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Dossier : Molecular and cellular effects of nutrients : global perspectives and modern aspects

Involvement of anti-inflammatory heme oxygenase-1 in the inhibitory effect of curcumin on the expression of pro-inflammatory inducible nitric oxide synthase in RAW264.7 macrophages

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Abstract

Curcumin, at high concentrations (>2 μM), inhibits the production of nitric oxide (NO) and the expression of inducible NO synthase (iNOS) through inactivation of nuclear factor (NF)-κB and, at low concentrations, induces the expression of heme oxygenase (HO)-1 in macrophages. Here, we demonstrated that curcumin at low concentrations (0.5–2 μM) can also inhibit NO production and iNOS expression in lipopolysaccharide (LPS)-activated RAW264.7 macrophages only when the cells were pretreated for at least 6 h with curcumin. Curcumin induced dose- and time-dependent HO-1 expression, and this was coincident with the inhibitory effects of low concentrations of curcumin on NO production and iNOS expression. Blockage of HO-1 activity or knockdown of HO-1 expression abolished the inhibitory effects of curcumin. Over-expression of HO-1 or exogenous addition of carbon monoxide, a byproduct derived from heme degradation, mimicked the inhibitory action of low concentrations of curcumin. Moreover, LPS-induced NF-κB was diminished in macrophages subjected to prolonged treatment with low concentrations of curcumin. Treatment with HO inhibitor abolished the inhibitory effect of curcumin on LPS-induced NF-κB activation. Collectively, we provide evidence to support the important role of HO-1 in inhibition of NO production and iNOS expression by curcumin even at low concentrations.

Keywords: Curcumin; Inducible nitric oxide synthase; Heme oxygenase-1; Nuclear factor-κB

1. Introduction

Inducible nitric oxide synthase (iNOS) is one of three key enzymes producing nitric oxide (NO) from the amino acid L-arginine. The iNOS-derived NO plays an important role in numerous physiological and pathological conditions, e.g., blood pressure regulation, inflammation, infection, and the onset and progression of malignant diseases [1]. The iNOS has been conjectured both as a marker and a therapeutic target in these situations. In macrophages, iNOS is a mediator of non-specific host defense and plays important roles in the clearance of bacterial, viral, fungal, and parasitic infections [2]. However, excess production of iNOS-derived NO by macrophages appears to be linked to tissue damage and organ dysfunction [3]. Recently, it has been shown that macrophages possess regulatory pathways where the regulatory mechanisms can operate to control pro-inflammatory responses via induction of the cytoprotective/anti-inflammatory enzymes and thus limit the destructive potential [4–6]. Among the cytoprotective/anti-inflammatory enzymes expressed in activated macrophages, inducible heme oxygenase (HO)-1 plays regulatory roles in the modulation of iNOS expression and NO

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production [7,8]. HO-1 is a rate-limiting enzyme in heme catabolism, leading to the formation of bilirubin, carbon monoxide (CO), and free iron [9]. This enzyme has been suggested to play a regulatory role in the resolution phase of inflammation and is considered as a potential therapeutic target for treating inflammatory diseases [10]. Moreover, it is well known that HO-1 and/or CO can inhibit iNOS expression and NO production in activated macrophages via inactivation of nuclear factor (NF)-κB [7–10].

Curcumin, the major constituent of the spice turmeric extracted from the root of *Curcuma longa* Linn, inhibits iNOS expression and NO production at least in part via direct interference with activation of NF-κB [11]. *In vitro*, curcumin inhibits iNOS expression and NO production by activated macrophages with an IC_{50} value of 6 μM [12]. Curcumin undergoes rapid metabolism by conjugation and reduction pathways [13]. The concentrations of curcumin achieved in plasma and target tissues are low (about 2 μM), probably due to its extensive metabolism [14]. Interestingly, curcumin at these low concentrations can induce in vitro HO-1 expression in macrophages [15]. Although a previous study has demonstrated that curcumin inhibits iNOS expression and NO production in activated macrophages through direct inhibition of NF-κB activation [11], the concentrations required for this activity are much higher than those detected in both human and animal plasma [14]. Nevertheless, a study using animal models has demonstrated *in vivo* the inhibitory effects of curcumin on iNOS expression and NO production [16]. These reports give rise to a possibility that curcumin at lower concentrations (about 2 μM) may inhibit iNOS expression and NO production through alternate mechanism(s).

In the present study, we assessed the effects of curcumin at low concentrations on iNOS expression and NO production in lipopolysaccharide (LPS)-activated RAW264.7 macrophages, and provided evidence to support the important role of HO-1 in inhibition of iNOS expression and NO production by curcumin even at low concentrations.

2. Materials and methods

2.1. Reagents and chemicals

Curcumin (>95% pure) was isolated from the rhizomes of turmeric, as described earlier [17]. Tricarbonyl dichlororuthenium (II) dimmer (RuCO), LPS, bilirubin, Dulbecco's modified Eagle's medium (DMEM), 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), hemin and ferrous citrate were purchased from Sigma–Aldrich (St. Louis, MO). Copper protoporphyrin (CoPP) and zinc protoporphyrin (ZnPP) were obtained from Porphyrin Products (Logan, UT). HO-1 antibody was obtained from Calbiochem (La Jolla, CA). Lipofectamine 2000™ was purchased from Invitrogen Life Technologies (Grand Island, NY). Small interfering RNA (siRNA) for HO-1 and antibodies to iNOS, phosphor (p)-IκBα, IκBα, p65, lammin B and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were obtained from Sigma-Aldrich. HO-1 cDNA was a kind gift from Dr. A.M.K. Choi (University of Pittsburgh, Pittsburgh, PA).

2.2. Cell culture and viability

RAW264.7 macrophages obtained from American Type Culture Collection (Manassas, VA) were cultured in DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell viability was determined by a modified MTT reduction assay, as described previously [18].

2.3. Nitrite assay

Nitrite production in the medium was assessed by measuring nitrite/nitrate, the stable degradation products of NO. The culture medium was incubated with coenzyme at 37 °C for 15 min. Next, the reaction mixture was incubated with nitrate reductase (Sigma–Aldrich) at 37 °C for 30 min. The Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) was added to the reaction mixture. The absorbance of the mixture at 530 nm was determined using a microplate reader, and nitrite concentration was determined using a dilution of sodium nitrite as a standard.

2.4. Western blot analysis

After treatment, cells were harvested, washed twice with ice-cold phosphate buffered saline (PBS), lysed with lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M sodium dodecyl sulfate (SDS), 0.3 mM bromophenol blue), and boiled for 5 min. Protein content was measured with BCA protein assay reagent (Pierce, Rockford, IL). The samples were diluted with lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein (20 μg of protein) were separated on 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBS-T) for 1 h, and incubated with antibodies against HO-1, p-IκBα, IκBα and p65 in PBS-T containing 1% nonfat milk for 4 h. After washing three times with PBS-T, the membranes were hybridized with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 40 min. Following triplicate washes with PBS-T, they were incubated with chemiluminescent solution for 5 min, and protein bands were visualized on X-ray film.

2.5. Preparation of cytosolic and nuclear fractions

Cells were harvested, washed ice-cold 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 μg/ml aprotinin, 5 μg/ml pepstatin, and 10 μg/ml leupeptin) and lysed by three freeze–thaw cycles. Cytosolic fractions were obtained by centrifugation at 12,000 × *g* for