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Carnosic acid, a pro-electrophilic compound, inhibits LPS-induced activation of microglia

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ABSTRACT

In the previous studies, we reported that carnosic acid (CA) protects cortical neurons by activating the Keap1/Nrf2 pathway, which activation is initiated by *S*-alkylation of the critical cysteine thiol of the Keap1 protein by the "electrophilic" *quinone*-type CA. Here, we found that the pro-electrophilic CA inhibited the *in vitro* lipopolysaccharide (LPS)-induced activation of cells of the mouse microglial cell line MG6. LPS induced the expression of IL-1β and IL-6, typical inflammatory cytokines released from microglial cells. CA inhibited the NO production associated with a decrease in the level of inducible NO synthase. Neither CA nor LPS affected cell survival at the concentrations used here. These actions of CA seemed to be mediated by induction of phase 2 genes (gclc, gclm, nqo1 and xct). We propose that an inducer of phase 2 genes may be a critical regulator of microglial activation. Thus, CA is a unique pro-electrophilic compound that provides both a protective effect on neurons and an anti-inflammatory one on microglia through induction of phase 2 genes.

1. Introduction

Microglia are the resident innate immune cells in the CNS and make up approximately 12% of the total number of cells in the brain [1,2]. In response to inflammatory triggers such as amyloid-β and lipopolysaccharide (LPS), microglial cells are readily activated and undergo dramatic morphological and physiological transformations [1,2]. Hyperactivation of microglia may result in deleterious and neurotoxic consequences by excessive production of pro-inflammatory mediators [1,2]. Reactive oxygen species generated by microglia help to eliminate pathogens in the extracellular milieu but also act on the microglia themselves, altering the intracellular redox balance and functioning as second messengers in the induction of proinflammatory genes [2]. Recent findings indicate that restoration of the proper redox balance may be a determinant in driving microglia back to the resting state [2]. Thus, activation of the transcription factor NF-E2-related factor-2 (Nrf2) [3,4], a master regulator of cellular redox homeostasis in microglial cells, results in the inhibition of inflammatory responses in the brain [5,6].

Electrophiles modulate a variety of cellular signaling processes involving several major anti-inflammatory and protective components via activation of the Nrf2/ARE pathway [5–9]. Electrophilic naturally-occurring products such as curcumin [5], sulforaphane [6], and carnosic acid (CA) [10] can act in this capacity by regulating redox reactions and phase 2 enzymatic activities [7–10]. In this present study, we focused on carnosic acid (CA) [10–13]. CA potently activates the Keap1/Nrf2 pathway, which regulates the cellular redox state, in several cells including neuronal, astroglial, and preadipocytic ones [10–13]. The Keap1/Nrf2 pathway comprises Keap1, a regulator protein, and Nrf2, a transcriptional factor that binds to the ARE [7–9]. Keap1 is an adapter protein for the ubiquitination of Nrf2 and thus drives the continuous degradation of this transcription factor [7–9]. When electrophiles such as NEurite outgrowth-Promoting Prostaglandin11 (NEPP11) [14], *tert*-butyl hyroquinone (TBHQ) [15] or CA [10] react with critical

Abbreviations: ARE, antioxidant-response element; CA, carnosic acid; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; gclc, glutamate-cysteine ligase, catalytic subunit; gclm, glutamyl cysteine ligase modifier subunit; HO-1, hemeoxygenase-1; iNOS, inducible nitric oxide synthase; IL, interleukin; LPS, lipopolysaccharide; PBS, Ca^{2+}, Mg^{2+}, (−)-phosphate-buffered saline; NEPP11, NEurite outgrowth-Promoting Prostaglandin11; NO, nitric oxide; nqo1, NADPH quinone oxidoreductase 1; Nrf2, NF-E2-related factor-2; PAT, pathologically-activated therapeutic; PED, pro-electrophilic drug; RT-PCR, reverse transcription polymerase chain reaction; TBHQ, *tert*-butyl hydroquinone; WST, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt; xct, Na^{+}-independent cystine-glutamate exchanger.

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cysteine residues on the Keap1 protein to form an adduct (*S*-alkylation), they perturb this system and stabilize Nrf2, thus allowing it to be translocated from the cytoplasm into the nucleus, where it binds to AREs and stimulates the expression of phase 2 genes. Here, we examined the effects of CA on microglial activation *in vitro* and found that CA inhibited microglial activation possibly through induction of phase 2 enzymes.

2. Materials and methods

2.1. Chemicals

The final concentration of DMSO in the culture medium was 0.1%. CA and LPS (L8274, *Escherichia* coli 026:B6-derived) were obtained from Sigma–Aldrich Chemical Co., Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine calf serum, and penicillin–streptomycin came from Life Technologies (Carlsbad, CA).

2.2. Cell culture

MG6 cells [16], obtained from RIKEN Bioresource Center, were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with 100 μM β-mercaptoethanol, 10 μg/ml insulin, 100 μg/ml streptomycin, and 100 U/ml penicillin in 100-mm Petri dishes (BD Falcon, Franklin Lakes, NJ). These cells are often used as an *in vitro* model of microglial activation [17–20] The MG6 cells were seeded in 24-well plates at a density of 1×10^5 cells/cm^2 in normal DMEM medium. The next day, after one wash with PBS, the medium was replaced with 500 μl of serum-free DMEM containing 0.02% BSA and 10 μg/ml LPS without or with CA at the indicated concentrations. After a 24-h incubation at 37 °C, the cells were subjected to the following procedures: the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST)-8 assay to assess viability, nitric oxide (NO) assay, Western blotting, and reverse transcription-polymerase chain reaction (RT-PCR), as described elsewhere [17–20].

2.3. WST-8 and NO assays

The viability and NO production of sister cultures of MG6 cells were determined by using the WST-8 assay (DOJINDO, Tokyo, Japan) and Griess reagent (Invitrogen, Carlsbad, CA), respectively. For the WST-8 assay, 10 μl of WST-8 was added to each well 1 h before spectrophotometric evaluation at 450 nm with a microplate spectrophotometer [21]. The generation of NO was determined by measuring the nitrite accumulation in the medium with modified Griess reagent. The culture supernatant and Griess reagent were mixed and incubated for 5 min, and subsequently the absorption was determined at 540 nm (SLT reader 340 ATTC). Sodium nitrite ($NaNO_2$) was used to generate a standard curve for quantitation [22].

2.4. Western blot analysis

The medium (250 μl) of MG6 cell cultures was collected into 1.5-ml tubes and briefly centrifuged to remove cellular debris, and the cells were lysed with 250 μl RIPA buffer. Equal volumes (20 μl) of supernatant (for interleukin-6 (IL-6) detection) or lysate (for β-actin detection) were separated by SDS–PAGE (12%) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon™-P, MILLIPORE, Bedford, MA). The membranes were blocked for 1 h with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). For detection of IL-6, the membranes were incubated with biotinylated anti-IL-6 (BD Pharmingen, San Diego, CA) antibody (1:1000) for 1 h followed by incubation with HRP–streptavidin conjugate (1:5000; Zymed, South San Francisco, CA) for 20 min. To detect β-actin, we incubated membranes with anti-β-actin antibody (1:5000; Chemicon, Temecula, CA) in TBST for 1 h followed by HRP-conjugated goat anti-mouse antibody (1:10,000) for 1 h. The membranes were washed three times with TBST after incubation with each antibody. The target proteins were detected by using an ECL Plus kit (GE Healthcare, Piscataway, NJ) and X-ray film.

2.5. RT-PCR

For RT-PCR analysis, total RNA of MG6 cells was obtained at 24 h of treatment with CA and/or LPS by use of TRIzol Reagent (Invitrogen). cDNA was synthesized by using the Superscript III system (Invitrogen). One one-hundredth of the cDNA was used for one PCR reaction. At the completion of the PCR, 10 μl of PCR products was mixed with 2 μl of loading buffer and electrophoresed in 1.5% agarose gel in the presence of 0.5 μg/ml of ethidium bromide. The following primers were used to amplify the cDNA fragments, which were visualized with a UV transilluminator:

β-actin: 287 bp
Forward 5′-ATC CGT AAA GAC CTC TAT GC-3′
Reverse 5′-AAC GCA GCT CAG TAA CAG TC-3′
Hemeoxygenase-1 (ho-1: 617 bp)
Forward 5′-AGG TGT CCA GAG AAG GCT T-3′
Reverse 5′-ATC TTG CAC CAG GCT AGC A-3′
Interleukin-1β (ilβ:152 bp)
Forward 5′-CAA CCA ACA AGT GAT ATT CTC CAT-3′
Reverse 5′-GAT CCA CAC TCT CCA GCT GCA GGG-3′
Inducible nitric oxide synthase (iNOS:95 bp)
Forward 5′-CAG CTG GGC TGT ACA AAC CTT-3′
Reverse 5′-CAT TGG AAG TGA AGC GTT TCG-3′
Glutamate-cysteine ligase, catalytic subunit (gclc: 561 bp)
Forward 5′-GTG GAG TAC ATG TTG GTG TC-3′
Reverse 5′-GTA GAT ATG GTC TGG CTG AG-3′
Glutamyl cysteine ligase modifier subunit (gclm: 422 bp)
Forward 5′-TTG GAG TTG CAC AGC TGG AC-3′
Reverse 5′-CCT GCT CTT CAC GAT GAC C-3′
NADPH quinone oxidoreductase 1 (nqo-1: 354 bp)
Forward 5′-CTC CAT GTA CTC TCT TCA GG-3′
Reverse 5′-TTG ATC TGG TTG TCA GCT GG-3′
Na+-independent cystine-glutamate exchanger (xct: 393 bp)
Forward 5′-TCA AGC TCG TGA CAG CTG TG-3′
Reverse 5′-GGA GTG TGC TTG TGG ACA TG-3′

2.6. Statistical analysis

Results were presented as means ± standard deviation (SD). Data were analyzed by using SAS software. Analysis of variance was performed by use of *t*-test procedures.

3. Results

3.1. Inhibition of NO production without an effect on cell survival

In order to confirm the absence of cytotoxicity at the concentrations used in the present study, we exposed MG6 cells to different concentrations of CA and LPS (applied alone or in combination). After a 24-h incubation with different CA concentrations (0, 1.0, 3.0, and 10.0 μM), the viability of the cells was determined by performing the standard WST-8 assay. As shown in Fig. 1A, the application of CA for 24 h at the indicated concentrations did not affect the viability of the cells, as indicated by the stable metabolic

activity. Furthermore, the application of LPS (10 μg/ml) alone or together with different CA concentrations did not affect the cell viability. Thus, none of the conditions used in the present study affected cell survival. A hallmark of microglial activation is the production of NO in response to LPS. Thus, we determine whether or not CA could modulate NO production in LPS-activated MG6 cells. As shown in Fig. 1B, LPS induced a strong increase in NO production in the MG6 cells 24 h after LPS-stimulation compared with the control level. CA depressed the LPS-induced increase in NO in a dose-dependent manner.

3.2. CA-mediated inhibition of production of IL-6, IL-1β and iNOS

We next analyzed the influence of CA on the expression of IL-6, a typical pro-inflammatory cytokine of MG6 cells after incubation with LPS [16–20]. The cells were treated or not with LPS at 10 μg/ml after pre-treatment or not with CA for 30 min IL-6 protein released into the culture medium was analyzed by Western blotting. As shown in Fig. 2A, the amount of IL-6 release by the non LPS-treated MG6 cells was very low. LPS treatment significantly increased the level of IL-6 in the supernatant. The presence of various concentrations (0, 1.0, 3.0, and 10.0 μM) of CA decreased the level of IL-6 in a dose-dependent manner (Fig. 2A and B). Next, we examined the effect of CA on other pro-inflammatory factors (IL-1β and iNOS) by performing RT-PCR (Fig. 2C). In the absence of LPS, the cells expressed very low levels of these genes; but in its presence there was a significant increase in their expression. CA at 10 μM partially decreased the mRNA levels of IL-1β and iNOS (Fig. 2C). These results suggest that CA inhibited the inflammatory response of the microglial cells activated by LPS.

3.3. Phase 2 induction

In order to examine the involvement of the Nrf2/ARE pathway, we performed RT-PCR analysis using primers for typical phase 2 enzymes (Fig. 3A). In the absence of LPS, CA induced the expression

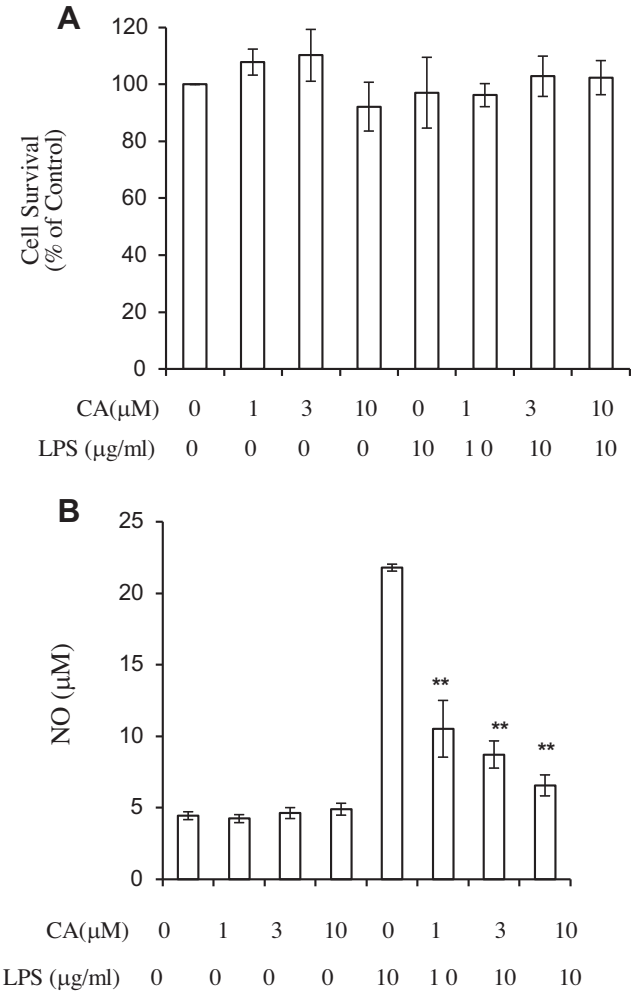

Fig. 1. (A) Cell survival. MG6 cells were incubated for 24 h with the various combinations of CA and LPS indicated in the figure. Cell viability was determined by using the WST-8 assay. Note that the viability of none of the treated cells was significantly ($p < 0.05$) different from the control. (B) NO production. LPS-induced NO production was inhibited by CA in MG6 cells. MG6 cells were treated for 24 h with the combinations of LPS and CA shown in the figure, and NO concentrations were then determined by use of the Griess reagent. The values are the mean ± SD from four separate measurements. **Significant difference ($p < 0.01$) compared with the LPS 10 μg/ml and CA 0 μM-treated cells.

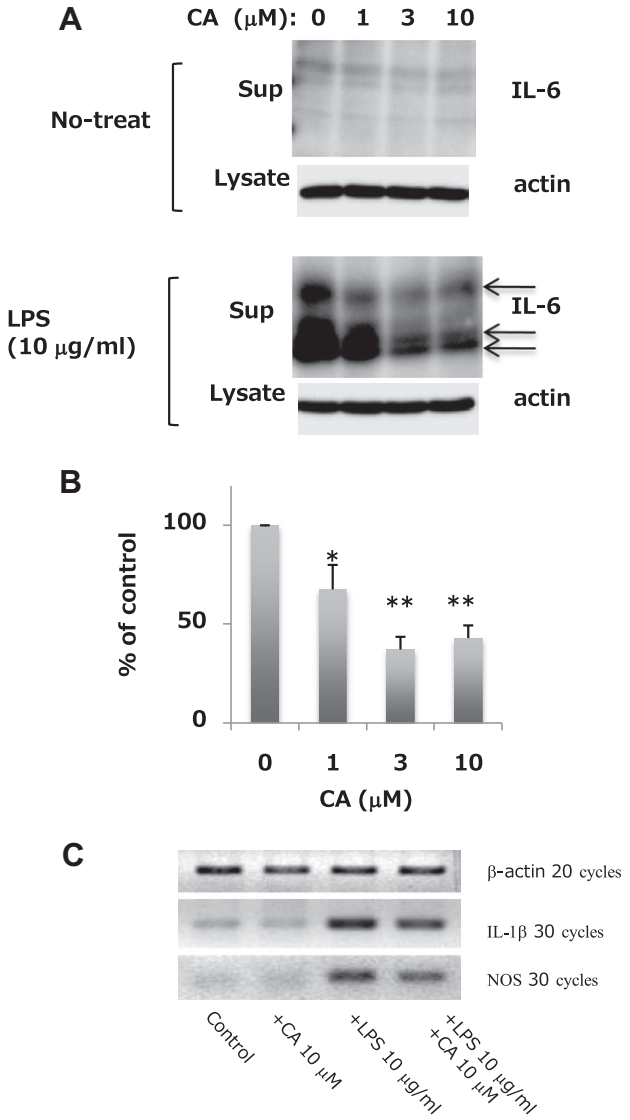

Fig. 2. (A and B) CA inhibited LPS-elicited IL-6 production detected by Western blotting (A) and densitometric quantitation of the bands (B). MG6 cells were treated with 10 μg/ml LPS or not with and without CA at the indicated concentrations for 24 h. The IL-6 released into the supernatant was determined by Western blotting. The values are the mean ± SD in "B". *,**Significant difference ($p < 0.05$ and 0.01, respectively) compared with the LPS 10 μg/ml and CA 0 μM-treated cells. (C) CA partially inhibited LPS-elicited IL-1β and iNOS production detected by RT-PCR. MG6 cells were left untreated or treated with CA (10 μM), LPS (10 μg/ml) or both for 24 h. The levels of IL-1β and iNOS mRNAs were determined by RT-PCR.

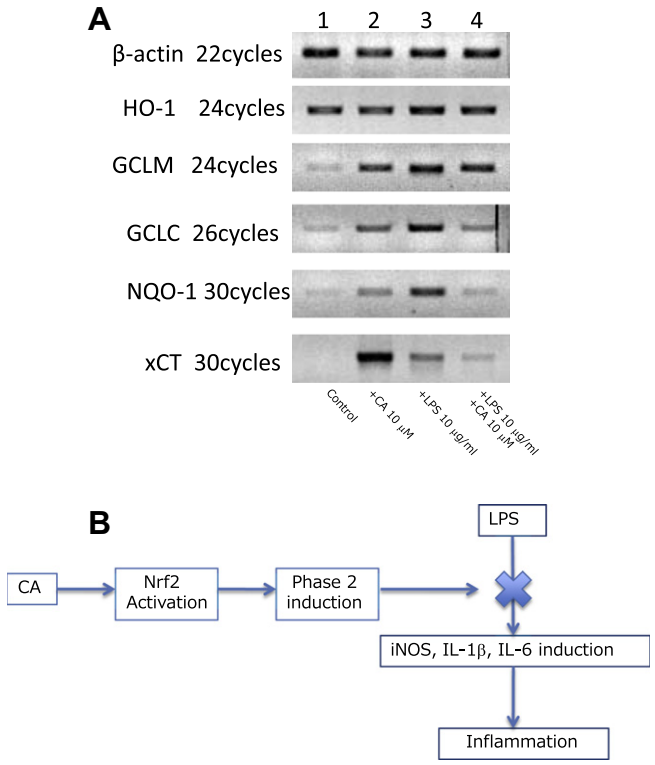

Fig. 3. (A) Induction of phase-2 genes by CA. MG6 cells were left untreated or treated with CA (10 μM), LPS (10 μg/ml) or both for 24 h. Total RNA was extracted from the cells and subjected to RT-PCR by using the primer sets for β-actin, ho-1, gclm, nqo-1, gclc and xct genes. Aliquots of the reaction products were subjected to electrophoresis after PCR amplification for the indicated number of cycles. (B) Proposed mechanism of the inhibition of microglial activation by CA.

of the genes of phase 2 enzymes (gclc, gclm, nqo-1 and xct). However, we could not see a clear induction of the gene of ho-1 because of high level of expression of control cells. In contrast, in the presence of LPS, all of the phase 2 genes are up-regulated even without CA (3rd lane). Although the genes of ho-1 and gclm maintained high level of expression, those of nqo-1 and xct were down-regulated by the presence of CA (4th lane).

4. Discussion

As shown in Fig. 3B, we propose the phase 2 induction-dependent CA-mediated inhibition of inflammatory actions of microglial cells. The induction of phase 2 genes greatly activates the redox-regulating mechanism of microglial cells, thus reducing the inflammatory action of these cells [7–14]. Because CA activated the Keap1/Nrf2 pathway in various biological systems and the activation leads to anti-inflammation [3–6], neuroprotection [9–12,23,24] and anti-obesity [13], we consider that the pathway is essential for the inhibition of microglial activation by CA. In the present study CA decreased the release of NO from LPS-stimulated microglia in a concentration-dependent manner and attenuated the LPS-induced increase in IL-1β, IL-6, and iNOS expression in MG6 cells. In order to examined whether CA could activate ARE in MG6 cells, we conducted a luciferase assay using MG6 cells transfected with ARE(GSTYa)-luciferase expression vector. However, we gave up the assay because the transfection efficiency was very low in MG6 cells by unknown reason (data not shown).

CA has a quite distinctive point [10] from other electrophiles such as curcumin and sulforaphane, both of which are also reported to inhibit microglial activation [25,26]. CA is not itself electrophilic but become electrophilic via oxidative conversion to its *quinone* form, which conversion occurs when CA encounters free radicals generated by cellular oxidative stress [10]. Thus, the *hydroquinone-type* compounds function as pro-electrophiles, which require conversion from *hydroquinone* to *quinone* in order to exert their anti-inflammatory effect [10]. We refer to these compounds as "pro-electrophilic drugs (PEDs)" [9,10]. The concept of PEDs may provide us with an important framework for the development of improved anti-inflammatory drugs that become electrophilic when they encounter oxidative damage [9,10]. Importantly, these PED compounds remain relatively innocuous in their pro-electrophilic form and only become active at the site of oxidative stress when converted from the hydroquinone to the quinone [9,10,27]. These compounds therefore represent a type of pathologically-activated therapeutic (PAT) drug [9,10,27]. As shown here, we believe that this chemical conversion is the key to understanding the molecular mechanism of the protective yet well-tolerated effects afforded by *hydroquinone-type* electrophilic compounds. There are two factors that affect this oxidation, i.e., the presence of copper ions and an electron acceptor, such as oxygen or oxygen radicals [28]. Thus, it is likely that this process is enhanced under oxidative stress. It would be expected that the more severe the oxidative stress, under which microglias are more activated [2], the more effective the conversion of a PED to the active electrophile [27,28].

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