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Broussochalcone A, a potent antioxidant and effective suppressor of inducible nitric oxide synthase in lipopolysaccharide-activated macrophages

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Abstract

The antioxidant properties of broussochalcone A (BCA) and its effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages were investigated in this study. BCA, isolated from *Broussonetia papyrifera* Vent., inhibited iron-induced lipid peroxidation in rat brain homogenate in a concentration-dependent manner with an IC_{50} of 0.63 ± 0.03 μM. It was as potent as butylated hydroxytoluene, a common antioxidant used for food preservation. In a diphenyl-2-picrylhydrazyl assay system, the radical-scavenging activity of BCA seemed to be more potent than that of α-tocopherol, its $IC_{0.200}$ being 7.6 ± 0.8 μM. BCA could directly scavenge superoxide anion and hydroxyl radicals. These results indicated that BCA was a powerful antioxidant with versatile free radical-scavenging activity. On the other hand, we found that BCA suppressed NO production concentration-dependently, with an IC_{50} of 11.3 μM in LPS-activated macrophages. This effect was not the consequence of a direct inhibitory action on the enzyme activity of inducible NO synthase (iNOS). Our results indicated that BCA exerts potent inhibitory effects on NO production, apparently mediated by its suppression of IκBα phosphorylation, IκBα degradation, nuclear factor-kappa B activation, and iNOS expression. Therefore, we conclude that the antioxidant activities of BCA and its inhibition of IκBα degradation and iNOS protein expression may have therapeutic potential, given that excessive free radicals and NO production have been associated with various inflammatory diseases.

Keywords: Broussochalcone A; Antioxidant; Superoxide; IκBα; NF-κB; iNOS

1. Introduction

Reactive oxygen free radicals (such as superoxide anion, hydroxyl radical, and hydrogen peroxide) have been known to participate in a variety of human diseases such as ischemia–reperfusion injury and inflammatory diseases [1]. Thus, radical scavengers for the pathogenic native radicals might be expected to act as therapeutic agents under these circumstances. NO is another important radical molecule that participates in the physiology and pathophysiology of many systems [2]. NO is produced by a family of enzymes known as NOS [3]. Upon stimulation by various cytokines and LPS, macrophages express iNOS, produce large amounts of NO, and sustain for a period from several hours to several days [4]. Under pathological conditions, macrophages produce large amounts of NO and superoxide simultaneously, resulting in the formation of peroxynitrite [5]. At the level of the whole organism, reactive peroxynitrite might be considered beneficial because of its cytotoxicity to bacteria or other invading organisms [6]. However, excessive production of peroxynitrite would damage normal tissue. Therefore, protection against peroxynitrite damage is important for normal tissue during inflammation [7]. Thus, modulation of NO synthesis or action may have therapeutic benefits in patients with inflammatory or autoimmune diseases [8,9].

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Abbreviations: NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; BCA, broussochalcone A; BHT, butylated hydroxytoluene; DPPH, diphenyl-2-picrylhydrazyl; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MDA, malondialdyhyde; PDTC, pyrrolidine dithiocarbamate; TBARS, thiobarbituric acid-reactive substances; PMSF, phenylmethylsulfonyl fluoride; and DTT, dithiothreitol.

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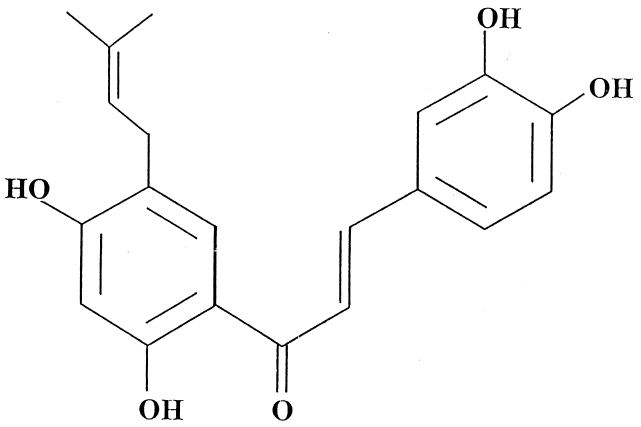

Fig. 1. The chemical structure of BCA.

Many flavonoids are known to possess antioxidant [10], free radical-scavenging, anti-inflammatory [11], and antitumor activity [12]. A number of derivatives of chalcone, a subclass of flavonoids, have also been found to inhibit several important enzymes in cellular systems, including xanthine oxidase [13], aldose reductase [14], and epoxide hydrolase [15]. A recent study demonstrated that chalcone derivatives also inhibited *de novo* iNOS and cyclooxygenase-2 synthesis in macrophages [16]. Thus, chalcone derivatives seem to possess several pharmacological properties that allow them to serve as natural biological response modifiers.

BCA, a prenylated chalcone (Fig. 1), was originally isolated from the cortex of *Broussonetia papyrifera* Vent. [17]. The cortex of this plant has been used as traditional medicine for diuresis, homeostasis, and the relief of edema and cough. In previous studies, BCA was found to be a potent inhibitor of platelet aggregation [18] and to act as an inhibitor of respiratory burst in neutrophils [19].

The aim of this study was to elucidate the antioxidant properties of BCA and its effects on NO production in the murine monocyte-macrophage cell line RAW 264.7 stimulated by LPS.

2. Materials and methods

2.1. Materials

BCA (Fig. 1) was isolated and purified as previously described [20]. BHT, α-tocopherol, 2-thiobarbituric acid (TBA), tetramethoxypropane, 2-deoxyribose, DPPH, xanthine oxidase (grade IV, from buttermilk), cytochrome *c* (type II, from horse heart), catalase, superoxide dismutase (type I, from bovine liver), D-mannitol, ascorbic acid, hydrogen peroxide (30% solution), bacterial LPS (*Escherichia coli* 055:B5), L-NAME, and PDTC were purchased from Sigma Chemical Co. All culture reagents were obtained from GIBCO BRL. L-[2,3,4,5-^{3}H]Arginine hydrochloride was obtained from Amersham Pharmacia Biotech. Rabbit polyclonal antibodies against iNOS and IκBα protein were obtained from Santa Cruz Biotechnology. Dowex AG 50W-X8 was purchased from Bio-Rad.

2.2. Anti-lipid peroxidation activity

Rat brain homogenate was prepared from the brains of freshly killed Wistar rats. Lipid peroxidation in the presence of BCA was measured by the thiobarbituric acid method, as described previously [21]. The results were expressed as MDA equivalents by linear regression analysis of a standard curve using tetramethoxypropane as a standard. The protein concentrations of rat brain homogenates were determined by Bio-Rad protein assay reagents according to the Bradford method [22].

2.3. DPPH-scavenging activity

An ethanol solution of the stable nitrogen-centered free radical DPPH (100 μM) was incubated with the test compounds for 30 min, and the absorbance was measured at 517 nm in a spectrophotometer (Hitachi, Model U3200). The concentration ($IC_{0.200}$) of the test compounds that induced a decrease of 0.200 in absorbance during a 30-min observation was taken as the free radical-scavenging potency [23]. In addition, the amount of reduced DPPH was estimated by using the millimolar extinction coefficient (ε) of 11.2 mM^{-1} cm^{-1} [23]. We were able to obtain the molar ratio: [reduced DPPH]/[oxidized BCA].

2.4. Superoxide-scavenging activity

Superoxide anion was generated by xanthine/xanthine oxidase and measured by the cytochrome *c* reduction method [24]. Test compounds were incubated in 50 mM KH_2PO_4/K_2HPO_4 (pH 7.4) buffer containing K_2H_2–EDTA (0.3 mM), cytochrome *c* (0.6 mM), xanthine (0.1 mM), and xanthine oxidase (0.02 U/mL). Production of superoxide anion was monitored spectrophotometrically at 550 nm. Superoxide dismutase (100 U/mL) was used as a reference inhibitor. The effect of test compounds on the activity of xanthine oxidase was determined by monitoring uric acid formation at 295 nm [24]. A molar extinction coefficient, 11,000 M^{-1} cm^{-1} for uric acid, was used for calculation.

2.5. Hydroxyl radical-scavenging activity

We used the deoxyribose method as described by Halliwell *et al.* [25] to measure the hydroxyl radical-scavenging activity. The second-order reaction constants for scavengers and hydroxyl radical were calculated from the slope of competition plots. The reaction constant of deoxyribose with hydroxyl radical was 3.1×10^9 M^{-1} sec^{-1} [25].

2.6. Cell culture

The murine monocyte-macrophage cell line RAW 264.7 was obtained from the Culture Collection and Research

Center (Hsinchu, Taiwan) and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum and an antibiotic mixture of penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL).

2.7. Nitrite assay

The nitrite concentration, an indicator of NO production, was measured in the culture medium according to the Griess reaction [26]. Briefly, 5×10^4 cells/0.2 mL/well of 96-well plates were incubated for 24 hr in phenol red-free DMEM with 10% heat-inactivated fetal bovine serum. In the presence or absence of BCA, cells were stimulated with LPS (1 μg/mL) for another 24 hr and then the nitrite in culture medium was quantified. Nitrite was measured by adding 60 μL of Griess reagents (20 μL 6.5 M HCl, 20 μL 37.5 mM sulfanilamide, and 20 μL 12.5 mM *N*-(1-naphthyl)ethylenediamine dihydrochloride) to 200 μl of cultured medium and incubating for 10 min. The absorbance at 550 nm was determined with an EL312e microplate reader (Bio-Tek). Nitrite concentrations were calculated by regression analysis of a standard curve using sodium nitrite as a standard.

2.8. Western blot analysis

Confluent macrophages in 6-well plates (about 2×10^6 cells/well) were stimulated by LPS (1 μg/mL) for 24 hr in the presence or absence of BCA. Then, cells were washed with ice-cold PBS and lysed in lysis buffer (20 mM Tris/HCl [pH 7.4], 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 5 μg/mL of leupeptin, and 20 μg/mL of aprotinin) [27] and then ultrasonicated at 4° for 20 sec. The lysate proteins (50 μg) were separated on polyacrylamide gels (7.5% for iNOS and 10% for IκBα) and transferred to PVDF membrane (Millipore). The membrane was incubated with blocking solution (5% non-fat dried milk and 0.1% Tween 20 in PBS) for 1 hr at room temperature and then incubated with anti-iNOS, anti-IκBα antibodies (Santa Cruz Biotechnology), or anti-IκBα-P polyclonal antibody (Biol Lab.) at 1:1000 dilutions at 4° overnight. Then, the membrane was incubated in diluted alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G at room temperature for 2 hr. Finally, the membrane was rinsed three times with PBST (0.1% Tween 20 in PBS) and twice with PBS before color development. The appropriate chromogenic substrate bromochloroindolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) was added to the membrane, and the progress of the reaction was monitored until the bands were of the desired intensity.

2.9. Assay of NOS activity

Cells were stimulated with LPS (1 μg/mL) for 10 hr, then pulse-labeled with [^{3}H]arginine (2 μCi/mL) for 1 hr in the presence or absence of BCA. The addition of ice-cold stop buffer (50 mM HEPES [pH 5.5], 2 mM EDTA, and 2 mM EGTA) stopped the reaction. Then, the cells were collected, sonicated, and centrifuged ($10{,}000 \times g$) at 4° for 10 min. The supernatants were passed through a small column containing 0.5 mL Dowex 50W-X8 (Na^+ form). The eluted [^{3}H]citrulline was then quantified by liquid scintillation spectroscopy and the enzyme activity determined [28].

2.10. Electrophorectic mobility shift assays (EMSA) for NF-κB

Nuclear extract was prepared as described by Chen *et al.* [29]. Briefly, cells were suspended in hypotonic buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 1 mM PMSF] for 10 min and vortexed for 10 sec. The nuclei were collected by centrifuging at 3300 *g* for 2 min. The pelleted nuclei were resuspended in hypertonic buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 1 mM PMSF] at 4° for 20 min. Then, the extracted nuclei were pelleted by centrifuging for 5 min at 11,000 *g*. The supernatants containing the solubilized nuclear proteins were stored at −70°.

For electrophoretic mobility shift assays, 5 μg of each nuclear extract was mixed with the ^{32}P-labeled double-stranded NF-κB oligonucleotide (5′-GATCAGTTGA-GGGGACTTTCCCAGGCC-3′) and incubated at room temperature for 30 min. The incubation mixture (15 μL) contained 2 μg of poly(dI-dC) (Amersham Pharmacia Biotech), 20,000 dpm of ^{32}P-labeled DNA probe, 10 mM Tris (pH 7.5), 1 mM EDTA, 4% Ficoll, 1 mM DTT, and 75 mM KCl. The DNA/protein complex was electrophoresed on 5% native polyacrylamide gels. For competition experiments, 50 ng of the labeled oligonucleotide was mixed with 1 μg of unlabeled oligonucleotide prior to protein addition. For supershift experiments, 4 μg of anti-p50 antibody was mixed with the nuclear extract protein. Radioactive bands were detected by autoradiography.

2.11. Statistical analysis

All experimental data are shown as means ± SEM and accompanied by the number of distinct experiments. Statistical analysis was performed using Student's *t*-test, and the significant difference was set at $P < 0.05$. The IC_{50} and $IC_{0.200}$ values were obtained by regression analysis.

3. Results

3.1. Inhibition of lipid peroxidation in rat brain homogenate

We examined the antioxidant activity of BCA on lipid peroxidation in rat brain homogenates. In the unstimulated

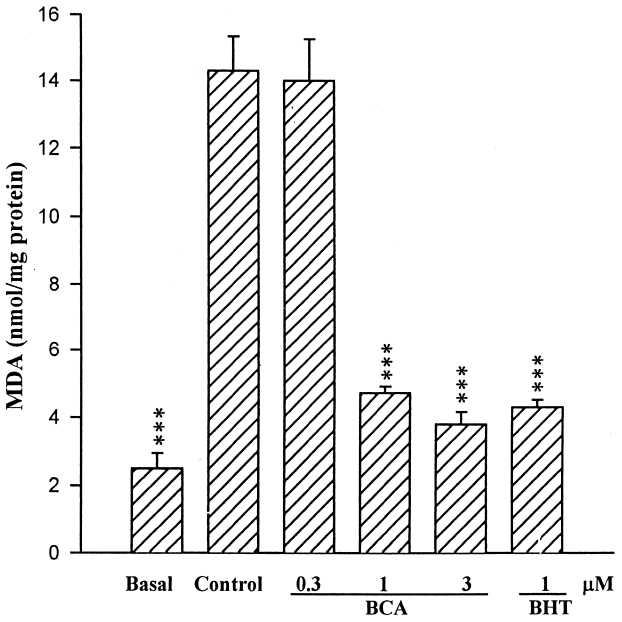

Fig. 2. The inhibitory effect of BCA on Fe^{2+}-induced lipid peroxidation. Rat brain homogenate was preincubated with DMSO (0.5%, basal and control), various concentrations of BCA (0.3, 1, 3 μM), or BHT (1 μM) at 37° for 10 min. Then, Fe^{2+} (200 μM) was added, except for basal, and incubated for another 30 min. Data are presented as means ± SEM (N = 6). ***: $P < 0.001$ as compared with the control.

experiments, the amount of TBARS formed in rat brain homogenate was 2.5 ± 0.5 nmol MDA/mg protein. After stimulation with 200 μM Fe^{2+}, the amount of TBARS increased to 14.3 ± 1.1 nmol MDA/mg protein. Apparently, BCA concentration-dependently inhibited iron-induced lipid peroxidation (Fig. 2), and its IC_{50} was calculated to be 0.63 ± 0.03 μM. BCA (1 μM) and BHT (1 μM) produced approximately 81% and 85% inhibition of iron-induced peroxidation, respectively, thereby demonstrating that their antioxidant potencies were comparable. A control experiment indicated that BCA did not affect the measurement of TBARS, because the absorbance at 532 nm was not affected by adding BCA to the brain homogenate that had already been oxidatively modified (data not shown).

3.2. DPPH radical-scavenging activity

The free radical-scavenging activity of BCA in the DPPH assay system was then examined. In this system, scavenging activity was expressed as $IC_{0.200}$ or molar ratio ([reduced DPPH]/[oxidized antioxidant]). The scavenging activities of BCA and α-tocopherol were both concentration-dependent (Fig. 3), with $IC_{0.200}$ values of 7.6 ± 0.8 and 10.8 ± 0.8 μM (N = 6), respectively. The molar ratios were calculated to be 2.50 ± 0.54 and 1.88 ± 0.27, respectively. BCA seems to be as potent as α-tocopherol in scavenging DPPH radicals.

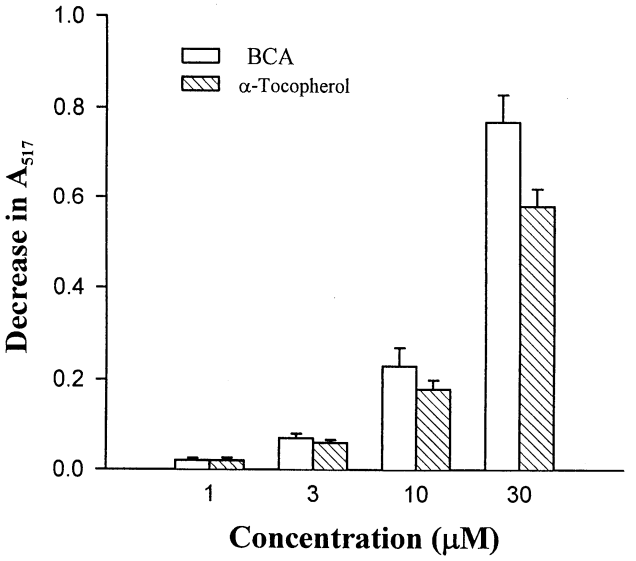

Fig. 3. The DPPH-scavenging activity of BCA. Various concentrations of BCA or α-tocopherol (1–30 μM) were incubated with DPPH (100 μM) at room temperature (25°) for 30 min, then the decrease in the absorbance at 517 nm was measured. Data are presented as means ± SEM (N = 5).

3.3. Superoxide anion-scavenging activity

Superoxide anion generated by the xanthine/xanthine oxidase system was monitored by the reduction of cytochrome *c*. The initial rate of cytochrome *c* reduction (0.20 ± 0.006 Δ A_{550}/min, N = 5) was completely inhibited by superoxide dismutase (100 U/mL). BCA (0.1 ~ 1 μM) inhibited cytochrome *c* reduction with an IC_{50} value of 0.5 ± 0.03 μM (Fig. 4). Since 10 μM BCA did not change the absorbance of cytochrome *c*, this indicated that BCA did not reduce cytochrome *c* directly. However, in addition to

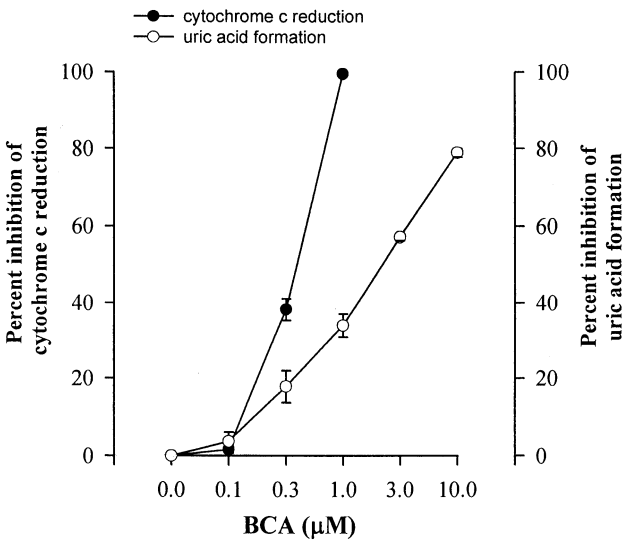

Fig. 4. Effect of BCA on the reduction of cytochrome *c* and the formation of uric acid in the xanthine/xanthine oxidase system. Data are presented as means ± SEM (N = 5).

superoxide anion scavenger, xanthine oxidase inhibitors may also affect cytochrome *c* reduction. Therefore, the effect of BCA on xanthine oxidase activity was assayed by monitoring uric acid formation. The initial rate of uric acid formation was 0.21 ± 0.01 ΔA_{295}/min (N = 6). BCA also inhibited xanthine oxidase activity in a concentration-dependent manner, with an IC_{50} value of 2.21 ± 0.13 μM (Fig. 4). If the data are plotted on a linear scale, the inhibition of cytochrome *c* reduction is seen to be linearly dependent upon the concentration of BCA. This is consistent with a free radical mechanism. In contrast, the dependence of uric acid formation upon the BCA concentration showed a saturation effect resembling a binding isotherm. Moreover, we found that 1 μM BCA could almost completely inhibit cytochrome *c* reduction, but only partially inhibit uric acid formation. These results imply that BCA-inhibited cytochrome *c* reduction could be largely attributed to its superoxide anion-scavenging activity and only partially to its inhibition of xanthine oxidase activity.

3.4. Hydroxyl radical-scavenging activity

The ability of BCA to scavenge hydroxyl radical was measured by the deoxyribose method. Our results showed that BCA concentration-dependently inhibited deoxyribose degradation. The rate constant for the reaction of BCA with hydroxyl radical was calculated to be 11.3 ± 0.9 × 10^9 M^{-1} sec^{-1} (N = 4). Mannitol, known to be a hydroxyl radical scavenger [25], had a rate constant of 3.2 ± 0.2 × 10^9 M^{-1} sec^{-1} (N = 3) in our experiments. It is apparent that BCA is more potent than mannitol in scavenging hydroxyl radical.

3.5. Inhibition of NO production and iNOS protein expression in LPS-activated macrophages

Stimulation of macrophages with LPS (1 μg/mL) for 24 hr increased nitrite production from 0.7 ± 0.06 to 33.1 ± 2.4 μM as measured by the Griess reaction. BCA (1 ~ 20 μM) concentration-dependently inhibited nitrite production (Fig. 5), with its IC_{50} value calculated to be 11.3 ± 0.8 μM. On the other hand, pretreatment with PDTC, an antioxidant that blocks NF-κB activation [30], also significantly inhibited nitrite production after LPS stimulation. Nevertheless, the potency of PDTC seemed to be less than that of BCA, as shown in Fig. 5. Based on both the lactate dehydrogenase release assay [31] and the MTT (3-[4,5-dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide) assay [32], these concentrations of BCA and PDTC had no cytotoxic effect on the macrophages in 24-hr incubation (data not shown). In addition, we analyzed the reversibility of the action of BCA by washout for 20 min before LPS stimulation. The preliminary results showed that the inhibition by BCA of NO production seemed to be reversible. Therefore, the reversible inhibition by BCA of nitrite production might account for why the IC_{50} of BCA on nitrite production was higher than that on lipid peroxidation. Moreover, we also examined the expression of iNOS protein after stimulation with LPS (1 μg/mL) in the presence of BCA (1 ~ 20 μM). Western blot analysis indicated that BCA also suppressed iNOS protein expression in a concentration-dependent manner (Fig. 6).

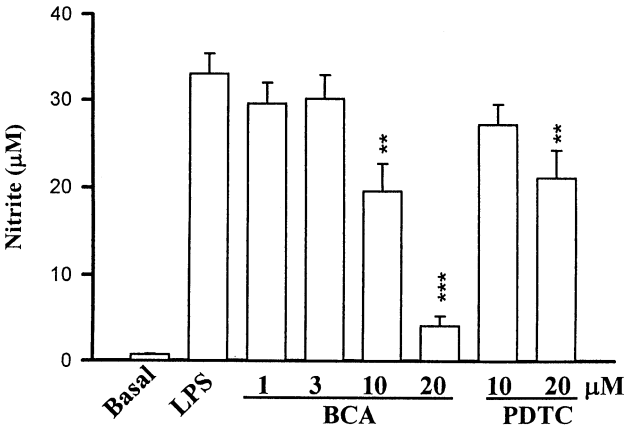

Fig. 5. Effect of BCA on nitrite production in LPS-activated RAW 264.7 macrophages. Cells were pretreated with indicated concentrations of BCA or PDTC for 30 min and then stimulated by LPS (1 μg/mL). After 24 hr, the level of nitrite in cultured medium was analyzed by the Griess reaction. Results are expressed as means ± SEM of four independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$, as compared with LPS alone.

3.6. Effect on enzyme activity of iNOS

To identify the mechanism responsible for inhibition of nitrite production by BCA, we examined whether BCA had a direct effect on the enzyme activity of iNOS. In the presence of BCA (10 and 20 μM), the enzyme activity of iNOS was not affected. Even a high concentration (100 μM) of BCA had only a minor effect on iNOS activity (Fig. 7). Addition of L-NAME (0.1 ~ 1 mM), a competitive inhibitor of NOS, into the assay medium inhibited NOS activity in a concentration-dependent manner (Fig. 7). These results suggest that the suppression of nitrite production by BCA may not be due to direct inhibition of NOS enzyme activity, but rather to suppression of iNOS protein expression.

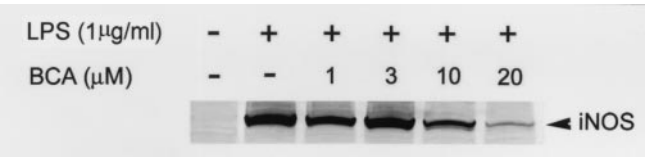

Fig. 6. Concentration-dependent inhibition of iNOS protein expression by BCA in LPS-activated RAW 264.7 macrophages. Cells were pretreated with BCA (BCA, 0 ~ 20 μM) for 30 min and then stimulated by LPS (1 μg/mL) for another 24 hr. Finally, the lysate protein (50 μg) was separated on a 7.5% polyacrylamide gel. The iNOS protein level was examined by Western blot analysis. Relative protein levels were quantified by densitometry. The figure is representative of three independent experiments.

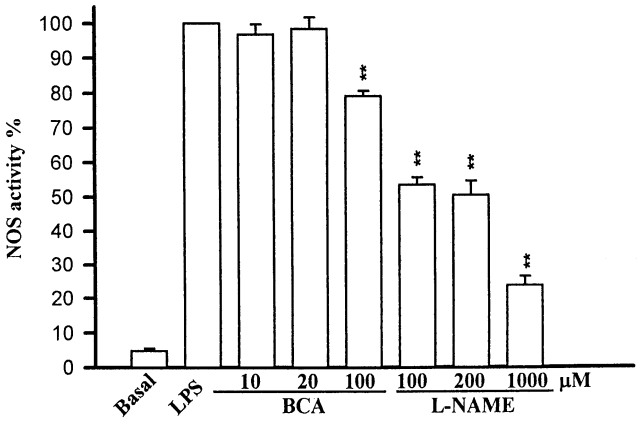

Fig. 7. Effect of BCA on the enzyme activity of iNOS. Cells were stimulated with LPS (1 μg/mL) for 10 hr and then pulse-labeled with [^{3}H]arginine (2 μCi/mL) for 1 hr in the absence or presence of different concentrations of BCA and L-NAME. Results are given as percentage of control (activated by 1 μg/mL of LPS). Data are presented as means ± SEM of four independent experiments performed in duplicate. $^{**}P < 0.01$, as compared with control.

3.7. Inhibition of NF-κB activation

Since NF-κB activation has been shown to be functionally important for iNOS induction [33], we further investigated whether BCA could inhibit LPS-induced NF-κB activation. Stimulation of macrophages with LPS (1 μg/mL) for 45 min markedly increased NF-κB-binding activity (Fig. 8, lane 5) compared to unstimulated cells (Fig. 8, lane 4), and this activation was inhibited by BCA (20 μM) (Fig. 8, lane 6). The specificity of NF-κB-binding activities was assayed by competition with a 20-fold excess of unlabeled NF-κB oligonucleotide (Fig. 8, lane 1) or AP-1 oligonucleotide (Fig. 8, lane 2) and by a supershift experiment which co-incubated with anti-p50 antibody. It was observed that BCA effectively inhibited NF-κB activation and that it was more potent than PDTC (Fig. 8, lane 7).

3.8. Inhibition of IκBα degradation

To investigate the upstream signaling effect of BCA, we tested whether BCA could prevent LPS-induced IκBα degradation. Following appropriate stimulation by LPS (1 μg/mL), cells were lysed and analyzed. Western blot analysis revealed a time-dependent degradation of IκBα (Fig. 9), which subsequently resulted in the activation of NF-κB [34]. As prior BCA (20 μM) treatment effectively prevented LPS-induced IκBα degradation (Fig. 9), it is suggested that BCA prevented LPS-induced IκBα degradation and NF-κB activation, and decreased iNOS protein expression and NO production.

3.9. Inhibition of IκBα phosphorylation

It has been reported that IκBα protein phosphokinase can phosphorylate IκBα and thereby target it for degradation

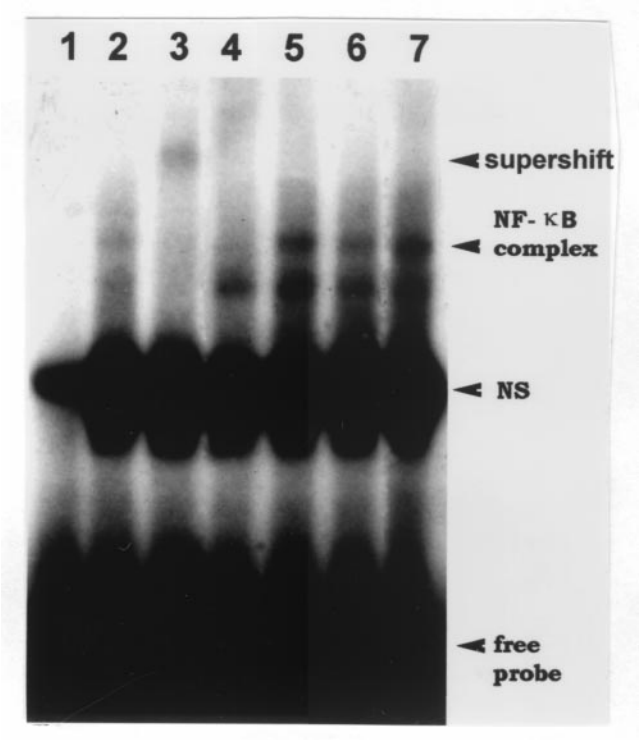

Fig. 8. Effect of BCA on NF-κB activation in RAW 264.7 cells. Nuclear extracts were prepared from cells unstimulated (lane 4) or stimulated with LPS (1 μg/mL) for 45 min (lanes 1–7, except 4) in the presence of 20 μM BCA (lane 6) or 20 μM PDTC (lane 7). EMSAs were carried out as described in Materials and Methods. The binding activity of NF-κB (from nuclear extract) to [^{32}P]NF-κB oligonucleotide is shown as NF-κB complex. Nuclear extracts (5 μg) from cells stimulated by LPS were either mixed directly with the labeled NF-κB oligonucleotide (lane 5) or first incubated with 20-fold unlabeled NF-κB oligonucleotide (lane 1), 20-fold unlabeled AP-1 oligonucleotide (lane 2), or anti-p50 antibody (lane 3). NS, non-specific binding.

through the ubiquitin–proteasome pathway [35]. To determine whether BCA-prevented IκBα degradation was due to down-regulated IκBα phosphorylation, we examined the cytosolic levels of IκBα-P by Western blot analysis. Incubation of cells with LPS for 7.5 min caused marked phosphorylation of cytosolic IκBα. From Fig. 10, it appeared that BCA (20 μM) down-regulated IκBα phosphorylation to near basal level. This suggests that BCA-prevented IκBα degradation was due to the down-regulated phosphorylation of IκBα but not to inhibition of proteasome proteolytic activity.

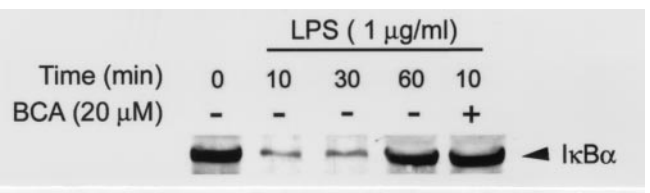

Fig. 9. Effect of BCA on IκBα degradation. After 0, 10, 30, and 60 min incubation with LPS (1 μg/mL) in the presence or absence of BCA (20 μM) pretreatment, cell lysate proteins were separated on 10% polyacrylamide gels. The figure is one of three experiments.

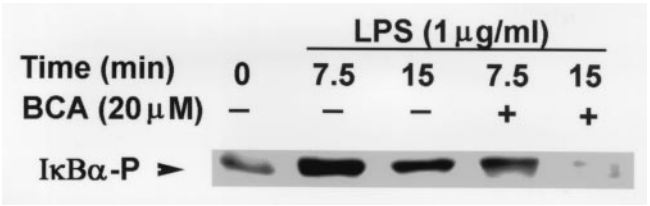

Fig. 10. Effect of BCA on IκBα phosphorylation. Cells were incubated with LPS (1 μg/mL) for 7.5 or 15 min in the presence or absence of BCA (20 μM). Total protein extracts were separated by 10% SDS–PAGE and analyzed by Western blot as indicated in Materials and Methods.

4. Discussion

Rat brain homogenates are usually used as a preparation to evaluate the antioxidant activities of compounds on lipid peroxidation [36]. It is known that transition metal ions are involved in both initiation and propagation of lipid peroxidation [37]. In this system, we found that BCA could effectively inhibit ferrous ion-induced lipid peroxidation. Its potency was comparable to that of BHT, a typical antioxidant in foods. BCA did not cause a spectral shift or absorbance change when incubated with ferrous ions (data not shown), indicating that its antioxidant mechanism seems to be associated with its free radical-scavenging but not its iron-chelating activity. According to the DPPH assay, BCA acted as a direct free radical scavenger with a potency ($IC_{0.200}$, 7.6 ± 0.8 μM) comparable to that of α-tocopherol, a chain-breaking antioxidant. In addition, BCA also exhibited superoxide- and hydroxyl radical-scavenging activities. Taken together, all these data suggest that the free radical-scavenging activity of BCA may contribute to its antiperoxidation activity in brain homogenates.

Recently, we found that BCA concentration-dependently inhibits NO production in RAW 264.7 macrophages stimulated by LPS. However, BCA had no direct inhibitory effect on iNOS enzyme activity. Therefore, the inhibition by BCA of NO production may be attributed to the suppression of iNOS protein expression. Our results are consistent with Herencia *et al.*'s observation that some novel anti-inflammatory chalcone derivatives inhibited iNOS protein synthesis but had no direct effect on iNOS enzyme activity [16].

NF-κB, a mammalian transcription factor, has been shown to be functionally important for iNOS induction [33]. The heterodimeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor complex with the inhibitory protein IκB family [38]. Among these inhibitory proteins, the best characterized is IκBα (37 kDa). Upon stimulation by an NF-κB activator such as LPS or cytokines, the inhibitory subunit IκBα is postulated to be phosphorylated and then degraded, thereby releasing it from the NF-κB-IκBα complex [34,39]. Then, the active NF-κB will translocate to the nucleus and transactivate the target genes [40,41]. Therefore, the degradation of IκBα plays an important role in NF-κB activation. In this study, we found that BCA could effectively prevent the degradation of IκBα and iNOS protein expression, which seems to indicate that preventing IκBα degradation would block NF-κB activation and iNOS protein expression. Ohata and colleagues observed similar results with 1′-acetoxychavicol acetate, an anticarcinogenic compound, in RAW 264 cells [42]. Moreover, dysregulation of NF-κB has been associated with various pathological processes such as acute and chronic inflammatory reactions, septic shock, and cancers [40,41]. Therefore, specific inhibitors of IκBα degradation might have potential for the treatment of various inflammatory diseases.

Furthermore, several lines of evidence suggest that NF-κB activation by various stimuli depends on intracellular reactive oxygen intermediate (ROI) generation [30,43–46]. Recently, Camhi and co-workers observed that LPS causes increased production of ROI in RAW 264.7 macrophages and that the antioxidants *N*-acetyl-L-cysteine and DMSO attenuate ROI production [47]. Our results indicate that BCA is a powerful antioxidant and free radical scavenger. Moreover, in Fig. 5, BCA showed a steep dose–response relationship similar to the free radical-scavenging reaction. Thus, we suggest that BCA prevents the degradation of IκBα presumably through its free radical-scavenging activity. In addition, it remains to be determined whether BCA affects the enzyme activities of IκB protein phosphokinase and phosphatase. Further studies need to be carried out to elucidate the effects of BCA on the pathway of IκBα degradation.

In conclusion, this study verifies that BCA is a powerful natural antioxidant and that its antioxidant activities may be primarily attributed to its free radical-scavenging activity. Moreover, BCA was also found to suppress LPS-induced iNOS protein expression by preventing IκBα degradation in RAW 264.7 macrophages. The free radical-scavenging activity of BCA and its inhibition of iNOS protein expression may have therapeutic potential, because excessive free radicals and NO production have been associated with various inflammatory diseases.

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