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Anti-angiogenic, anti-inflammatory and anti-nociceptive activity of 4-hydroxybenzyl alcohol

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Abstract

4-Hydroxybenzyl alcohol (HBA), one of the well-known phenolic compounds in diverse plants, displayed a significant inhibition in the chick chorioallantoic membrane (CAM) angiogenesis assay. HBA was shown to contain an anti-inflammatory activity in carrageenan-induced air pouch model in rats and acetic acid-induced permeability model in mice. Anti-nociceptive activity of HBA was also assessed using the acetic acid-induced writhing test in mice. HBA was able to suppress production of nitric oxide (NO) and expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated RAW264.7 macrophages. In the macrophages, the level of reactive oxygen species (ROS) was diminished by HBA. Taken together, HBA possesses anti-angiogenic, anti-inflammatory and anti-nociceptive activity possibly via its down-regulating activity on NO production, which may be partly responsible for the pharmacological efficacy of several folkloric medicines.

Introduction

4-Hydroxybenzyl alcohol (HBA, Figure 1) is known to play a protective role against oxidative-damage-related diseases, such as coronary heart disease, stroke and cancer. It is widely spread throughout diverse plants, including carrots (Kobayashi et al 2003), *Gymnadenia conopsea* (Cai et al 2006) and *Coeloglossum viride* var. *bracteatum* (Huang et al 2004). HBA, together with vanillin and 4-hydroxybenzaldeyde, was also identified as one of the major constituents in the rhizome of *Gastrodia elata*, widely used as a folkloric medicine for the treatment of headache, tetanus, inflammatory disease and convulsive disorders in Oriental countries (Ha et al 2001; Jung et al 2006). *G. elata* rhizome has recently been shown to possess anti-inflammatory and anti-angiogenic activity (Ahn et al 2007).

Recently, some pharmacological actions of HBA have been assessed against neuronal disorders. The anticonvulsive effect of *G. elata* is attributed to the antioxidant action of HBA and other phenolic compounds (Liu & Mori 1993). HBA prevents brain damage through the increased expression of several genes encoding antioxidant proteins after transient focal cerebral ischaemia (Yu et al 2005). HBA possesses an anxiolytic-like effect via the serotonergic nervous system (Jung et al 2006). HBA is able to ameliorate cycloheximide-induced impairment of passive avoidance response in rats (Hsieh et al 1998). Since the anti-inflammatory and related activity of HBA has not been assessed, this study demonstrates that HBA possesses anti-angiogenic, anti-inflammatory and anti-nociceptive activity.

Materials and Methods

Materials and animals

4-Hydroxybenzyl alcohol (HBA) was purchased from Sigma Chemical Co. (St Louis, MO). HBA was dissolved in 1% CMC in saline for animal experiments, while it was dissolved in ethyl alcohol for egg and in-vitro experiments. Male ICR mice, about 25 g, were obtained from Samtaco Animal Farm (Osan, Korea). The animal room was maintained at 23 ± 2°C with a 12-h light–dark cycle. Food and tap water were freely available. Fertilized brown Leghorn eggs were obtained from Pulmuone Food Co. (Seoul, Korea). Ethical guidelines,

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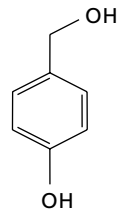

Figure 1 Chemical structure of 4-hydroxybenzyl alcohol (HBA), a major phenolic compound widely spread throughout the plant kingdom.

described in the NIH Guide for Care and Use of Laboratory Animals, were followed throughout the experiments.

Cell culture

RAW264.7 cells, a murine macrophage cell line, were obtained from American Type Culture Collection (Manassas, VA). The mammalian cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES (pH 7.5), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The RAW264.7 cells were plated at a density of 1×10^6, pre-incubated for 24 h at 37°C and maintained in a humidified atmosphere containing 5% CO_2. For all experiments, the cells were grown to 80–90% confluence, and subjected to no more than 20 cell passages.

Chorioallantoic membrane (CAM) assay

Anti-angiogenic activity was measured using CAM assay as previously described (Song et al 2003). The fertilized chicken eggs used in this work were kept in a humidified egg incubator at 37°C. After a 3.5-day incubation, about 2 mL of albumin was aspirated from the eggs through a small hole drilled at the narrow end of the eggs, allowing the small chorioallantoic membrane and yolk sac to drop away from the shell membrane. The shell covering the air sac was punched out and removed by forceps and the shell membrane on the floor of the air sac was peeled away. In the 4.5-day-old chick embryo, an HBA (0.3, 1.0 or 3.0 μg)-loaded Thermanox coverslip was applied to the CAM surface. Two days after returning the chick embryo to the incubator, an appropriate volume of a 10% fat emulsion (Intralipose, 10%) was injected into a 6.5-day-old embryo chorioallantois. The eggs were then evaluated using a score system under a microscope. The vascular response of each egg was graded as 0, 1^+ or 2^+. Convergence of a few vessels toward the CAM surface was denoted as 1^+, while 2^+ reflected an increased density and length of vessels toward the CAM face. Each group contained at least 20 fertilized eggs. The CAM assay was independently repeated three times.

Acetic-acid-induced vascular permeability

According to a modification of the method of Whittle (1964), an acetic-acid-induced vascular permeability test was performed. One hour after oral administration of vehicle (1% CMC in saline), HBA (50 or 100 mg kg^{-1}) or aminopyrine (AP, 100 mg kg^{-1}), 0.1 mL/10 g body weight of 1% Evans blue solution was injected intravenously in each mouse. Thirty minutes later, 0.1 mL/10 g body weight of 0.7% acetic acid in saline was intraperitoneally injected. Thirty minutes after the administration of acetic acid, the mice were killed by cervical dislocation. After 10 mL of saline was injected into the peritoneal cavity, the washings were collected in test tubes. The concentration of Evans blue in the peritoneal cavity was measured by the absorbance at 630 nm. The vascular permeability was represented in terms of the absorbance (A_{630}) that leaked into the cavity. The vascular permeability test was independently repeated three times.

Carrageenan-induced air pouch formation

According to a modification of the procedure of Ghosh et al (2000), λ-carrageenan-induced air pouch formation was performed. Six days before drug treatment, the air pouch was formed in the intrascapular region of rats by initial subcutaneous injection of 20 mL sterile air and successive injections of 10 mL sterile air every 3 days to sustain its patency. On day 0, vehicle (1% CMC in saline), HBA (0.05, 0.5 or 1.0 mg/pouch) or dexamethasone (0.01 mg/pouch) was administered into the pouch 1 h before the λ-carrageenan injection (0.1 mL of 1.0% solution). After 15 min, the pouch cavity was opened and the exudates were collected. The exudate volumes were measured using a graduate tube. Samples were diluted with Turk solution, and the polymorphonuclear leucocytes were counted in a standard haemocytometer chamber. The air pouch experiment was independently repeated three times.

Acetic-acid-induced writhing response

As previously described (Olajide et al 2000), the response to an intraperitoneal injection of acetic acid solution, manifesting as a contraction of the abdominal muscles and stretching of hind limbs, was measured. Nociception was induced by intraperitoneal injection of 0.7% acetic acid solution at a dose of 0.1 mL/10 g body weight. Each experimental group of mice was treated orally with vehicle (1% CMC in saline), HBA (50 or 100 mg kg^{-1}) or aminopyrine (AP, 100 mg kg^{-1}) as a positive control. From 10 min later, the number of writhes during the following 10-min period was counted. The writhing experiment was independently repeated three times.

Nitrite analysis

Accumulated nitrite (NO_2^-) in the media obtained from the cell cultures was determined using a colorimetric assay based on the Griess reaction (Sherman et al 1993). The samples (100 μL) were reacted with 100 μL Griess reagent (6 mg mL^{-1}) at room temperature for 10 min, and then NO_2^- concentration was determined by measuring the absorbance at 540 nm. The standard curve was constructed using known concentrations of sodium nitrite. This experiment was independently repeated three times.

Determination of intracellular ROS

For analysis of intracellular ROS, the redox-sensitive fluorescent probe DCFH-DA was used as previously described

(Royall & Ischiropoulos 1993). After pre-incubation with varying concentrations of HBA for 1 h, the 1×10^6 RAW264.7 cells were treated with LPS for 24 h. Then, they were incubated with 5 μM dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37 °C. The harvested cells were immediately analysed by flow cytometry. This experiment was independently performed three times.

Immunoblot analysis

The 1×10^6 RAW264.7 cells were incubated with LPS (1 μg mL^{-1}) in the presence or absence of HBA for 24 h and then washed twice with ice-cold phosphate-buffered saline (PBS). The cells were lysed in a buffer containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.3 M NaCl, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1 μg mL^{-1} leupeptin and 1 μg mL^{-1} pepstatin. Western blotting was performed as previously described (Kim et al 2002). For immunoblotting, anti-inducible nitric oxide synthase (anti-iNOS; Transduction Laboratories, Lexington, KY), anti-cyclooxygenase (anti-COX-2; Transduction Laboratories, Lexington, KY) and anti-β-actin (Sigma-Aldrich, St Louis, MO) antibodies were used. This experiment was independently repeated three times.

MTT reduction assay

The cell viability was quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Freshney 1994). Briefly, 1×10^5 cells incubated with various concentrations of HBA were treated with 10 μL of MTT (Sigma-Aldrich Co., St Louis, MO) solution (5 mg mL^{-1}) for 2 h. The cells were then lysed with isopropyl alcohol and the absorbance was read at a wavelength of 540 nm. This experiment was independently repeated three times.

Statistical analysis

The results were expressed as mean ± s.e. Comparison between experimental groups was performed by analysis of variance followed by the Tukey's multiple range tests. $P < 0.05$ was considered to be significant. The IC50 values (dose required for half-maximal response) were calculated from the dose–response linear regression plots.

Results and Discussion

Anti-angiogenic activity

Down-regulation of angiogenesis has been considered to be advantageous for the prevention of neoplastic growth and inflammation. Some anti-angiogenic substances are effective in animal models of arthritis, whereas several antirheumatic drugs, such as indometacin, methotrexate and corticosteroids, possess anti-angiogenic activity. The chick chorioallantoic membrane (CAM) assay was used for examining the inhibitory activity of HBA on vascular development, and retinoic acid was used as a positive control for the assay. The disc weight did not give any changes in vascular density, indicating that it was unable to affect the growth of blood vessels in the CAM assay (data not shown). After the 2-day treatment, retinoic acid at 1 μg/egg gave rise to an inhibition of 77.8% in the branching patterns of blood vessels (Figure 2). When 0.3, 1.0 and 3.0 μg/egg of HBA was applied in the CAM assay, the inhibition percentages in CAM angiogenesis were 20.0%, 30.0% and 55.6%, respectively (Figure 2). This indicates that HBA inhibits angiogenesis in a dose-dependent manner. The dose required for half-maximal inhibition (IC50) of HA was determined to be 2.56 μg/egg. In brief, HBA possesses anti-angiogencic activity, which might provide a pharmacological basis for the traditional use of several plants, including *G. elata*. However, the anti-angiogenic activity of HBA needs to be confirmed using in-vivo angiogenesis models.

Anti-inflammatory activity

In vascular permeability assay as a model typical of the first stage inflammatory reactions, the inflammatory mediators, released following stimulation, lead to dilation of arterioles and venules and increased vascular permeability (Vogel & Vogel 1997). HBA at oral doses of 50 and 100 mg kg^{-1} body weight showed an inhibition of 16.3% and 40.7% on vascular permeability, respectively (Table 1). The suppressing effect of HBA at 100 mg kg^{-1} was comparable with that of aminopyrine (AP, 100 mg kg^{-1}) used as a positive control (Table 1). This result implies that acute inflammatory activity of HBA partly arises from its prevention of the release of inflammatory mediators at the first stage. The carrageenan-induced air pouch model, another inflammatory model used in this study, is known to be an excellent acute inflammatory model in which fluid extravasation, leucocyte migration and biochemical parameters in the exudate involved in the inflammatory response can be easily detected. The injection of carrageenan into a subcutaneous air pouch on the dorsal surface of rats initiates an inflammatory process. In the carrageenan-induced air pouches, dexamethasone (0.01 mg/pouch), a non-selective cyclooxygenase inhibitor, reduced the volume of the exudates

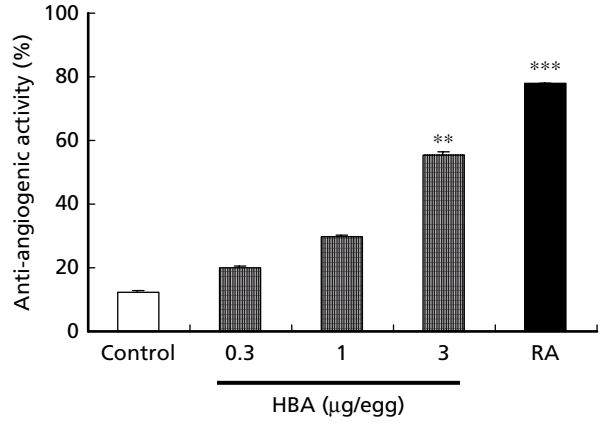

Figure 2 Dose-dependent anti-angiogenic activity of HBA in the chick embryo chorioallantoic membrane (CAM) assay. Retinoic acid (RA, 1 μg/egg) was used as a positive control. Each group contained at least 20 eggs. Each column represents mean ± s.e. of the three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control.

Table 1 Inhibitory effect of HBA in acetic-acid-induced vascular permeability and writhing response in mice

Group	Dose (mg kg^{-1})	Absorbance at 630 nm	No. of writhings
Control	—	1.23 ± 0.17	25.9 ± 2.90
HBA 50	50	1.03 ± 0.14 (16.3)	17.6 ± 3.29** (32.0)
HBA100	100	0.73 ± 0.11** (40.7)	14.5 ± 3.45** (44.0)
AP	100	0.49 ± 0.18** (60.2)	2.00 ± 1.29** (92.3)

Aminopyrine (AP, 100 mg kg^{-1} body wt.) was used as a positive control. HBA (50 and 100 mg kg^{-1}) was orally administered. Vascular permeability was represented by the absorbance at 630 nm. The results are expressed as mean ± s.e. Each group contained 7 mice. This experiment was performed in triplicate. Figures in parentheses indicate inhibition percentage with respect to the control group treated only with 1% CMC in saline. $^{**}P < 0.01$, compared with the control group.

by 71.7% (Table 2). Treatment with HBA at 0.5, 1.5 and 4.5 mg/pouch gave rise to an inhibition of 6.1%, 24.5% and 29.4%, respectively, with respect to the control exudate volume (Table 2). The total number of polymorphonuclear leucocytes in the air pouches was also diminished by treatment with HBA at the dosages used, the inhibitory percentages of which were 30.9%, 49.6% and 56.0%, respectively (Table 2). Although the effective dosage of HBA in the air pouch model appears to be relatively high, HBA is estimated to contain an acute anti-inflammatory activity.

Anti-nociceptive activity

In the acetic-acid-induced writhing response, which is a visceral pain model, the anti-nociceptive mechanism of abdominal writhing induced by acetic acid involves the process or release of arachidonic acid metabolites via cyclooxygenase, and prostaglandin biosynthesis (Franzotti et al 2000). As shown in Table 1, HBA at 50 and 100 mg kg^{-1}, orally, caused inhibition by 32.0% and 44.0%, respectively, on the writhing response induced by acetic acid. This result suggests that HBA also possesses anti-nociceptive activity in addition to anti-inflammatory activity, subsequently suggesting that the same mediator(s) might be commonly involved in the activity of HBA.

Inhibition of NO production

Nitric oxide (NO) is a well-known pro-inflammatory mediator in the pathogenesis of inflammation. For the expression of inducible NOS (iNOS), the mammalian cells should be triggered by specific stimulants, such as pro-inflammatory cytokines and bacterial LPS (Chesrown et al 1994). Since iNOS-derived NO is involved in various pathological conditions, such as inflammation, and leads to cellular injury (Singh et al 2000), suppression of iNOS is closely linked with anti-inflammatory action. The inhibitory effect of HBA was examined on LPS-induced NO production in RAW264.7 macrophages (Table 3). The accumulated nitrite, determined by the Griess method, in the medium was used as an index for NO level. After treatment with LPS, nitrite content notably increased about 7.0 fold. When the macrophage cells were treated with 0.05, 0.5 and 1.0 mM HBA, NO production induced by LPS was significantly inhibited in a dose-dependent manner (Table 3). No cytotoxicity was observed in the macrophages at the used concentrations of HBA, which was determined by MTT assay (Figure 3). HBA, up to 1 mM, was unable to decrease the cell viability. The suppressive effect of HBA on the production of NO was confirmed in the in-vivo air pouch model. As shown in Table 2, HBA gave rise to a marked decrease in the content of nitrite in the exudates obtained from the carrageenan-induced air-pouch model, which corresponds with the in-vitro results obtained using the macrophages (Table 3). With the assumption that inhibition of NO production by HBA would be caused by a decrease in the iNOS protein level, the effect of HBA on the iNOS expression was examined in the macrophages cells treated with LPS. As shown in Figure 4, HBA dose-dependently suppressed iNOS induction without changes in the levels of β-actin, an internal control, indicating the specific inhibition of iNOS expression by HBA. However, HBA, at the used concentrations, was unable to modulate expression of cyclooxygenase-2 (COX-2) in LPS-stimulated macrophages (Figure 4). Although this result looks to be unexpected, it might imply that HBA manifests its anti-inflammatory activity independent of COX-2. Another possibility is that HBA is able to modulate COX-2 at the activity level, which is less probable. Taken together, HBA is able to suppress in-vitro and in-vivo production of NO, which would support anti-inflammatory activity of HBA.

Table 2 Effect of HBA on carrageenan-induced air-pouch model in rats

Group	Dose (mg/pouch)	Exudate (mL)	Total leucocytes (×10^5 cells)	Nitrite content (μM)
Control	—	6.13 ± 0.85	1508 ± 339	18.49 ± 1.99
HBA0.5	0.5	5.75 ± 0.41 (6.1)	1042 ± 190* (30.9)	12.23 ± 3.12* (33.9)
HBA1.5	1.5	4.63 ± 1.03 (24.5)	759 ± 138** (49.6)	9.05 ± 1.61** (50.5)
HBA4.5	4.5	4.33 ± 0.70* (29.4)	663 ± 126** (56.0)	8.66 ± 1.85** (53.2)
Dexamethasone	0.01	1.73 ± 0.50** (71.7)	117 ± 46** (92.2)	1.55 ± 0.60** (90.4)

The results are expressed as mean ± s.e., n = 8. Figures in parentheses indicate inhibitory percentages with respect to the corresponding control. Dexamethasone was used as a positive control. The control group was treated only with 1% CMC in saline. This experiment was repeated in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group.

Table 3 Effect of HBA on nitric oxide (NO) and reactive oxygen species (ROS) levels in RAW264.7 macrophage cells activated with LPS

Group	LPS treatment	HBA concn (mM)	Nitrite content (μM)	DCF fluorescence
Control 1	—	—	7.2 ± 0.60	14.3 ± 0.74
Control 2	—	1.0	6.5 ± 0.43	10.4 ± 1.65
LPS only	+	0	50.7 ± 4.65 (100)	70.1 ± 8.33 (100)
HBA0.05	+	0.05	39.8 ± 5.60 (78.5)	61.5 ± 4.39 (87.8)
HBA0.5	+	0.5	32.4 ± 4.90* (63.8)	41.8 ± 4.22** (59.6)
HBA1.0	+	1.0	21.7 ± 0.50** (42.7)	34.1 ± 3.41** (48.6)

The 1×10^6 mammalian cells were incubated for 24 h with LPS (1 μg mL^{-1}) in the presence or absence of indicated concentrations of HBA. Nitrite content and DCF fluorescence are indexes of NO and ROS levels, respectively. The values are mean ± s.e. of three independent experiments performed in triplicate. Figures in parentheses indicate relative values. *$P < 0.05$, **$P < 0.01$, compared with LPS only.

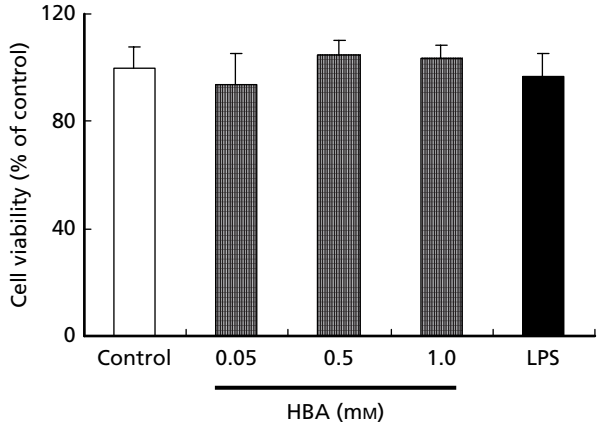

Figure 3 Effect of HBA on the viability of RAW264.7 macrophage cells. Cell viability was measured by the MTT assay. The 1×10^5 mammalian cells were treated with indicated concentrations of HBA or 1 μg mL^{-1} LPS for 24 h. Data indicate mean ± s.e. from three independent experiments performed in triplicate.

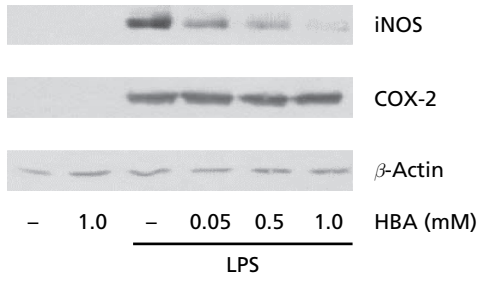

Figure 4 Inhibitory effect of HBA on LPS-induced expression of iNOS and COX-2 in RAW264.7 macrophage cells. The mammalian cells were incubated for 24 h with LPS (1 μg mL^{-1}) in the presence or absence of indicated concentrations of HBA. After 24-h incubation, the cell lysates (30 μg protein) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and blotted with appropriate antibodies. β-Actin was used an internal control. This western blot is a representative of the three independent experiments.

Suppressing effect on ROS level

Although ROS at low concentrations play the role of an intracellular messenger of various physiological events, including cell proliferation and apoptosis, the large amount of ROS is considered cytotoxic and associated with various disorders. As shown in Table 3, when the macrophage cells were treated with LPS only, the intracellular ROS level was increased about 5 fold. However, HBA was able to significantly suppress the ROS level, which was elevated by LPS in the macrophages (Table 3). This suppressing activity of HBA might correspond with the previous finding that HBA has an ability to scavenge superoxide and hydroxyl radicals (Liu & Mori 1993). Since HBA can decrease the intracellular ROS level, HBA might be involved in various ROS-related phenomena.

Although novel pharmacological actions of HBA, such as anti-angiogenic, anti-inflammatory and anti-nociceptive activity, were assessed in this study, some of them were observed at relatively high concentration. Our study also showed that HBA, at the used concentrations, didn't give rise to any toxicity on the cell viability in-vitro. However, its in-vivo cytotoxicity needs to be determined. It is further required to modify HBA and screen out its analogues to decrease effective concentrations before clinical application. Topical application could be another plausible way for its clinical use.

Conclusions

HBA is one of the well-known phenolic compounds widely distributed in various kinds of plants, including some used in folkloric medicine. Specially, it is known to be an active ingredient in *Gastrodia elata* Blume. It sometimes exists as an aglycone in several glycosides, such as gastrodin. Throughout this work, some pharmacological actions of HBA have been assessed. It contains anti-angiogenic activity, which may be beneficial for the treatment of inflammatory diseases and cancer. HBA also has anti-inflammatory activity, which was demonstrated using carrageenan-induced air pouch model in rats and acetic-acid-induced vascular permeability in mice. Additionally, it possesses anti-nociceptive activity. HBA also possesses an inhibitory activity on in-vitro NO production in stimulated mammalian macrophage cells and in-vivo NO production in the air pouch. It is capable of

suppressing expression of iNOS in LPS-stimulated RAW264.7 cells. However, HBA, at the concentrations used, had no effect on the expression of COX-2 in the stimulated macrophages. HBA also contains a reducing activity on ROS level in LPS-activated macrophages. These findings provide additional pharmacological information on the therapeutic efficacy of HBA. Anti-inflammatory and related activity of HBA are possibly mediated by its inhibitory effect on NO production and antioxidant activity.

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