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Cudratricusxanthone A from *Cudrania tricuspidata* suppresses pro-inflammatory mediators through expression of anti-inflammatory heme oxygenase-1 in RAW264.7 macrophages

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ABSTRACT

Cudratricusxanthone A (CTXA), isolated from the roots of *Cudrania tricuspidata* Bureau (Moraceae) has an isoprenylated xanthone skeleton that is known to exert a variety of biological activities. In the present study, we demonstrated that CTXA inhibited cyclooxygenase-2 (COX-2) and inducible nitric oxide (NO) synthase (iNOS) expression, and thereby reduced COX-2-derived prostaglandin E2 (PGE_2) and iNOS-derived NO production in lipopolysaccharide (LPS)-stimulated macrophages. Similarly, CTXA suppressed tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) production. Moreover, CTXA inhibited the induced phosphorylation and degradation of IκB-α as well as the LPS-induced increase in p65 in the nuclear fraction of macrophages. CTXA also induced heme oxygenase-1 (HO-1) expression and increased heme oxygenase (HO) activity in RAW264.7 macrophages. We also demonstrated that the effects of CTXA on LPS-induced PGE_2, NO, TNF-α, and IL-1β production were partially reversed by the HO-1 inhibitor tin protoporphyrin, suggesting that CTXA-induced HO-1 expression was partly responsible for the resulting anti-inflammatory effects of the drug. Thus CTXA was shown to be an effective HO-1 inducer, capable of inhibiting macrophage-derived pro-inflammatory mediators.

1. Introduction

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme in heme catabolism, leading to the formation of carbon monoxide (CO), iron and biliverdin/bilirubin [1]. This enzyme has been shown to play a regulatory role as a potential therapeutic target in the treatment of inflammatory diseases [2]. HO-1 and its by-products are known to function in a wide range of processes that could be important during the resolution phase of inflammation, with macrophages acting as the critical target [3,4]. Studies have shown that HO-1 expression or CO treatment inhibits the production of pro-inflammatory cytokines and chemokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in activated macrophages [5–8]. Furthermore, the upregulation of HO-1 expression or administration of CO suppresses the expression of the pro-inflammatory cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), and thereby reduces COX-2-drived prostaglandin E_2 (PGE_2) and iNOS-derived nitric oxide (NO) production [9,10]. It is also well known that HO-1 and/or CO can inhibit iNOS expression and NO production in activated macrophages through the inactivation of nuclear factor (NF)-κB [4–12]. Owing to these studies, a number of therapeutic agents have been reported to upregulate the expression of HO-1 and exert anti-inflammatory activities through HO-1 induction [13–18].

The common names of *Cudrania tricuspidata* Bureau (Moraceae) are cudrang, mandarin melon berry and silkworm thron. The root bark of this species has been used as a traditional Chinese medicine for the treatment of lumbago, hemoptysis, and contusion [19]. Cudratricusxanthone A (CTXA), isolated from the roots of *C. tricuspidata* has potent hepatoprotective, anti-proliferative, and mono-amine oxidase inhibitory effects [20–22]. CTXA can also scavenge oxygen radicals such as hydroxyl radicals and superoxide [20,23]. Previously, we demonstrated that CTXA was able to protect mouse hippocampal cells against glutamate-induced neurotoxicity via the induction of HO-1 [24]. In the present study, we investigated whether CTXA could also downregulate the expression of pro-inflammatory COX-2 and inducible nitric oxide synthase in RAW264.7 macrophages. We further provided evidence to support the important role of HO-1 in mediation of the anti-inflammatory effects of CTXA.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco

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BRL Co. Tin protoporphyrin IX (SnPP IX), an inhibitor of HO activity, was obtained from Porphyrin Products (Logan, UT). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). unless indicated otherwise. CTXA was isolated from *C. tricuspidata* as described previously [20]. CTXA was obtained as yellowish solid, showed a $[M-H]^-$ peak at *m*/*z* 395.6 in the (−)-ESIMS, corresponding to a molecular formula of $C_{23}H_{24}O_6$. The purity of CTXA was checked by ^1H and ^{13}C NMR spectra, and its spectra showed highly pure signals without any other impurities. RAW264.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA). Primary antibodies, including HO-1, COX-2, iNOS, IκB-α, p-IκB-α, p65 and responsible secondary antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enzyme-linked immunosorbent assay (ELISA) kits for PGE_2, TNF-α, and IL 1-β were purchased from R&D system (Minneapolis, MN).

2.2. Cell culture and viability assay

RAW264.7 macrophages were maintained at 5×10^5 cells/mL in DMEM medium supplemented with 10% heat-inactivated FBS, penicillin G (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. For determination of cell viability, 50 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to 1 mL of cell suspension (1×10^5 cells/mL in 96-well plates) for 4 h, and the formazan formed was dissolved in acidic 2-propanol; optical density was measured at 590 nm.

2.3. Nitrite assay

The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent; absorbance of the mixture at 525 nm was determined with an ELISA plate reader.

2.4. PGE_2 assay

Macrophages were cultured in 24-well plates, pre-incubated for 12 h with different concentrations of CTXA and then stimulated for 18 h with LPS. One hundred microliters of supernatant of culture medium was collected for the determination of PGE_2 concentrations using the ELISA kit.

2.5. TNF-α and IL-1β assay

Macrophages were cultured in 24-well plates, pre-incubated for 12 h with different concentrations of CTXA and then stimulated for 18 h with LPS. The concentrations of TNF-α and IL-1β in the supernatant obtained from the culture medium were assayed using ELISA kits according to the manufacturer's instructions.

2.6. Preparation of cytosolic and nuclear fractions

Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS) buffer and kept on ice for 1 min. The suspension was mixed with buffer A (10 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin, and 10 mg/mL leupeptin) and lysed by three freeze–thaw cycles. Cytosolic fractions were obtained after centrifugation at 12,000 ×g for 20 min at 4 °C. The pellets were re-suspended in buffer C (20 mM HEPES, pH 7.5, 0.4 M NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin, and 10 mg/mL leupeptin), kept on ice for 40 min and then centrifuged at 14,000 ×g for 20 min at 4 °C. The resulting supernatant was used as soluble nuclear fractions.

2.7. Western blot analysis

Western blot analysis was performed by lysing cells in 20 mM Tris–HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethanesulfonyl fluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 1 mg/mL chymostatin). Protein concentration was determined using the Lowry protein assay kit (P5626; Sigma). An equal amount of protein for each sample was resolved using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Heracules, CA). The membrane was blocked with 5% skim milk and sequentially incubated with primary antibody (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ).

2.8. HO activity

The method used for the determination of HO activity follows the protocol published by Motterlini et al. [25]. Briefly, after the incubation process, the cells were washed twice with PBS, gently scraped off the dish, and centrifuged (1000 ×g for 10 min at 4 °C). The cell pellet was suspended in $MgCl_2$ (2 mM) phosphate (100 mM) buffer (pH 7.4), frozen at −70 °C, thawed 3 times, and finally sonicated on ice before centrifugation at 18,000 ×g for 10 min at 4 °C. The supernatant (400 L) was added to an NADPH-generating system containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate-L-dehydrogenase, and 2 mg protein of rat liver cytosol prepared from the 15,000 ×g supernatant fraction as a source of biliverdin reductase, potassium phosphate buffer (100 mM, pH 7.4), and hemin (10 M) in a final volume of 200 L. The reaction was conducted for 1 h at 37 °C in the dark and terminated by addition of 1 mL chloroform. The extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm using a quartz cuvette (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin).

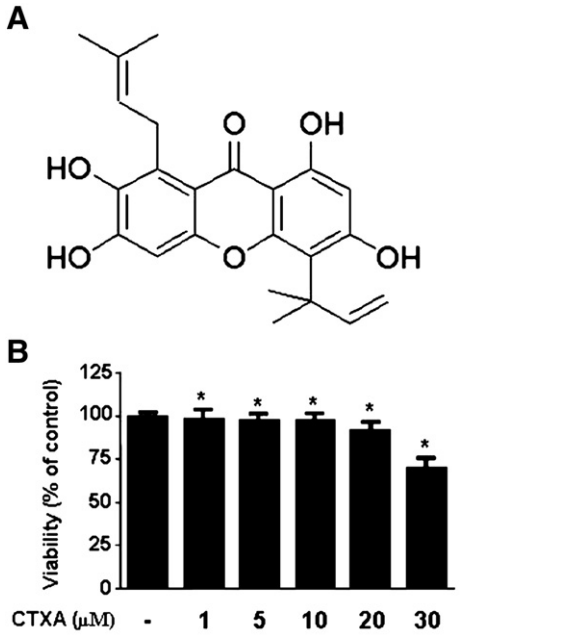

Fig. 1. Chemical structure of CTXA (A) and effects of CTXA on cell viability (B). RAW264.7 macrophages were incubated for 24 h with various concentrations of CTXA (1–30 μM). Cell viability was determined as described under Materials and methods. Data represent the mean values of three experiments ± SD. *$p<0.05$ compared to control.