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ScienceDirect
Life Sciences 80 (2007) 1420–1430
Life Sciences
www.elsevier.com/locate/lifescie

Chalcone inhibits the activation of NF-κB and STAT3 in endothelial cells via endogenous electrophile

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Received 6 September 2006; accepted 27 December 2006

Abstract

Chalcone, an α,β-unsaturated flavonoid, possesses anti-inflammatory properties. In our present study, we have demonstrated chalcone inhibited IL-6- and LPS-induced ICAM-1 gene expression. In adhesion assay, chalcone reduced the LPS-induced adhesion of THP-1 cells to endothelial cells (ECs). Chalcone was found to abrogate the activation of STAT3 and NF-κB in a dose- and time-dependent manner, in IL-6- and LPS-treated ECs. Other flavonoids, quercetin and cyanidin, which lack α,β-unsaturated carbonyl group, showed weaker or no inhibitory effect on both IL-6-induced STAT3 phosphorylation and LPS-induced p65 translocation. However, the electrophilic compounds curcumin and crotonaldehyde, which also contain an α,β-unsaturated carbonyl moiety, mimic the inhibitory effects of chalcone with different efficiencies. In addition, *N*-acetyl-L-cysteine (NAC) could reverse the inhibition of STAT3 phosphorylation when preincubated with chalcone. The use of buthionine sulfoximine (BSO) to decrease intracellular GSH levels further enhanced the effects of chalcone. On the other hand, in ECs treated with BSO only no abrogation of IL-6-induced STAT3 phosphorylation was observed. We also found that chalcone could reduce the GSH level *in vitro*. Furthermore, the cellular GSH levels were rapidly reduced after 25 μM chalcone treatment. Following 6 h exposure, however, chalcone treatment rescued the GSH levels in ECs, coincident with the inhibition of STAT3 and NF-κB activation. In contrast, chalcone induced expression of thioredoxin reductase and heme-oxygenase genes after prolonged treatment. Furthermore, chalcone upregulated the levels of the transcription factor Nrf2 in nuclear extracts and increased antioxidant response element (ARE)-luciferase activity and thioredoxin reductase promoter activity. Hence, our present findings indicate that chalcone suppresses both IL-6- and LPS-induced signaling pathways through the thiol-dependent intracellular redox state. In addition, chalcone may provide distinct cytoprotective effects at different durations of pretreatment.

Keywords: Chalcone; ICAM-1; STAT3; NF-κB; Glutathione; Electrophile

Introduction

Atherogenesis is a chronic inflammatory response (Ross, 1993) and many pathophysiologic studies have shown that the first step in its development is the adhesion of monocytes to the vascular endothelium. The recruitment and subsequent migration of leukocytes to the site of inflammation is in part regulated by the major adhesion molecule, ICAM-1, on the surface of the vascular endothelium (Poston et al., 1992). Cytokines have been suggested to play a key role in monocyte/endothelial cell interactions and thus contribute to atherosclerosis (Erren et al., 1999; Libby, 2002). A previous report has indicated that ICAM-1 is upregulated when endothelial cells (ECs) are exposed to various inflammatory cytokines (Van de Stolpe and Van der Saag, 1996). A previous study from this laboratory further demonstrated that the induction mechanisms underlying ICAM-1 expression are distinct, in which ICAM-1 activation by tumor necrosis factor-α (TNFα) and interleukin (IL)-6 is mediated via nuclear factor-κB (NF-κB) and activator of transcription-3 (Stat3), respectively (Wung et al., 2005b).

Chalcone and its derivatives have been identified in *Angelica keiskei* (Akihisa et al., 2003), a Japanese plant which is traditionally used as a vegetable and that has diuretic, laxative, analeptic and lactagogue effects (Baba et al., 1998). Chalcone and its derivatives have been reported to possess various pharmacological activities, including anti-inflammatory (Hsieh et al., 1998) anti-cancer (Zhu et al., 2005) and anti-oxidant (Anto

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0024-3205/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2006.12.040

et al., 1995) actions. The anti-inflammatory effects of chalcones have been further shown to regulate NO and cytokine production in macrophages (Alcaraz et al., 2004; Ban et al., 2004), and prevent TNF-α- and lipopolysaccharide (LPS)-induced neutrophil adhesion (Madan et al., 2000). In addition, it has also been shown that chalcone suppresses the activity of cyclo-oxygenase-2 and 5-lipoxygenase (Araico et al., 2006). A number of recent studies have indicated that the anti-inflammatory effect of chalcones is due to the inhibition of the NF-κB pathway, which mediated by IκB degradation and the phosphorylation of c-*Jun* N-terminal kinase (JNK) and c-*Jun*. (Lee et al., 2006; Ban et al., 2004). Nevertheless, to date it is not clear if chalcone is also functionally related to other inflammatory cytokine-induced single pathways and gene expression. Because chalcone has been found to be pharmacologically important, we were interested in further elucidating its mechanism of chalcone on ICAM-1 induction in endothelial cells.

Chalcone possesses a highly electrophilic α,β-unsaturated carbonyl moiety, which has been reported to result in Nrf2 activation and the induction of phase II detoxifying enzyme expression (Foresti et al., 2005; Wu et al., 2006a). It has also been shown that this moiety can act as an electrophile and react with free sulfhydryl groups of thioredoxin and cysteine residues in proteins (Shibata et al., 2003). Foresti et al. (2005) indicated that electrophilic phytochemicals could give rise to thiyl radicals. The electrophile-induced thiyl radical could also interact with sulfhydryl residues of intracellular targets, including NF-κB (Heiss et al., 2001; Rossi et al., 2000; Cernuda-Morollón et al., 2001). Our recent data indicated that the electrophilic activity of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) plays a crucial role in its anti-inflammatory effects (Wung et al., 2006b). We also found that an electrophilic toxin, acrolein could induce heme-oxygenase (HO-1) expression at sublethal concentrations (Wu et al., 2006a). In the present study, we propose that the endogenous electrophilic activity, through its α,β-unsaturated carbonyl moiety, is involved in the anti-inflammatory properties of chalcone. Our present findings therefore provide new insights into the anti-inflammatory mechanisms of chalcone.

Materials and methods

Materials

Bacterially derived IL-6 was purchased from Calbiochem (San Diego, CA.). The ICAM-1 promoter (I540-Luc) was generated by PCR, as described previously (Wung et al., 2005b). The p3xARE/Luc vector was also constructed as described previously (Wu et al., 2006b). Antibodies raised against native STAT3 and STAT3 phosphorylated on tyrosine 705, and against p65 and Nrf2, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL reagents were purchased from Pierce (Rockford, IL, USA). Luciferase assay kits were purchased from Promega (Madison, WI, USA). Peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Amersham (Arlington Heights, IL, USA). Nitrocellulose was obtained from Schleicher & Schuell (Dassel, Germany). All other reagents were purchased from Sigma (St. Louis, MO, USA).

Endothelial cell cultures

Bovine aortic endothelial cells (BAECs) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of air and 5% CO_2. Cells were grown in Petri dishes for 3 days and allowed to reach confluence (Wung et al., 1997). The culture medium was then replaced with serum-free DMEM and the cells were incubated for 12 h prior to experimental treatment.

Coculture and in vitro monocyte adhesion assay

The treatment with chalcone might inhibit monocyte recruitment. To test this, LPS-stimulated BAECs were pretreated with chalcone for 1 h prior to the 24 h exposure to 500 ng/ml LPS (Go and Jones, 2005). Human monocytic leukemic cells of the THP-1 cell line (American Type Culture Collection) were labeled with 5 μM calcein AM (Molecular Probes Inc.) in RPMI 1640 medium containing 10% FBS. In the coculture system, the labeled THP-1 cells were seeded at a density of 5.0×10^5 cells onto ECs and incubated for 2 h. The quantitative results were obtained by using a Fluoroscan enzyme-linked immunosorbent assay (ELISA) plate reader (FLx800, Bio-Tek, USA) at $\lambda=485$ nm excitation and $\lambda=538$ nm emission (Braut-Boucher et al., 1995).

RNA isolation and northern blot analysis

Total RNA was isolated from ECs using a GIT/phenol/chloroform method. RNA samples were separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane by vacuum blotting. After hybridization with ^{32}P-labeled ICAM-1 cDNA probes, the results were developed by autoradiography.

Plasmids, transfections and luciferase assays

Two oligonucleotide primers, TTAAAAGCTTCTCT-GAGTTTTTCTT and 5′-AATGCTCGAGTGAAGAAAACT-GAGG, were designed based on the human TrxR1 promoter sequence, which has been described in detail elsewhere (Sakurai et al., 2005). These primers were used in PCR reactions with Taq polymerase using normal human genomic DNA as template. The resulting PCR product of 646 bp, incorporating the nucleotide positions −646nt to +49 of the transcription initiation site, was subcloned into the TA cloning PCR II vector (Invitrogen, Leek, The Netherlands). This insert was then subcloned into the luciferase reporter gene vector PGL3-basic (Promega, Madison, WI, USA). All transfection experiments were performed using lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For luciferase assays, the cell lysate was first mixed with luciferase substrate solution (Promega), and the resulting luciferase activity was measured using a luminometer. For each experiment, luciferase activity was determined in triplicate and normalized for each sample by β-galactosidase activity.