H_2O_2	H_2O_2 + Apocynin
A431 epidermoid	Tf	76.3 ± 8.3	92.5 ± 17.5
A431 epidermoid	TTR	61.4 ± 10.1	73.9 ± 4.9
Primary liver	Tf	85.6 ± 2.2	88.7 ± 12.6

2.4. Endocytosis assay

Equal amounts of cell suspension were pre-treated for 30 min at 37 °C with hydrogen peroxide in Eppendorf tubes. Biotin-labeled ligand (transferrin, 20 μg/ml final concentration) was then added to the cell suspension on ice, and the tubes were incubated at 37 °C for 10 min to allow endocytosis. Tubes were then returned to ice and centrifuged to pellet cells. Cell pellets were rinsed with 100 μl of pH 3-acetate buffer and re-centrifuged. The cells were again washed with 100 μl of the low-pH buffer by re-suspending the pellet three times using a pipette. The tubes were then re-centrifuged as above, and the low pH rinse repeated. Final cell pellets were re-suspended in 100 μl of lysis buffer (1% Triton X-100, 0.1% SDS in 1 mM EDTA, 50 mM NaCl and 10 mM Tris-HCl, pH 7.4) and stored frozen until further analysis. Endocytosis assays performed with cell membrane preparations involved the same procedure as above except for the presence of cytosol and ATP along with biotin-ligand during the 10 min, 37 °C endocytosis event, [20] and references therein.

The internalized ligand was captured on anti-ligand IgG-coated plates and, after rinsing, tagged with streptavidin-peroxidase as previously described [20]. The biotin-ligand was quantified using the standard ELISA color reaction. This reaction involves 0.4 mg/ml *o*-phenylenediamine dihydrochloride (OPD) and 0.02% hydrogen peroxide in 50 mM Na2PO4 and 30 mM sodium citrate, pH 5. The color reactions were terminated with 2 N sulfuric acid. Absorbance was measured at 490 nm using an ELISA plate reader. Lysates from endocytosis assays performed with HRP ligand were analyzed directly by the OPD color reaction.

2.5. RNS levels

Nitrite assays were performed as previously described [21]. Briefly, cells were treated for 4 h with aTTR (2.4 μM pre-aggregation concentration) to induce RNS production in the presence or absence of apocynin (20 μM). Culture media (100 μl) from these treatments as well as nitrite standards (0 to 200 μM) were added to wells of a 96-well microplate and mixed with 100 μl of Griess reagent containing 1% sulfanilamide and 0.05% *N*-(1-napthyl)-ethylenediamine for 45 min at room temperature. Absorbance was then measured using an ELISA plate reader. For the total nitrate + nitrite assay, 100 μl of 8 mg/ml vanadium trichloride (VCl_3) was added to 100 μl of nitrate standards (0–200 μM) and to the 100 μl of culture media of the samples. The standards and samples were then processed by the same method as for the nitrite above.

2.6. Hydrogen peroxide production

The production of hydrogen peroxide was assayed using the amplex red (*N*-acetyl-3, 7-dihydroxyphenoxazine) method according to the manufacturer's instruction (Molecular Probes, Canada). Cells were treated with 50 μl of aTTR or TTR at 2.4 μM (or no-TTR control; for aTTR, concentration represents pre-aggregate state) in Krebs–Ringer phosphate buffer (KRPG) for 30 min at 37 °C. In parallel, some cells were treated with aTTR plus 20 μM apocynin. Reaction mixture (100 μl) containing 50 μM amplex red reagent and 0.1 U/ml HRP in KRPG buffer was added to the above samples, hydrogen peroxide controls (0 to 10 μM), and blanks (KRPG buffer