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Apocynin inhibits NADPH oxidase in phagocytes but stimulates ROS production in non-phagocytic cells

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Abstract

Apocynin is a naturally occurring methoxy-substituted catechol, experimentally used as an inhibitor of NADPH oxidase. Since it acts as a potent inhibitor in studies with neutrophils and macrophages, no inhibitory effect can often be found in non-phagocyte cells. In our experiments, apocynin even stimulated reactive oxygen species (ROS) production by vascular fibroblasts. Even when added to macrophages, apocynin initially caused an increase in ROS production. The inhibition of ROS formation followed, suggesting that in the presence of leukocyte myeloperoxidase and hydrogen peroxide, apocynin is converted to another compound. Apocynin pre-activated with H_2O_2 and horseradish peroxidase (HRP) inhibited ROS production immediately. In non-phagocytes, apocynin stimulated ROS production and no inhibition was observed even after 60 min. Apocynin treated with H_2O_2 and HRP, however, decreased ROS production in the same manner as in macrophages. The stimulatory effect on ROS production can be abolished by tiron and superoxide dismutase (SOD), suggesting that superoxide was the produced species. The effect of apocynin was inhibited by diphenylene iodinium (DPI), a non-scavenging NADPH oxidase inhibitor. It can be summarized that apocynin stimulates cell superoxide production. In the presence of peroxidase and hydrogen peroxide, however, it is converted into another compound that acts as an inhibitor of superoxide production. It strongly suggests that under conditions in vivo, apocynin can have opposite effects on phagocytes and non-phagocyte cells. It acts as an inhibitor of phagocyte NADPH oxidase but also as a ROS production stimulator in non-phagocyte cells.

Keywords: Apocynin; NADPH-oxidase; Reactive oxygen species

1. Introduction

Apocynin (4-hydroxy-3-methoxyacetophenone, acetovanillone, CAS 498-02-2) is a methoxy-substituted catechol originally extracted from the roots of *Picrorrhiza kurroa* [1], a small perennial herb that grows in the Himalayas. Like other methoxy-substituted catechols [2,3], it can decrease the production of superoxide ($O_2^{\cdot-}$) by activated neutrophils and macrophages while the ability of phagocytosis remains unaffected. The formation of superoxide and consequently of other reactive oxygen species is the key to microcidal and cytotoxic function of phagocytes; however, it also contributes to the collateral damage of tissues in inflammation. In traditional medicine, various extracts of *P. kurroa* are used for treating diseases associated with chronic inflammation. Nowadays, this compound is investigated as a possible anti-inflammatory drug that could be useful, e.g. in the treatment of arthritis [1]. The positive effects of apocynin in treating atherosclerosis [4] or preventing ischemia-reperfusion lung injury [5] were proved in experiments with animals.

Apocynin inhibits the formation of superoxide by phagocyte NADPH oxidase. The likely mechanism of this effect is the inhibition of enzyme assembly, probably by blocking sulfhydryl groups [2]. Apocynin itself does not act as a ROS scavenger [6].

Abbreviations: ROS, reactive oxygen species; DCFH, dichlorodihydrofluorescin; DCF, dichlorofluorescin; HRP, horseradish peroxidase; SOD, superoxide dismutase; DPI, diphenylene iodinium; HBSS, Hank's balanced salt solution; DCFH-DA, dichlorodihydrofluorescin diacetate; MEM, minimal essential medium; STZ, serum treated zymosan; PBS, phosphate buffered saline; CPS, counts per second; F12K, Nutrient mixture F12 Ham Kaighn's modification

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Apocynin is used as an experimental tool to inhibit phagocyte NADPH oxidase. We intended to use this compound, like other authors [7,8], to inhibit NADPH oxidase in non-phagocytes. The addition of apocynin, however, did not lead to a decrease in ROS formation but surprisingly to a significant increase. Some authors suggested earlier that apocynin does not act directly as an inhibitor but that it must be pre-activated by hydrogen peroxide and a peroxidase [2]. In this way it is converted into a symmetrical dimer by means of the formation of a 5,5′ carbon–carbon bound [9]. In the locus of inflammation, hydrogen peroxide as well as myeloperoxidase are secreted by phagocytes; in non-phagocyte cells, however, they are practically absent. Therefore we tested the hypothesis that apocynin has different effects on immune stimulated phagocytes and on non-phagocyte cells. In the first case it acts as an inhibitor of NADPH oxidase and its presence leads to a decrease in ROS formation; in the latter case, apocynin on the contrary increases ROS production.

2. Materials and methods

All reagents were obtained from regular commercial sources and were of at least an analytical grade. Apocynin, cell culture media, Hank's balanced salt solution (HBSS), zymosan A from *Saccharomyces cerevisiae*, Histopaque, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), catalase, superoxide dismutase, diphenylene iodinium, hydrogen peroxide and tiron (4,5-dihydroxy-benzenedisulfonic acid) were purchased from Sigma-Aldrich Corp., USA. Horseradish peroxidase (HRP) was purchased from ICN Biochemicals, Inc.

Vascular fibroblasts were prepared as described in the literature [10]. Briefly, thoracic and abdominal aorta (male Wistar, 4 weeks old, weight 100–120 g) was explanted, washed in serum-free culture media F12K with penicillin 50 U ml^{-1} and streptomycin 50 μg ml^{-1} and aseptically cut into thin rings. The rings were placed onto a sterile polystyrene Petri dish and drops of culture media F12K with 10% fetal calf serum, penicillin and streptomycin (50 U ml^{-1} and 50 μg ml^{-1} respectively) were put on every ring. Two days later, the same culture medium was added and was replaced twice per week. At the first subculture (3 weeks from the establishment of the culture), antibiotics were given up. Fibroblasts were sub-cultured once per week in the ratio 1:4. Cells from passage 7 to 12 were used for experiments. Cells were harvested with trypsin 0.25% EDTA 0.2 mmol l^{-1} in HBSS.

Rat monocytes were prepared from blood collected from adult male animals (Wistar) using Histopaque 1083 and Histopaque 1077 (Sigma) following the protocol provided by the manufacturer. Briefly, in four polypropylene centrifugal tubes, 3 ml of Histopaque 1083 was overlaid with 3 ml of Histopaque 1077. The discontinuous gradient created in this way was subsequently overlaid with 6 ml of heparinized blood diluted 1:3 with saline. A layer of granulocytes at the interface of Histopaque 1083 and 1077 and another layer of monocytes at the interface of Histopaque 1077 and serum were separated after rotating at 700×*g* for 30 min. Blood cells were washed twice in salt solution and re-suspended in minimal essential medium (MEM) with Hank's salts and without phenol red. The type of collected cells was checked using smear stained with hematoxylin.

Serum treated zymosan (STZ) was prepared from zymosan A from *S. cerevisiae*. The suspension of zymosan A in HBSS (approx. 5 mg ml^{-1}) was boiled in a water bath for 20 min. Then the suspension was centrifuged and the supernatant was discarded. Swelled zymosan was incubated in rat serum (5 mg of zymosan to 1 ml of serum) at 37 °C for 30 min. This step was repeated three times. Finally, zymosan was washed in HBSS and re-suspended to a final concentration 10 mg ml^{-1}. Aliquots were stored at −85 °C.

DCFH-DA was dissolved in 96% ethanol to a concentration 4 mmol l^{-1} and further diluted with distilled water to 250 μmol l^{-1}. This stock solution was stored in aliquots at −85°C under nitrogen.

2′,7′-Dichlorofluorescin (DCFH) was prepared by deesterification of DCFH-DA as described [11]. Briefly, 0.5 ml DCFH-DA 1.0 mmol l^{-1} in methanol was mixed with 2 ml of 10 mmol l^{-1} NaOH. After incubation for 30 min at room temperature, the solution was neutralized by the addition of 10 ml of 25 mmol l^{-1} phosphate buffer, pH 7.4. The solution was kept in the dark on ice until used.

Apocynin pre-activated with peroxidase and hydrogen peroxide was prepared as follows: 0.4 ml of apocynin 4 mmol l^{-1} was mixed with 2 ml of phosphate buffered saline (PBS), 3 units of HRP and 100 μl of diluted hydrogen peroxide (3 ppm). After incubation for 1 h at room temperature, 8 kU of catalase was added and the mixture was incubated for another 15 min at 37 °C. Short boiling denatured the proteins. MEM was added to the cooled solution to the final volume of 4 ml and precipitate was removed by centrifugation.

Statistics The results are represented as means of at least three measurements and standard deviations. Student's *t*-test was used to determine significance.

3. Determination of ROS production

2′,7′-Dichlorofluorescein diacetate was employed to measure ROS formation in intact cells. This fluorescence probe passes through the cell membrane to cytoplasm where it is cleaved by cell esterases to impermeable 2′,7′-dichlorofluorescin (DCFH). DCFH can be easily oxidized by ROS to fluorescent DCF [11,12].

Fibroblasts or leukocytes were re-suspended in MEM without phenol red supplemented with 10 μmol l^{-1} DCFH-DA. After incubation for 30 min at 37 °C, the cells were