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Effects of phenylethanoid glycosides from *Digitalis purpurea* L. on the expression of inducible nitric oxide synthase

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Abstract

We have isolated four different phenylethanoid glycosides (purpureaside A, desrhamnosyl acteoside, calceolarioside B and plantainoside D) from the leaves of *Digitalis purpurea* (foxglove). The effects of these glycosides on activator protein-1 (AP-1)-mediated inducible nitric oxide synthase (iNOS) gene expression in the Raw264.7 macrophage cell line have been studied. Of these four glycosides, purpureaside A potently inhibited iNOS induction by lipopolysaccharide (LPS). Increase in iNOS mRNA by LPS was completely suppressed by purpureaside A. Purpureaside A did not significantly affect LPS-inducible nuclear factor-κB (NF-κB) activation or the nuclear translocation of p65. Moreover, a reporter gene assay using AP-1 specific luciferase reporter revealed that the enhanced activity of AP-1 by LPS was completely abolished in cells treated with purpureaside A. These results demonstrated that purpureaside A inhibited LPS-inducible iNOS expression in macrophages through the suppression of AP-1, but not of NF-κB.

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Introduction

Foxglove (*Digitalis purpurea*) is a biennial or perennial herb of the Scrophulariaceae family, and has been commonly used to treat congestive heart failure since Withering clarified its use in 1785 (Willius & Keys 1941). Moreover, recent research has demonstrated in-vitro anticancer effects of digitalis compounds, and has suggested their possible use in oncology (Lopez-Lazaro et al 2003).

We have isolated four different phenylethanoid glycosides (purpureaside A, desrhamnosyl acteoside, calceolarioside B and plantainoside D) from the leaves of *D. purpurea*. Previously, we had demonstrated that acteoside had antioxidant effects on $FeCl_2$-ascorbate-induced lipid peroxidation in a mouse liver homogenate, and that it protected against liver toxicity induced by CCl_4 (Lee et al 2004). Calceolarioside B and plantainoside D showed moderate binding to HIV glycoprotein41 (Kim et al 2002) and inhibitory activity against PKCα (Zhou et al 1998). However, the pharmacological effects of purpureaside A have not been clarified.

Nitric oxide (NO), which is a radical produced from L-arginine by NO synthase (NOS), plays a dual role as a beneficial and detrimental molecule in the process of inflammation. NO produced by constitutive NOS (cNOS) is critical for maintaining cellular function (Porsti & Paakkari 1995), whereas NO produced by inducible NOS (iNOS) is an important mediator of acute or chronic inflammation (Kubes & McCafferty 2000). Nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) are immediately activated after macrophage exposure to inflammatory signals, such as lipopolysaccharide (LPS). The activation of NF-κB and AP-1 act co-operatively on the induction of the iNOS gene in cells, which is followed by the sustained production of NO (Sherman et al 1993; Xie et al 1993).

In an effort to screen for the pharmacological effects of *D. purpurea* glycosides, we were interested in the effects of glycosides from *D. purpurea* on NF-κB- and AP-1-mediated iNOS expression and NO production induced by LPS. In this study, for the

first time we have shown that purpureaside A was an inhibitor of LPS-induced iNOS expression, and that this was achieved via the specific blocking of AP-1 activation.

Materials and Methods

Isolation of pure glycosides from *D. purpurea*

Melting points were measured using a Fisher Scientific melting point apparatus and were uncorrected. IR spectra were recorded on an IMS 85 (Bruker), and NMR spectra on a Varian Unity Inova 500 (500 MHz) spectrometer. ^{1}H-^{1}H COSY, DEPT, HMQC, and HMBC NMR spectra were obtained with pulse sequences. HR-FABMS was determined on a JMS 700 (JEOL). Semi-preparative HPLC was performed on a Waters 600E equipped with a UV Detector (Waters model 2487) using a μ Bondpak C_{18} (3.9 mm × 300 mm, Waters) column. TLC and column chromatography were carried out on precoated Si Gel F_{254} plates (Merck, art. 5715), RP-18 F_{254} plates (Merck, art. 15423), and Si gel 60 (Merck, 230–400 mesh), respectively.

The leaves of *D. purpurea* (Scrophulariaceae) were obtained from the Herbarium of the College of Pharmacy, Chosun University, and authenticated by the Department of Pharmacognosy, Chosun University. Voucher specimens were deposited in the Herbarium of the College of Pharmacy, Chosun University (997-17). Dried leaves (180 g) of *D. purpurea* were extracted with MeOH at room temperature to afford 80.6 g of residue. The residue was suspended in water and then partitioned sequentially with dichloromethane, ethyl acetate, and *n*-butanol. A 5.5-g sample of EtOAc fraction was subjected to column chromatography over silica gel (350 g, 4.6 × 46 cm) and eluted with a $CHCl_3$–MeOH–H_2O (8:1:0.1→6:1:0.1→4:1:0.1→2:1:0.1→1:1;0.1, MeOH only) gradient system. The fractions were combined based on their TLC patterns to yield subfractions, designated as E1–E10. Subfraction E7 (543 mg) was further purified by column chromatography through Sephadex LH20 by eluting with MeOH to afford five subfractions (E71–E75).

Subfraction E74 (186 mg) was further purified by repeated column chromatography through silica gel by eluting with a $CHCl_3$–MeOH–H_2O gradient system to give compounds **3** (calceolarioside B, 13 mg) and **2** (desrhamnosyl acteoside, 32 mg). Subfraction E9 (2074.77 mg) was purified by column chromatography through Sephadex LH 20 to afford seven subfractions (E91–E97). Subfraction E94 (514.4 mg) was finally purified by semi-prep HPLC (Waters 600E) by eluting with *i*-PrOH/MeOH/H_2O (3:24:73), which afforded compound **1** (purpureaside A 38.4 mg), and compound **4** (plantainoside D, 4.6 mg) (Miyase et al 1991; Zhou et al 1998). Chemical structures (Figure 1) were confirmed by spectroscopic analyses including NMR (Miyase et al 1991; Zimin & Zhongjian 1991; Kim et al 2001).

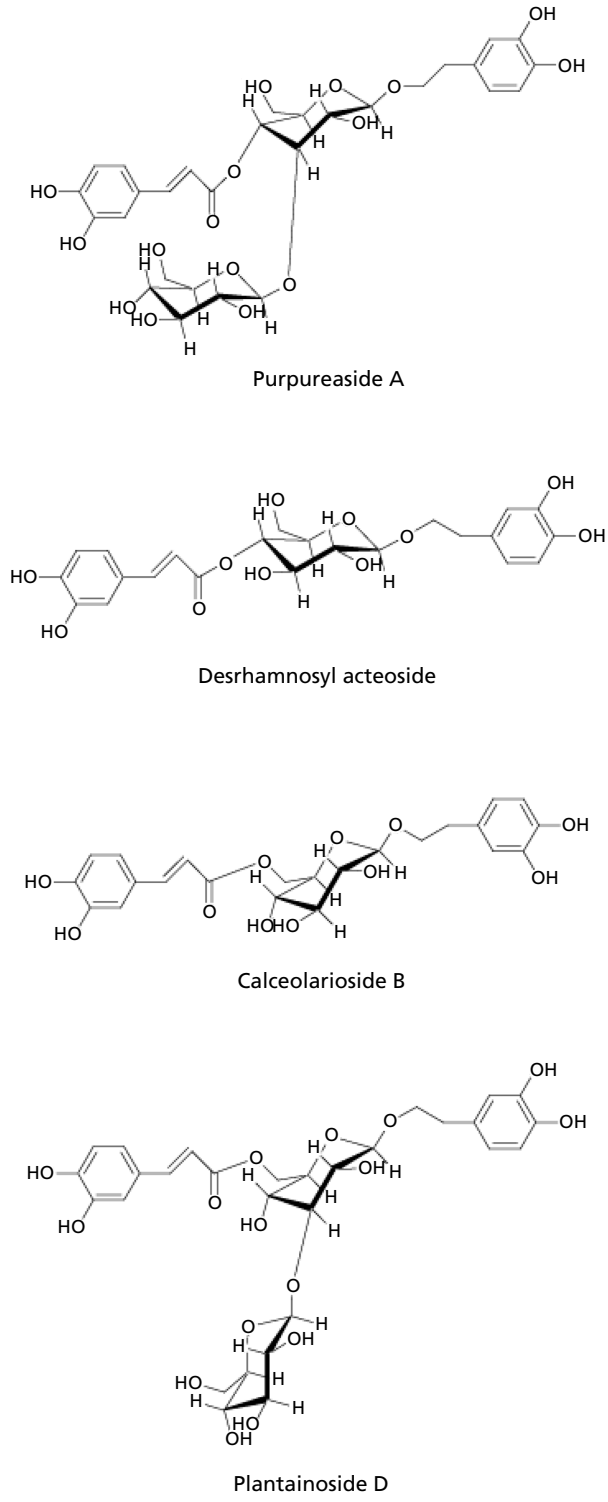

Figure 1 Structures of the four phenylethanoid glycosides isolated from the leaves of *D. purpurea*.

Purpureaside A

^{1}H NMR (CD_3OD, 500 MHz): d6.71 (1H, d, J = 2.0 Hz, H-2), 6.68 (1H, d, J = 8.0 Hz, H-5), 6.56 (1H, dd, J = 8.0, 2.0 Hz, H-6), 2.80 (2H, m, H-7), 4.05 (1H, m, H-8), 3.74 (1H, m, H-8), 4.41 (1H, d, J = 8.0 Hz, H-1′), 3.55 (1H, t, J = 8.5 Hz, H-2′), 3.95 (1H, t, J = 9.3 Hz, H-3′), 4.94 (1H, t, J = 9.3 Hz, H-4′), 3.51 (1H, m, H-5′), 3.55 (1H, m, H-6′), 3.72 (1H, m, H-6′), 4.54 (1H, d, J = 1.5 Hz, H-1″), 3.15 (1H, m,

H-2″), 3.34 (1H, t, $J=8.5$ Hz, H-3″), 3.26 (1H, t, $J=9.3$ Hz, H-4″), 3.19 (1H, m, H-5″), 3.51 (1H, m, H-6″), 3.64 (1H, m, H-6″), 7.08 (1H, d, $J=2.0$ Hz, H-2‴), 6.79 (1H, d, $J=8.0$ Hz, H-5‴), 6.98 (1H, dd, $J=8.0$, 2.0 Hz, H-6‴), 7.58 (1H, d, $J=16.0$ Hz, H-7‴), 6.32 (1H, d, $J=16.0$ Hz, H-8‴); ^{13}C NMR (CD_3OD, 125 MHz): d131.57 (C-1), 117.28 (C-2), 146.23 (C-3), 144.78 (C-4), 116.46 (C-5), 121.43 (C-6), 36.66 (C-7), 72.34 (C-8), 104.10 (C-1′), 75.96 (C-2′), 84.37 (C-3′), 71.01 (C-4′), 75.17 (C-5′), 62.41 (C-6′), 105.88 (C-1″), 76.12 (C-2″), 77.78 (C-3″), 71.32 (C-4″), 78.00 (C-5″), 62.51 (C-6″) 127.80 (C-1‴), 115.30 (C-2‴), 146.97 (C-3‴), 149.89 (C-4‴), 116.71 (C-5‴), 123.28 (C-6‴), 147.58 (C-7‴), 115.40 (C-8‴), 168.68 (C-9″).

Desrhamnosyl acteoside

^{1}H NMR (CD_3OD, 500 MHz): d6.69 (1H, d, $J=2.0$ Hz, H-2), 6.67 (1H, d, $J=8.0$ Hz, H-5), 6.56 (1H, dd, $J=8.0$, 2.0 Hz, H-6), 2.80 (2H, m, H-7), 3.71 (1H, dd, $J=16.0$, 8.0 Hz, H-8), 4.04 (1H, dd, $J=16.0$, 8.0 Hz, H-8), 4.37 (1H, d, $J=8.0$ Hz, H-1′), 3.32 (1H, dd, $J=9.3$, 8.0 Hz, H-2′), 3.61 (1H, t, $J=9.3$ Hz, H-3′), 4.83 (1H, t, $J=9.3$ Hz, H-4′), 3.51 (1H, t, $J=9.3$ Hz, H-5′), 3.52 (1H, m, H-6′), 3.62 (1H, m, H-6′), 7.05 (1H, d, $J=2.0$ Hz, H-2″), 6.77 (1H, d, $J=8.0$ Hz, H-′5″), 6.95 (1H, dd, $J=8.0$, 2.0 Hz, H-6″), 7.59 (1H, d, $J=16.0$ Hz, H-7″), 6.30 (1H, d, $J=16.0$ Hz, H-8″); ^{13}C NMR (CD_3OD, 125 MHz): d131.61 (C-1), 117.26 (C-2), 146.26 (C-3), 144.80 (C-4), 116.44 (C-5), 121.41 (C-6), 36.70 (C-7), 72.34 (C-8), 104.50 (C-1′), 75.37 (C-2′), 75.96 (C-3′), 72.65 (C-4′), 76.24 (C-5′), 62.59 (C-6′), 127.83 (C-1″), 114.87 (C-2″), 146.97 (C-3″), 149.86 (C-4″), 116.66 (C-5″), 123.22 (C-6″), 147.77 (C-7″), 115.35 (C-8″), 168.75 (C-9″).

Calceolarioside B

^{1}H NMR (CD_3OD, 500 MHz): d6.67 (1H, d, $J=2.0$ Hz, H-2), 6.63 (1H, d, $J=8.0$ Hz, H-5), 6.54 (1H, dd, $J=8.0$, 2.0 Hz, H-6), 2.78 (2H, m, H-7), 3.70 (1H, dd, $J=16.0$, 8.0 Hz, H-8), 3.95 (1H, dd, $J=16.0$, 8.0 Hz, H-8), 4.33 (1H, d, $J=8.0$ Hz, H-1′), 3.27 (1H, t, $J=8.0$ Hz, H-2′), 3.52 (1H, dd, $J=7.5$, 2.0 Hz, H-3′), 3.36 (1H, t, $J=9.3$ Hz, H-4′), 3.38 (1H, t, $J=9.3$ Hz, H-5′), 4.34 (1H, dd, $J=12.0$, 6.0 Hz H-6′), 4.50 (1H, dd, $J=12.0$, 2.0 Hz H-6′), 7.03 (1H, d, $J=2.0$ Hz, H-2″), 6.77 (1H, d, $J=8.0$ Hz, H-5″), 6.89 (1H, dd, $J=8.0$, 2.0 Hz, H-6″), 7.56 (1H, d, $J=16.0$ Hz, H-7″), 6.29 (1H, d, $J=16.0$ Hz, H-8″); ^{13}C NMR (CD_3OD, 125 MHz): d131.54 (C-1), 117.22 (C-2), 146.27 (C-3), 144.80 (C-4), 116.50 (C-5), 121.41 (C-6), 36.84 (C-7), 72.54 (C-8), 104.69 (C-1′), 75.22 (C-2′), 75.60 (C-3′), 71.88 (C-4′), 78.08 (C-5′), 64.79 (C-6′), 127.83 (C-1″), 115.00 (C-2″), 146.94 (C-3″), 149.78 (C-4″), 116.68 (C-5″), 123.30 (C-6″), 147.39 (C-7″), 115.20 (C-8″), 169.32 (C-9″).

Plantainoside D

^{1}H NMR (CD_3OD, 500 MHz): d6.68 (1H, d, $J=2.0$ Hz, H-2), 6.63 (1H, d, $J=8.0$ Hz, H-5), 6.54 (1H, dd, $J=8.0$, 2.0 Hz, H-6), 2.79 (2H, m, H-7), 3.97 (1H, m, H-8), 3.72 (1H, m, H-8), 4.38 (1H, d, $J=8.0$ Hz, H-1′), 3.44 (1H, t, $J=8.5$ Hz, H-2′), 3.42 (1H, t, $J=9.3$ Hz, H-3′), 3.47 (1H, t, $J=9.3$ Hz, H-4′), 3.57 (1H, m, H-5′), 4.35 (1H, dd, $J=12.0$, 6.0 Hz, H-6′), 4.52 (1H, dd, $J=12.0$, 2.0 Hz, H-6′), 4.58 (1H, d, $J=1.5$ Hz, H-1″), 3.31 (2H, m, H-2″, 4″), 3.41 (1H, t, $J=8.5$ Hz, H-3″), 3.33 (1H, m, H-5″), 3.63 (1H, dd, $J=12.0$, 6.0 Hz, H-6″), 3.90 (1H, dd, $J=12.0$, 2.0 Hz, H-6″), 7.03 (1H, d, $J=2.0$ Hz, H-2‴), 6.77 (1H, d, $J=8.0$ Hz, H-5‴), 6.89 (1H, dd, $J=8.0$, 2.0 Hz, H-6‴), 7.56 (1H, d, $J=16.0$ Hz, H-7‴), 6.27 (1H, d, $J=16.0$ Hz, H-8‴); ^{13}C NMR (CD_3OD, 125 MHz): d131.47 (C-1), 117.23 (C-2), 146.27 (C-3), 144.80 (C-4), 116.50 (C-5), 121.41 (C-6), 36.81 (C-7), 72.54 (C-8), 104.20 (C-1′), 75.21 (C-2′), 87.82 (C-3′), 70.49 (C-4′), 74.58 (C-5′), 64.73 (C-6′), 105.41 (C-1″), 75.68 (C-2″), 77.94 (C-3″), 71.71 (C-4″), 78.32 (C-5″), 62.77 (C-6″) 127.79 (C-1‴), 114.95 (C-2‴), 146.94 (C-3‴), 149.81 (C-4‴), 116.68 (C-5‴), 123.31 (C-6‴), 147.40 (C-7‴), 115.19 (C-8‴), 169.22 (C-9″).

Materials

Horseradish peroxidase-conjugated donkey anti-rabbit, anti-mouse, and alkaline phosphatase-conjugated donkey anti-mouse IgGs were purchased from Jackson Immunoresearch laboratories (West Grove, PA, USA). Anti-p65 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-murine iNOS antiserum was obtained from Transduction Laboratories (Lexington, KY, USA). pNF-κB-luciferase and pAP-1-luciferase plasmids were obtained from Stratagene (La Jolla, CA, USA). Reagents for the molecular studies were obtained from Sigma (St Louis, MO, USA).

Cell culture

The macrophage cell line Raw264.7 (ATCC No.TIB-71) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 μg mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin, in 5% CO_2 at 37°C. Raw264.7 cells were incubated with 1 μg mL^{-1} LPS (*Escherichia coli*, serotype 0127B8, Sigma Co., St Louis, MO, USA) to activate NF-κB/AP-1 and to stimulate iNOS gene expression. Each glycoside was dissolved in dimethyl sulfoxide and added to the incubation medium 10 min before LPS addition.

Assay of NO production

NO production was monitored by measuring the nitrite level in the culture medium. This was performed by mixing medium with Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylenediamine dihydrochloride, and 2.5% phosphoric acid). Absorbance was measured at 540 nm after incubating for 10 min.

Preparation of nuclear fractions

Nuclear extracts were prepared from the cells as described by Schreiber et al (1990). Briefly, cells in dishes were washed with ice-cold phosphate-buffered saline. They were then scraped, transferred to microtubes, and allowed to swell after adding 100 μL lysis buffer containing 10 mM

HEPES (pH 7.9), 0.5% Nonidet P-40, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride. Cell membranes were disrupted by vortexing, and lysates were incubated for 10 min on ice and centrifuged at 7200 *g* for 5 min. Pellets containing crude nuclei were resuspended in 80 μL extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonylfluoride, and then incubated for 30 min on ice. Samples were centrifuged at 15 800 *g* for 10 min to obtain supernatants containing the nuclear extracts, which were then stored at -70°C until use.

Immunoblot analysis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to Laemmli (1970), and immunoblot analysis was performed according to a previously published procedure (Kim et al 1997). Samples were fractionated by 10% gel electrophoresis and electrophoretically transferred to nitrocellulose paper. This was then incubated with polyclonal rabbit anti-iNOS antibody (1:4000, Transduction Laboratories, KY, USA), monoclonal mouse anti-actin antibody (1:20 000, Sigma, MO, USA), and monoclonal mouse anti-p65 antibody (1:1000), and then incubated with alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibody. Blots were finally developed using 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium or an ECL chemiluminescence detection kit (Kang et al 2000).

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was isolated using the guanidium-isothiocyanate procedure, as described by Kang et al (2000). Total RNA (1.0 μg) obtained from the cells was reverse-transcribed using an oligo(dT) 20mer as a primer and M-MLV reverse transcriptase (Bioneer, Eumsung, Korea). PCR was performed using selective primers for mouse iNOS (sense primer: 5′-ATGTCCGAAGCAAACATCAC-3′, antisense primer: 5′-TAATGTCCAGGAAGTAGGTG-3′) (449 bp) and the S16 ribosomal protein (S16r) gene (sense: 5′-TCCAAGGGTCCGCTGCAGTC-3′, antisense: 5′-CGTTCACCTTGATGAGCCCATT-3′) (152 bp). PCRs were carried out over 39 cycles using the following conditions: denaturation at 98°C for 10 s, annealing at 50°C for 0.5 min, and elongation at 72°C for 1 min. The band intensities of amplified DNA were compared after visualization on an UV transilluminator.

Transient transfection and NF-κB and AP-1 reporter gene assay

Cells were plated at a density of 3×10^5 cells/12-well dish and transfected the following day. To determine promoter activity we used a dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, cells were transiently transfected with 1 μg pNF-κB-luciferase or pAP-1-luciferase plasmid and 20ng pRL-SV plasmid (*Renilla* luciferase expression for normalization) (Promega, Madison, WI) using Lipofectamine 2000 Reagent (Life Technologies, Gaithersburg, MD) and then exposed to LPS for 18 h. Firefly and *Renilla* luciferase activity in cell lysates were measured using a luminometer (Turner Designs; TD-20, CA). Relative luciferase activity was calculated by normalizing NF-κB or AP-1 promoter-driven firefly luciferase activity vs that of *Renilla* luciferase.

Scanning densitometry and statistical analysis

Scanning densitometry was performed using an Image Scan & Analysis System (Alpha-Innotech, San Leandro, CA). The results were statistically analysed using Kruskal–Wallis one-way analysis of variance to determine whether significant differences existed at the 95% confidence level. When differences were significant, a multiple comparison test for testing pairs of groups was adjusted according to Dunn's Test honestly significant difference (HSD) test (SPSS Version 10; SPSS Inc., Chicago, IL, USA). A non-parametric test was used due to the small sample size.

Results

Effects on nitrite formation

NO production was monitored in macrophages stimulated by LPS with or without each glycoside. Nitrite (the oxidized form of NO) in the medium increased 16-fold, as compared with control, after exposure of cells to LPS (1 μg mL^{-1}) for 24 h (Figure 2A). Purpureaside A at 100 μM significantly suppressed LPS-inducible NO production, resulting in 55% inhibition (Figure 2A). Desrhamnosyl acteoside, calceolarioside B, and plantainoside D inhibited NO production at 100 μM, but the inhibition percentages were markedly lower than that of purpureaside A (i.e. 15–27%) (Figure 2A).

Effects on iNOS expression induced by LPS

We determined whether the inhibition of NO formation by the glycosides was the result of the inhibition of iNOS gene expression. Treatment of cells with 100 μM purpureaside A substantially suppressed the iNOS protein increase by LPS (12 h), verifying that the compound inhibited iNOS induction (Figure 2B). Calceolarioside B and plantainoside D did not affect the intensity of iNOS protein. Levels of actin protein, which were used for comparative purposes, were comparable among the samples (Figure 2B). We next assessed the inhibitory effects of different concentrations of purpureaside A on the induction of iNOS protein. Treatment of cells with purpureaside A (30 or 100 μM) significantly inhibited iNOS protein levels in a concentration-dependent manner (Figure 2C). A concentration of 100 μM was chosen for subsequent experiments, and the expression of iNOS mRNA was additionally assessed by RT-PCR analysis. Treatment of macrophages with LPS (1 μg mL^{-1}) increased the level of

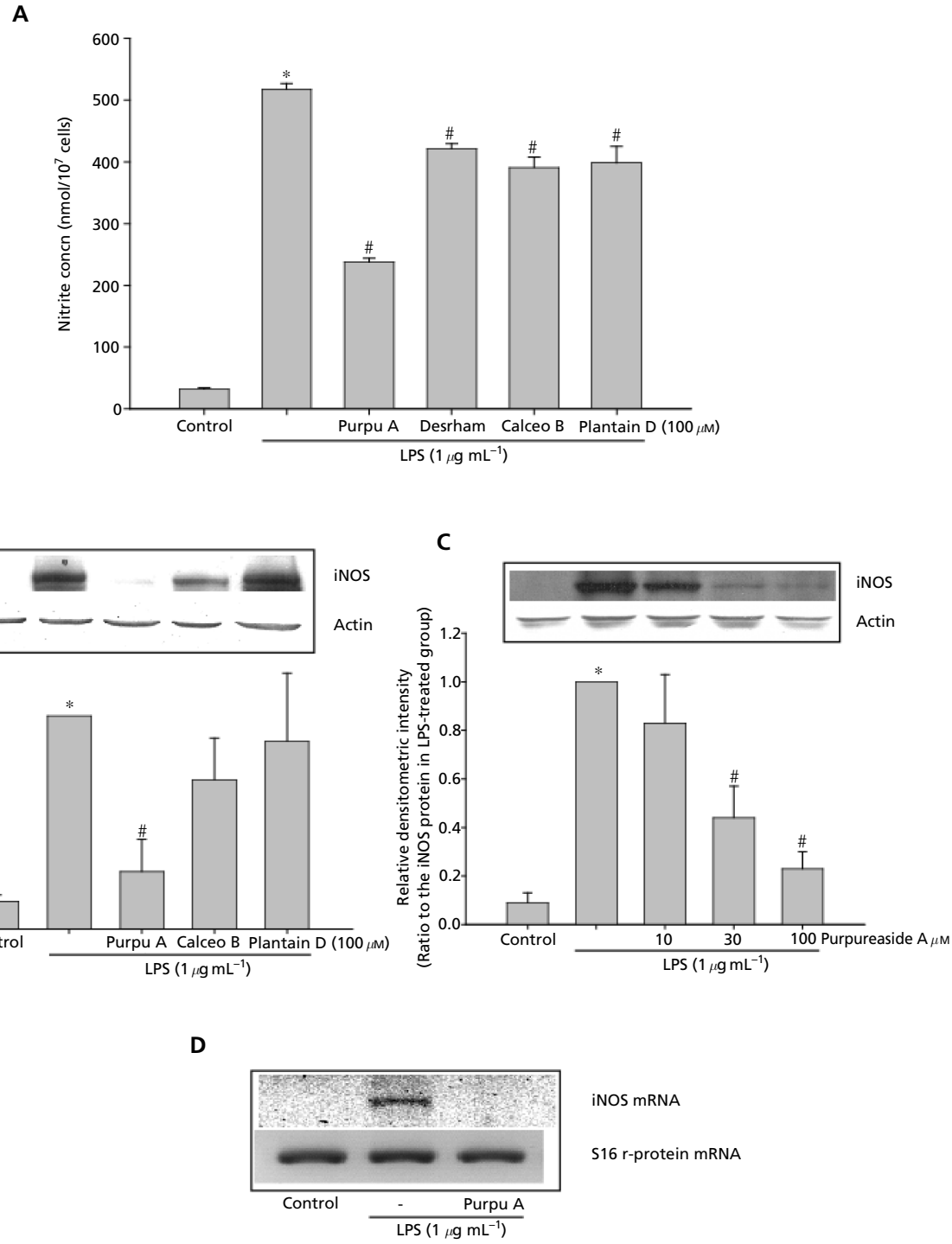

Figure 2 Effect of purpureaside A on the expression of iNOS. A. Effects of glycosides (100 μM, Purpu A, purpureaside A; Desrham, desrhamnosyl acteoside; Calceo B, calceolarioside B; Plantain D, plantainoside D) isolated from the leaves of *D. purpurea* on LPS (1 μg mL^{-1})-induced nitrite production. Data represent the mean ± s.e.m. of four different samples (*significant as compared with control; #significant as compared with LPS-treated group). B. Effects of digitalis glycosides (100 μM) on LPS (1 μg mL^{-1})-induced iNOS expression. The density ratios of iNOS to actin were calculated, and the density ratio in the LPS control group was set at 1.0. Each lane was loaded with 20 μg protein. Data represent the mean ± s.d. of three different experiments (*significant as compared with control; #significant as compared with LPS-treated group). C. Concentration-dependent inhibition of iNOS induction by purpureaside A (10–100 μM). Data represent the mean ± s.d. of three different experiments (*significant as compared with control; #significant as compared with LPS-treated group). D. iNOS mRNA expression was determined by RT-PCR. Cells were incubated in the presence of 100 μM purpureaside A for 10 min and then treated with LPS at 1 μg mL^{-1} for 4 h.

iNOS mRNA at 4 h, and 100 μM purpureaside A completely inhibited iNOS mRNA production (Figure 2D). The mRNA levels of S16 ribosomal protein were comparable in the samples (Figure 2D). These results indicated that purpureaside A potently inhibited iNOS mRNA increase by LPS, which was in agreement with the observed reduction in iNOS protein levels by the glycoside.

Effects on NF-κB activation by LPS

LPS and other inflammatory cytokines activate NF-κB, and the expression of iNOS is controlled by the transcription factor (Xie et al 1994). To determine whether the inhibition of iNOS induction by purpureaside A was associated with the suppression of NF-κB activation, a reporter gene assay was conducted using a luciferase plasmid containing NF-κB promoter. The exposure of Raw264.7 cells, transiently transfected with the plasmid, to LPS (18 h) resulted in a 2.7-fold increase in luciferase activity (Figure 3A). Moreover, pretreatment with 100 μM purpureaside A did not change the LPS-inducible increase in luciferase activity.

Since p65 was the major component of NF-κB activated by LPS in macrophages, we also examined p65

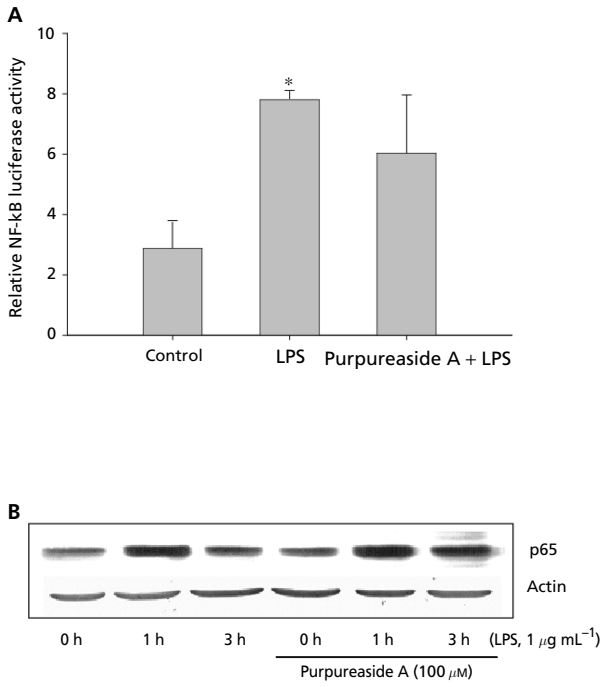

Figure 3 Effects of purpureaside A (100 μM) on the LPS (1 μg mL^{-1})-induced activation of NF-κB. A. NF-κB reporter gene analysis. Induction of luciferase activity by LPS in Raw264.7 cells transiently transfected with pNF-κB-luciferase construct was confirmed using a luminometer. Data represent the means ± s.d. of four separate experiments (*significant as compared with control). B. Effect of purpureaside A (100 μM) on the nuclear translocation of p65. Subcellular fractionation and subsequent Western blot analyses show nuclear p65 in cells exposed to LPS. Each lane was loaded with 15 μg protein and equal loading was confirmed by Ponceau-S staining. Results were confirmed by repeating experiments.

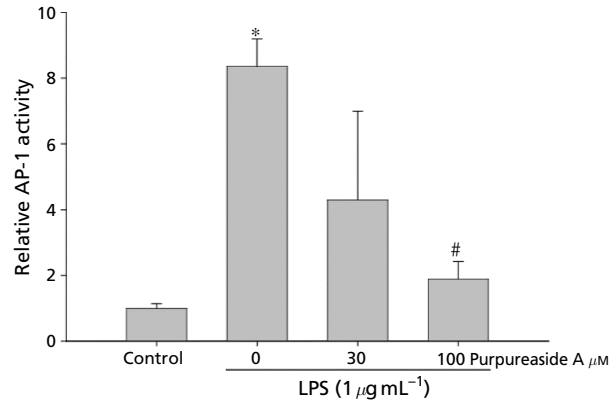

Figure 4 Effect of purpureaside A (30 or 100 μM) on the LPS (1 μg mL^{-1})-induced activation of AP-1. Data represent the means ± s.d. of four separate experiments (*significant as compared with control; #significant as compared with LPS-treated group).

translocation into the nucleus by subcellular fractionation and subsequent immunoblot analysis using an antibody immunoreactable to p65 protein. Immunoblot analysis showed that nuclear p65 protein levels increased from 1 to 3 h after treating cells with LPS (1 μg mL^{-1}) (Figure 3B) and pretreatment with purpureaside A did not affect the nuclear translocation of p65 stimulated by LPS (Figure 3B). These results suggested that the inhibition of iNOS induction by purpureaside A might not be related to NF-κB activation.

Effects on AP-1 activation by LPS

The expression of iNOS is controlled by the transcription factor AP-1 and by NF-κB (Xie et al 1993). The activation of AP-1 was assessed by reporter gene assay using a luciferase plasmid containing AP-1 promoter. LPS treatment (18 h) caused an 8.3-fold increase in luciferase activity (Figure 4). The pretreatment of cells with 30 or 100 μM purpureaside A completely blocked LPS-inducible AP-1 reporter activity (Figure 4). These results provided strong evidence that purpureaside A inhibited the activation of AP-1, which might be associated with the inhibitory effect of the glycoside on iNOS induction.

Discussion

Excessive pro-inflammatory cytokine and NO production by activated macrophages plays a critical role in severe inflammatory diseases such as sepsis (Szabo 1998). Rosselet et al (1998) reported that selective iNOS inhibition was better than noradrenaline (norepinephrine) for the treatment of endotoxic shock in rats. These reports suggested that the inhibition of pro-inflammatory cytokines and iNOS gene expression in inflammatory cells, including macrophages, offered a new therapeutic strategy against inflammation.

In this study, we isolated four different glycosides from *D. purpurea* and estimated their potential for blocking

iNOS induction in macrophage cells treated with LPS. We observed that purpureaside A inhibited the production of NO in LPS-stimulated Raw264.7 cells, whereas desrhamnosyl acteoside, calceolarioside B and plantainoside D only had attenuated suppressing effects. We found that the expression of iNOS protein was potently inhibited by purpureaside A, but was less or not affected by the other glycosides. In parallel with Western blot analysis, RT-PCR analysis revealed that purpureaside A completely blocked increased iNOS mRNA levels in cells treated with LPS. Hence, the mechanistic basis for the inhibition of NO formation by purpureaside A could be explained by the inhibition of the transcription of the iNOS gene by the glycoside.

The iNOS gene promoter contains several homologous consensus sequences for the binding of transcription factors, including NF-κB, AP-1, and C/EBP (Cieslik et al 2002; Lee et al 2003). Of these transcription factors, NF-κB and AP-1 are essentially required for the iNOS transcription (Xie et al 1993). NF-κB plays an important role in various inflammatory processes, including iNOS induction. The NF-κB heterodimer of p65 and p50 is located in the cytoplasm as an inactive complex bound to I-κBα. Activated NF-κB is transported into nuclei, where it binds to an NF-κB binding consensus sequence to activate the expression of target genes (Gilmore 1999). This study showed that purpureaside A did not block the nuclear translocation of p65 by LPS in macrophages. In addition, the glycoside did not suppress the increased reporter activity of NF-κB promoter in the lysates of cells treated with LPS. We examined the effect of purpureaside A on the activation of AP-1, because the promoter region of the iNOS gene contains a putative binding site for the transcription factor AP-1 (Lowenstein et al 1993). As shown in Figure 4, reporter gene analysis showed that the LPS-induced activation of AP-1 was completely blocked by purpureaside A. Thus, the inhibition of AP-1 activation by purpureaside A presented a possible mechanism for the iNOS blocking effect of the glycoside.

The aglycone structure (3, 4-dihydroxyphenylethyl alcohol) and functional residue (caffeic acid) of the four glycosides were the same. Hence, the differential NO inhibiting potency of the four digitalis glycosides would be due to the number of sugars or location of the caffeoyl group. It has been reported that 14-hydroxyhypocretenolide-β-D-glucoside-4′-14″-hydroxyhypocretenoate exhibited inhibitory effects on NO production in macrophages, whereas the different glucosides of the compound were not or only weakly active (Zidorn et al 1999). In addition, sugar conjugates of flavonoids can be taken up through the unique transporter (Walgren et al 2000) in cells and the uptake ratio is dependent on the type of sugar moiety. Hence, it would be possible that the sugar moiety of the *Digitalis* glycoside determined the ratio of cellular uptake and the pharmacological potency.

In summary, this study demonstrated that purpureaside A isolated from *Digitalis purpurea*, suppressed the LPS-induced production of nitric oxide in macrophages by inhibiting AP-1 activation. Since AP-1 is a critical transcription factor that regulates the formation of many pro-inflammatory proteins in activated macrophages during inflammatory processes, the inhibition of this transcription factor by purpureaside A could offer a potential therapeutic approach against severe inflammatory disease.

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