NADPH Oxidase Inhibition of Novel Apocynin Dimer Derivatives

ABSTRACT

Apocynin is a widely studied inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which has been regarded as a target for developing anti-oxidative and anti-inflammatory therapeutic agents. Diamycin, dimer of apocynin, was found to have higher NADPH oxidase inhibition activity than apocynin. To seek for dimeric apocynin analogues of better efficacy, in our previous work, 14 novel apocynin dimer derivatives were designed and synthesized based on the chemical structure of apocynin dimer analogue JJA-D0. The biological activities of the derivatives were evaluated in RAW 264.7 cells. All compounds protected RAW 264.7 cells from lipopolysaccharide (LPS)-induced cytotoxicity, and significantly lowered down the intracellular ROS level induced by LPS. JJA-D26 were more potent than apocynin and JJA-D0, which retained 71.2% of cell viability, and decreased 24.8% of intracellular ROS level at the concentration of 200 μM. JJA-D26 also greatly inhibited the expression of pro-inflammatory cytokine TNF-α, and significantly reduced the expression of subunits p47phox and gp91phox of NADPH oxidase complex (P < 0.05 and P < 0.01 respectively) compared to apocynin. This work may provide useful information for the research and development of potent anti-oxidation and anti-inflammation drugs targeting NADPH oxidase.

KEYWORDS apocynin dimer derivatives, NADPH oxidase, reactive oxygen species, anti-oxidation, anti-inflammation

INTRODUCTION

NADPH oxidases (NOXs) are a group of transmembrane enzymes found in a variety of cells, including phagocytes, which catalyze the transfer of electrons across the cell membrane. Seven isoforms of NADPH oxidase have been described in mammals, including NOX1-NOX5, dual oxidase (DUOX) 1 and DUOX2. NADPH oxidase consists of a core catalytic subunit and several regulatory subunits, such as cytochrome b558 (a heterodimer composed of gp91phox and p22phox), p47phox, p67phox, p40phox. NOX activator 1, NOX organizer 1, Rap 1α, nrc, DUOX activator 1 and 2. Cytochrome b558 is anchored to the cell membrane, while p47phox, p67phox, p40phox, and nrc reside in the cytoplasm of quiescent cells. Upon activation of NADPH oxidase, the cytosolic subunits translocate to dock with cytochrome b558. The binding of cytosolic subunits with cytochrome b558 results in conformational change of this flavocytochrome, and initiates electron flow across the membrane to reduce oxygen to superoxide anion (O2−), which is released into phagosome or the extracellular medium.

The superoxide anion is the primary free radical reactive oxygen species (ROS) and serves as the precursor of a vast assortment of secondary ROS, including oxidized halogens, free radicals and singlet oxygen. The O2− was generated by NOX in a burst-like manner in phagocytes to kill invading microorganisms or in a slow and sustained fashion in non-phagocytes to act as intracellular signaling molecules. The superoxide anion and its secondary oxidants were reported to influence physiological function to exert protection in some cases or to cause injury in other cases. The development of various diseases results from ROS accumulation originated from O2−. NOXs were thus regarded as a valuable target for research and development of new anti-oxidation and anti-inflammation drugs.

Apocynin (4-hydroxyl-3-methoxycetophenone, compound 1) has been widely studied in NADPH oxidase inhibition researches and found to efficiently reduce ROS production. Thus, apocynin has been investigated in the...
treatment of different kinds of diseases for its action of attenuating oxidative and inflammatory injury\textsuperscript{13-18}. Apocynin demonstrated potential in the treatment of rheumatoid arthritis\textsuperscript{16}, osteoarthritis\textsuperscript{17}, asthma\textsuperscript{13}, stroke\textsuperscript{18} and diabetes neuropathy\textsuperscript{19} to a certain extent although the action was generally not so great. Diapocynin, a dimer converted from apocynin by myeloperoxidase in phagocytes and supposed to be the actual active compound to exert NADPH oxidase inhibition activity, was reported to achieve greater efficacy than apocynin\textsuperscript{4}. However, there is only limited information about the pharmacological activity of diapocynin so far. In our previous work, JJA-D\textsubscript{0}, an analogue of diapocynin with more functional groups, was yielded for further structural modification. 14 apocynin dimer derivatives were designed and synthesized on the basis of chemical structure of JJA-D\textsubscript{0} (Fig. 1). In this work, their anti-oxidation, anti-inflammation and NADPH oxidase inhibition activities were investigated \textit{in vitro} to seek for agents of better anti-oxidative and anti-inflammatory efficacy.

**MATERIALS AND METHODS**

**Materials**

Compounds JJA-D\textsubscript{0}, JJA-D\textsubscript{12-21}, JJA-D\textsubscript{25-28} were synthesized in our laboratory. All reagents were obtained from commercial suppliers as follows. Apocynin was purchased from Beijing J&K Scientific Co., Ltd (China). Dulbecco’s Modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Inc. (USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich, Inc. (USA). ROS Assay kit was purchased from Jiangsu Beyotime Biotechnology (China). TNF-\textalpha and \textbeta-actin antibodies were purchased from Cell Signaling Technology, Inc. (USA). p47\textsubscript{phox} and gp91\textsubscript{phox} antibodies were purchased from Abcam (USA). Goat anti-rabbit IgG(H+L) HRP was purchased from Bioworld Technology, Inc. (USA).

**Cell culture**

The RAW 264.7 macrophage cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were cultured in DMEM containing 10\% FBS, 100 units/mL penicillin and 100 mg/mL streptomycin. The cells were incubated in an atmosphere of air containing 5\% CO\textsubscript{2} at 37\degree C.

**Cell protection of compounds measured by MTT assay**

The MTT method was used to evaluate the activity of 14 apocynin dimer derivatives against LPS-induced oxidative injury in RAW 264.7 macrophage cells. The RAW 264.7 cells were seeded (10\textsuperscript{4} well, 100 \mu L) in 96-well plates and then cultivated at 37\degree C for 24 h under 5\% CO\textsubscript{2}. The cells were pretreated with the tested...
compounds (50, 100, 200 μM) for 1 h. The supernatant was removed, and the cells were washed with PBS for three times. Then the cells of model and test groups were treated with 100 μg/mL of LPS for 24 h. MTT was then added into each well to make a final concentration of 0.5 mg/mL. After incubation for 4 h, the supernatant was removed and DMSO (150 μL) was added. The absorbance of each well was recorded at 570 nm using a Synergy HT Multi-Detection Microplate Reader (BioTek, USA), and the cell viability was calculated.

**Fluorescence-based ROS-scavenging assay**

The RAW 264.7 cells were seeded (10^4/well, 100 μL) in 96-well plates. After 24 h culture at 37°C under 5% CO₂, the cells were pretreated with the apocynin dimer derivatives (50, 100, and 200 μM) for 1 h. The supernatant was removed, and the cells were washed with PBS for three times. Then, the cells of model and test groups were treated with 100 μg/mL of LPS for 24 h. Then, the cells were cultured with 10 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 20 min at 37°C under 5% CO₂. After that, the cells were washed by PBS for three times and the fluorescence intensity was quantitatively assayed by a Synergy HT Multi-Detection Microplate Reader (BioTek, USA) at excitation and emission wavelengths of 488 and 530 nm, respectively. The ROS level was expressed as the fluorescence intensity ratio of the test group to the blank control group, whose fluorescence intensity was set as 100%.

**Western blot assay for measurement of expression of TNF-α, p47^phox^ and gp91^phox^**

The RAW 264.7 cells were pretreated with apocynin (200 μM), JJA-D0 (100 μM) and JJA-D26 (200 μM) for 1 h and then stimulated with LPS (100 μg/mL) for 24 h. The medium was removed and the cells were washed three times with PBS. The cells were then added with 100 μL of RIPA lysate (Jiangsu Beyotime Biotechnology, China) for 10-min lysis at 4°C. After centrifugation, the supernatant was collected. The protein concentration in the supernatant was measured by bicinchoninic acid (BCA) assay (Pierce, USA). Sample proteins were added with 5 × SDS loading buffer and heated in boiling water for 10 min to ensure denaturation, and the samples were then stored at −20°C. An equal amount of protein from each sample was resolved on 12% SDS-polyacrylamide gel by electrophoresis, and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were incubated in blocking solution (5% (w/v) nonfat dry milk in Trisbuffered saline with 0.1% Tween 20) for 2 h at room temperature, and then incubated with primary antibody of TNF-α, p47^phox^ or gp91^phox^, and β-actin (1:1000) at 4°C over night. After washing with TBST, the membranes were then incubated with the appropriate horseradish peroxidase-linked secondary antibodies (1:2000) for 1 h at room temperature. Protein bands were detected using ECL western blotting reagents (Pierce, USA). The optical density of each band was measured with a quantitative analyzer (Carestream, USA). β-actin was used as an internal loading control. The results were expressed as the zone intensity ratio of TNF-α, p47^phox^ or gp91^phox^ to β-actin.

**RESULTS AND DISCUSSION**

LPS was reported to signal NADPH oxidase activation, and then cause ROS generation that can amplify the production of proinflammatory cytokines, such as TNF-α. RAW 264.7 cells with LPS insult were selected to investigate the antioxidation, anti-inflammation and NADPH oxidase inhibition activities of the new derivatives. The RAW 264.7 cells were pretreated for 1 h with JJA-D0 and its derivatives at concentration of 50, 100 and 200 μM, respectively, and then stimulated with 100 μg/mL of LPS for 24 h. Cell viability was measured by the MTT staining method. The intracellular ROS level was assayed using fluorescence probe DCFH-DA. The protein samples were prepared from cell lysate to test the expression level of TNF-α and NADPH oxidase subunits p47^phox^ and gp91^phox^ using western blot assay.

**Effects of apocynin dimer derivatives against LPS-induced cytotoxicity and intracellular ROS level increase in RAW 264.7 cells**

The viability of RAW 264.7 cells was illustrated in Fig. 2. Most of the dimer derivatives showed better efficacy than apocynin. Compared to JJA-D0, compounds with alkylated phenolic hydroxyl group (JJA-D14~JJA-D21) showed lower or comparable cell viability in most cases. Compounds with branched alkyl on phenolic hydroxyl group (JJA-D16 and JJA-D20) had better activity than those with linear alkyl group (JJA-D17 and JJA-D21). Amidation of the secondary amidogroup of JJA-D0 (JJA-D12 and JJA-D13) reduced the cell protection activity. The sulfonic ester derivatives of JJA-D0 (JJA-D25~JJA-D28) conferred improvement at 50 μM, while exerted promoting or detrimental influence on the activity at 100 and 200 μM. The sulfonation of phenolic hydroxyl group in motif 1 resulted in better protection than that in motif 2. Among them, JJA-D26 showed better activity than JJA-D0 at all tested concentration and was the most potent compound in this assay, which acted in a dose-dependent manner and retained 71.2% of cell viability at the concentration of 200 μM.

RAW 264.7 cells treated only with LPS showed strong fluorescence intensity indicating burst generation of intracellular ROS. JJA-D0 and its derivatives reduced the intracellular ROS level and showed better activity than apocynin at all tested concentrations (50, 100 and 200 μM) (Fig. 3). Compounds with alkylated phenolic hydroxyl group showed similar ROS scavenging activity as JJA-D0. Compounds with modified secondary amidogroup were less active than JJA-D0. No apparent
tendency was observed in the influence of sulfonic esterification of phenolic hydroxyl on ROS scavenging. Although JJA-D26 showed weaker activity than JJA-D0 at 50 and 100 μM, it achieved the lowest intracellular ROS level with a ROS reduction rate of 24.8% at 200 μM.

The above results indicated that the secondary amido-group and the phenolic hydroxy group in motif 2 were important for the bioactivity of JJA-D0 and its derivatives. This was evidenced by the poor activity of amido-group substituted derivatives (JJA-D12 and JJA-D13), and the higher efficacy of derivatives with sulfonation of phenolic hydroxyl in motif 1 (JJA-D25 and JJA-D26) than in motif 2 (JJA-D27 and JJA-D28). The bioactivity results were in accordance with the results of preliminary molecular docking simulation between NADPH oxidase and JJA-D0, which indicated the importance of the two groups in NADPH oxidase inhibition. Molecular docking results demonstrated that hydrogen bond was formed between the secondary amido-group of JJA-D0 and the CYS378 residue of NADPH oxidase. Furthermore, there was hydrogen bond formed between the phenolic hydroxyl in motif 2 of JJA-D0 and the LYS383 residue of NADPH oxidase, while no hydrogen bond formed between the phenolic hydroxyl in motif 1 and NADPH oxidase.

Inhibition of LPS-induced p47phox and gp91phox protein expression in RAW 264.7 cells

Inhibition of the expression of important subunit provides a feasible way to inhibit NADPH oxidase activity. Both catalytic subunit gp91phox and cytosolic subunit p47phox are crucial in the activation of NADPH oxidase. Their expression level can reflect the activity of NADPH oxidase. They thus become the key targets of development of NADPH oxidase inhibitors. The expression of the two subunits gp91phox and p47phox was measured herein and the results were shown in Fig. 4. Apocynin, JJA-D0 and JJA-D26 markedly reduced the expression of p47phox and gp91phox protein induced by LPS (P < 0.05 and 0.01 respectively vs. model group). JJA-D0 showed higher activity than apocynin, while JJA-D26 led to the lowest expression level of p47phox and gp91phox (P < 0.05 for p47phox and < 0.01 for gp91phox vs. apocynin), indicating JJA-D26 to be a promising NADPH oxidase inhibitor. The similar results were achieved in other researches with higher tested concentration of apocynin and diapocynin in different cell lines. Barbieri et al. reported that apocynin at 600 μM effectively decreased p47phox expression in human monocytes treated with zymosan and phorbol myristate acetate. Kanegae et al. observed that apocynin and diapocynin decreased gp91phox mRNA expression in LPS-induced peripheral blood mononuclear cells (PBMC) at 3 mM and 0.5 mM, respectively, which indicated that diapocynin inhibited NADPH oxidase more effectively than apocynin.

Inhibition of LPS-induced TNF-α expression in RAW 264.7 cells

The relationship between oxidative stress and inflammation was complicated. TNF-α is an important...
Fig. 4 Effects of apocynin, JJA-D0 and JJA-D26 on the expression of p47phox and gp91phox protein induced by LPS in RAW 264.7 cells. **P < 0.01 vs. control (Ctrl) group; *P < 0.05, **P < 0.01 vs. model group; \( \Delta P < 0.05, \Delta \Delta P < 0.01 \) vs. apocynin group.

Fig. 5 Inhibition effects of apocynin, JJA-D0 and JJA-D26 on TNF-\( \alpha \) expression induced by LPS in RAW 264.7 cells. **P < 0.01 vs. control (Ctrl) group; *P < 0.05 vs. model group; \( \Delta P < 0.05 \) vs. apocynin group.

pro-inflammatory cytokine of important roles in various cell processes, such as cell survival, apoptosis and necroptosis as well as intercellular communication.\(^2\) TNF-\( \alpha \) was found to induce recruitment of NOX, thus to result in activation of NADPH oxidase followed by production of ROS.\(^2,4\) Kanegae et al. found that apocynin inhibited TNF-\( \alpha \) production and diapocynin had a much more pronounced effect, and considered that inhibiting TNF-\( \alpha \) release could be another contribution of apocynin and diapocynin in decreasing oxidative stress.\(^4\) In this report, JJA-D0 and JJA-D26 was found to significantly diminish TNF-\( \alpha \) expression (\( P < 0.05 \) vs. model group) (Fig. 5). JJA-D26 resulted in the lowest expression of TNF-\( \alpha \) and had significant difference from apocynin (\( P < 0.05 \)).

CONCLUSION

In summary, the 14 novel apocynin dimer derivatives tested herein demonstrated significant anti-oxidative, anti-inflammatory and NADPH oxidase inhibition activities. The results were in consistence with other researches that apocynin dimer had better efficacy than apocynin. It indicated that diapocynin analogue JJA-D0 is a hopeful pharmacophore with potential NADPH oxidase inhibition. Additionally, the structural modification of JJA-D0 provided more potent anti-oxidative and anti-inflammatory agents for further development. Among the 14 derivatives investigated, the sulfonic ester derivative JJA-D26 demonstrated the best activity, and may be used as lead compound to develop therapeutics for oxidative stress and inflammation related diseases like acute lung injury, asthma, cardiovascular and cerebrovascular diseases and diabetes mellitus.

REFERENCES