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3-(4′-hydroxyl-3′,5′-dimethoxyphenyl) Propionic Acid Suppresses NO Production and Elevates GSH Levels in Murine Macrophages

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Abstract

Previous studies have shown that *kimchi* and *kimchi*-derived 3-(4′-hydroxyl-3′,5′-dimethoxyphenyl) propionic acid have anti-oxidative and hypolipidemic effects in rats and rabbits. This study was designed to investigate whether chemically synthesized 3-(4′-hydroxyl-3′,5′-dimethoxyphenyl) propionic acid (HDMPPA) may ameliorate oxidative stress through the regulation of nuclear factor κB (NFκB) activation in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage cells. Treatment of RAW 264.7 cells with 400 μM of HDMPPA significantly reduced LPS-stimulated nitric oxide (NO) production. Treatments with HDMPPA at 100 μM to 400 μM concentrations significantly elevated glutathione (GSH) level. However, cell viability and thiobarbituric acid-reactive substances (TBARS) concentrations were not affected by the concentrations of HDMPPA used. The specific DNA binding activities of NFκB, a transcription factor which is sensitive to oxidative stress, were not down-regulated by HDMPPA treatments. These results suggest that HDMPPA may have weak anti-oxidative activity against LPS challenge by scavenging NO and stimulating GSH production.

Key words: 3-(4′-hydroxyl-3′,5′-dimethoxyphenyl) propionic acid, nitric oxide, glutathione

INTRODUCTION

Nitric oxide (NO) is produced from L-arginine by constitutive and inducible nitric oxide synthase (iNOS) in various mammalian cells and tissues. iNOS is induced by either bacterial lipopolysaccharide (LPS) or cytokines, including tumor necrosis factor, α and interferon-γ, in macrophages (1-3). NO produced by iNOS reacts with superoxide and yields peroxynitrite, which contributes to etiology of cardiovascular disease and cancer by promoting oxidative stress and inflammatory processes (4-6). A high NO level along with decreased superoxide dismutase (SOD) activity leads to peroxynitrite formation, while the degradation of hydrogen peroxide by catalase and glutathione peroxidase (GSH-px) ameliorates cytotoxic effects of reactive oxygen species (ROS) (7). Furthermore, it is known that NO interacts with thiol-containing glutathione (GSH). Depletion of GSH was observed to increase the sensitivity of cells to the toxic effects of NO (8).

Nuclear factor κB (NFκB), an inducible transcription factor, exists in a latent form in the cytoplasm of unstimulated cells as a transcriptionally active dimer bound to an inhibitor protein, IkB. NFκB is activated by the disassociation with IkB in response to various extracellular stimuli, including cytokines (9), lipopolysaccharide (LPS) (10), and oxidative stress (11). Following activation, NFκB is translocated to the nucleus where it regulates the expression of many target genes involved in immune and inflammatory responses. It has been reported that NFκB is sensitive to oxidative modification of a particular cysteine at position 62 in p50, which is essential for DNA binding (12). Furthermore, antioxidants such as carnosol (13), anthocyanins (14), selenium (15), and organosulfur compounds (16) have been reported to suppress NO production in macrophages through mechanisms dependent on their abilities to inhibit the activation of NFκB. Several animal studies have demonstrated that intraperitoneal injection of LPS elevated oxidative stress, and that pretreatment with antioxidants before LPS challenge ameliorated oxidative damage by decreasing the production of ROS (17-19).

Our previous studies have shown that the supplementation of *kimchi*-derived 3-(4′-hydroxyl-3′,5′-dimethoxyphenyl) propionic acid has anti-oxidative and hypolipidemic effects in rats and rabbits (20-22). Thus, the aim of this study was to determine the effect of chemically synthesized 3-(4′-hydroxyl-3′,5′-dimethoxyphen-

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yl) propionic acid (HDMPPA, Fig. 1) on oxidative stress in LPS-stimulated RAW 264.7 murine macrophage cell line. In this study we observed the suppressive effect of HDMPPA on NO production and its ability to increase GSH concentrations in LPS-stimulated RAW 264.7 macrophges.

MATERIALS AND METHODS

Cell culture and treatment

The murine macrophage cell line RAW 264.7 was obtained from ATCC and cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Cells in 10 mm dishes (5×10^6 cells/dish) or 24 well plates (8×10^5 cells/well) were pre-incubated with and without indicated concentrations of HDMPPA (100, 200, 400 μM) for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. Untreated cells were used as a negative control without LPS treatment, while positive control cells were activated with LPS but not treated with HDMPPA.

Cell viability

Cell viability was assessed by measuring the uptake of the supravital dye neutral red by viable cells according to the procedure of Fautz et al. (23). Cells (8×10^5 cells/well) in 24-well plates were preincubated with and without indicated concentrations of HDMPPA for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. The medium was removed and replaced with 0.5 mL of fresh medium containing 1.14 mmol/L neutral red. After incubation for 3 h, the medium was removed and the cells were washed twice with phosphate buffered saline solution (PBS, pH 7.4). The incorporated neutral red was released from the cells by incubation for 15 min at room temperature in the presence of 1 mL of the cell lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L dithiothreitol and Triton X-100 (1%, v/v)] containing acetic acid (1%, v/v) and ethanol (50%, v/v). To measure the dye taken up, the cell lysis products were centrifuged and absorbance of the supernatant was measured spectrophotometrically at 540 nm.

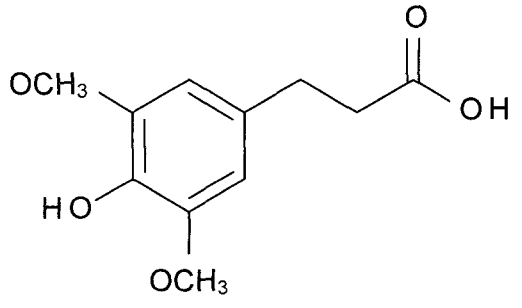

Fig. 1. Structure of 3-(4′-hydroxyl-3′,5′-dimethoxyphenyl) propionic acid.

NO production

The nitrite accumulated in the culture medium is an indicator of NO production as assayed using the Griess reaction (24). Cells (8×10^5/well) in 24-well plates were preincubated with and without indicated concentrations of HDMPPA for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. One hundred μL of each medium supernatant was mixed with 50 μL of 1% sulphanilamide (in 5% phosphoric acid) and 50 μL of 0.1% naphthylenediamine dihydrochloride and then incubated at room temperature for 10 min. The absorbance at 550 nm was measured with $NaNO_2$ and a serial dilution standard curve was used to estimate nitrite production.

Lipid peroxidation

Lipid peroxidation was quantified by measuring the production of TBARS as described by Fraga et al. (25). Cells (8×10^5/well) in 24-well plates were preincubated with and without indicated concentrations of HDMPPA for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. Two hundred μL of each medium supernatant was mixed with 400 μL of TBARS assay solution and then boiled at 95°C for 30 min. The absorbance at 532 nm was measured with 1,1,3,3-tetraethoxypropane and a serial dilution standard curve was used to estimate TBARS expressed as nmole of malondialdehyde equivalents.

GSH concentration

GSH was measured by an enzymatic recycling procedure described by Tietze (26), in which GSH is sequentially oxidized by 5,5″-dithiobis (2-nitrobenzoic acid) and reduced by NADPH in the presence of glutathione reductase. Cells (5×10^6 cells) in 10 mm dishes were preincubated with and without indicated concentrations of HDMPPA for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. The medium was removed and the cells were washed twice with PBS. One mL of PBS was added and cells were scraped from the dishes and collected. Cell suspensions were sonicated 3 times for 5 s each time on ice and then cell sonicates were centrifuged at 4,500 rpm for 10 min. Four hundred μL of cell supernatant was mixed with 200 μL of 5% sulfosalicylic acid and then centrifuged at 4,500 rpm for 10 min. Fifty μL of supernatant was mixed with 100 μL of reaction mixture [100 mM sodium phosphate buffer with 1 mM EDTA (pH 7.5), 1 mM dithiolnitirobezene, 1 mM NADPH, 1.6 U glutathione reductase]. The rate of 2-nitro-5-thio-benzoic acid formation was monitored spectrophotometrically at 412 nm. GSH content was determined by comparision of the rate to a standard curve generated with known

amount of GSH.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein was extracted with slight modification of the method of Dignam et al. (27). Cells (5×10^6 cells) in 10 mm dishes were preincubated with and without indicated concentrations of HDMPPA for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. Cells were lysed with buffer containing 0.6% igepal, 0.15 M NaCl, 10 mM Tris pH 7.9, 1 mM EDTA and 0.1% protease inhibitor cocktail; mixed, kept on ice for 5 min, and centrifuged at 2,300 rpm for 5 min at 4°C. Pelleted nuclei were resuspended in 50 μL of extraction buffer [10 mM Hepes, pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 1 mM DTT, and 0.33% protease inhibitor cocktail]. Following gentle mixing and keeping on ice for 20 min, samples were centrifuged at 2,300 rpm for 5 min at 4°C. The supernatant fractions were transferred to new tubes and stored at -70°C. Protein concentration was determined by the Bradford assay (28). For the EMSA, NFκB-specific oligonucleotide was end-labeled with [r -^{32}P]-ATP using T_4 polynucleotide kinase (Promega, Madison, WI, USA) and purified using a microspin G-25 column (Amersham Inc., Piscataway, NJ, USA). An EMSA was performed according to Promega's instruction manual. Five mg of nuclear protein, 2 μL binding buffer, 1 μL ^{32}P-labeled NFκB, and loading buffer were incubated for 30 min at room temperature. In competition assays, a 100-fold excess unlabeled NFκB was added 10 min before addition of radiolabeled probe. DNA-protein complexes were separated from unbound DNA probes by electrophoresis through 4% polyacrylamide gel by using 0.5X TBE as the running buffer. The gels were dried and exposed to an X-ray film for 2 h at room temperature and the bands were quantitated by a phospho imager (Packard, USA).

Statistical analysis

All data are expressed as the means±SD. The statistical analyses were performed on an SPSS program. One-way ANOVA and Duncan's multiple range test were used to examine the difference between groups. P values <0.05 were considered significant, if not otherwise stated.

RESULTS AND DISCUSSION

NO production

Multiple studies have implicated elevated NO production in activated macrophages in inflammation, the development of atherosclerosis, and cancer (4,6,11). This study was designed to investigate the effect of HDMPPA on NO production and intracellular oxidative stress, and the inhibition of NFκB activation as its possible mechanism of antioxidant activity in LPS-stimulated RAW 264.7 murine macrophage cells. LPS treatment significantly elevated nitrite generation, the oxidative product of NO, above that of LPS-untreated negative controls, which has also been documented by other investigators (13,14,16). HDMPPA at a 400 μM concentration suppressed NO production to 76% of the LPS-treated control (Fig. 2). Cell viability was >95% at the concentrations used, as assessed by the neutral red assay, which clearly demonstrates that HDMPPA suppression of NO production is not due to cell death.

NO is involved in phagocytosis at the physiological level. However, NO can lead to amplification of inflammation and tissue injury when produced in large amounts. NO reacts rapidly with superoxide to form peroxynitrite, a powerful oxidants, that damages many biological molecules, including DNA (29,30), and upregulates DNA-binding activity of NFκB in macrophages activated by pro-inflammatory stimulants (6). Pre-incubation of the cells with HDMPPA before LPS treatment did not affect TBARS status (Fig. 3), suggesting that either it does not attenuate lipid peroxidation in LPS-stimulated macrophages or that TBARS is not a sensitive method to analyze the status of intercellular oxidative stress in cell systems.

GSH concentration

Since GSH plays an important role in the protection of cells against oxidative stress, we also measured the

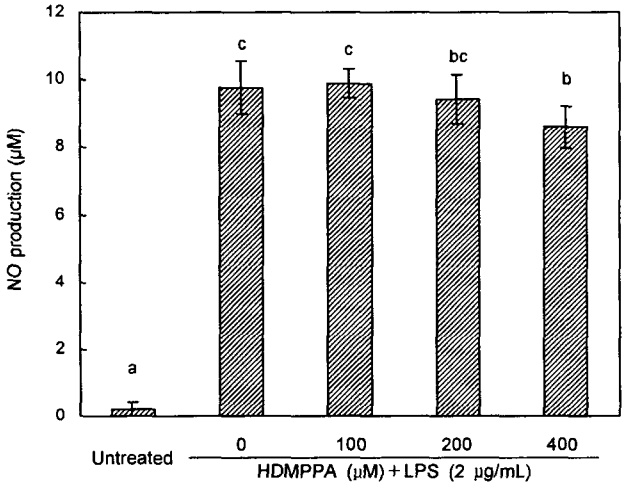

Fig. 2. Inhibitory effect of HDMPPA on NO production in LPS-stimulated RAW 264.7 cells. Cells (4×10^5/plate) in 24-well plates were first incubated with and without indicated concentrations of HDMPPA for 2 hr, and then incubated with LPS (2 μg/mL) for 20 hr. Untreated is negative control without LPS treatment. Data represent the means±SD of triplicate experiments. Values sharing same superscript are not significantly different at $p<0.05$.

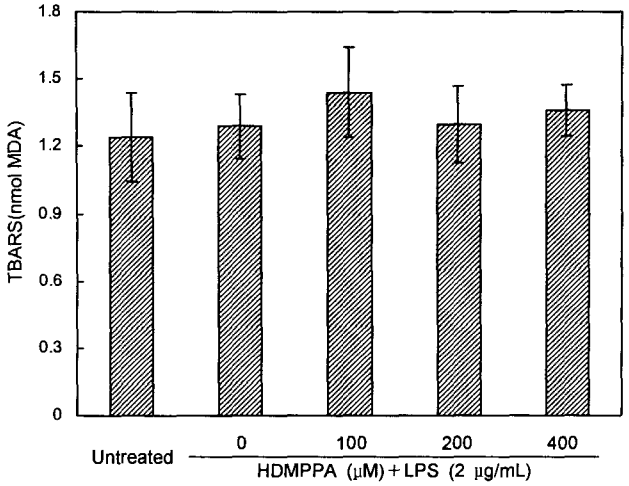

Fig. 3. Inhibitory effect of HDMPPA on TBARS generation in LPS-stimulated RAW 264.7 cells. Cells (4×10^5 cells) in 24-well plates were first incubated with and without indicated concentrations of HDMPPA for 2 hr, and then incubated with LPS (2 μg/mL) for 20 hr. Untreated is negative control without LPS treatment. Data represent the means±SD of triplicate experiments.

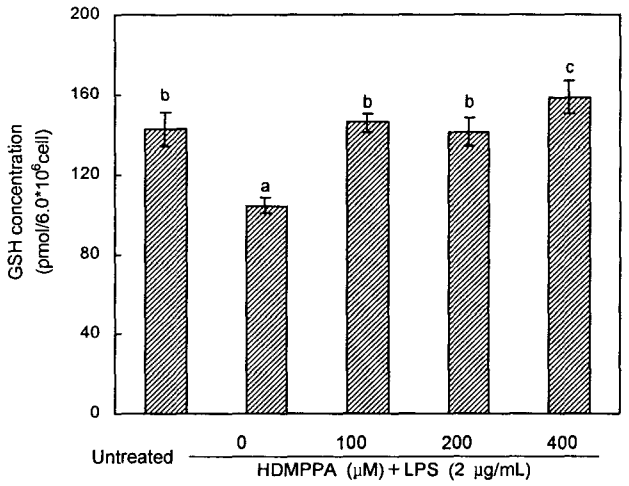

Fig. 4. Effect of HDMPPA on GSH concentration in LPS-stimulated RAW 264.7 cells. Cells (5×10^6 cells) in 10 mm dishes were first incubated with and without indicated concentrations of HDMPPA for 2 hr, and then incubated with LPS (2 μg/mL) for 20 hr. Untreated is negative control without LPS treatment. Data represent the means±SD of triplicate experiments. Values sharing same superscript are not significantly different at $p<0.05$.

level of GSH in LPS-stimulated RAW 264.7 cells. As shown in Fig. 4, exposure of RAW 264.7 cells to LPS significantly decreased GSH concentrations. GSH levels remained at the level of LPS-untreated controls when treated at 100 and 200 μM concentrations and were even higher with 400 μM HDMPPA. Depletion of GSH by oxi-LDL treatment and recovery from the oxidative injury by the pre-incubation with aged garlic extract possessing antioxidative activity was reported in cultured endothelial cells (31). Furthermore, Mizutani et al. (30). suggested that increased GSH level by phytoestrogen treatment in vascular smooth muscle cells was due to enhanced expression of the rate-limiting enzyme for GSH synthesis, glutamylcysteine synthetase. Thus, an increase in GSH content by HDMPPA treatment could lead to decreased intracellular oxidative stress, and thus is part of the mechanism for the antioxidative effect of HDMPPA.

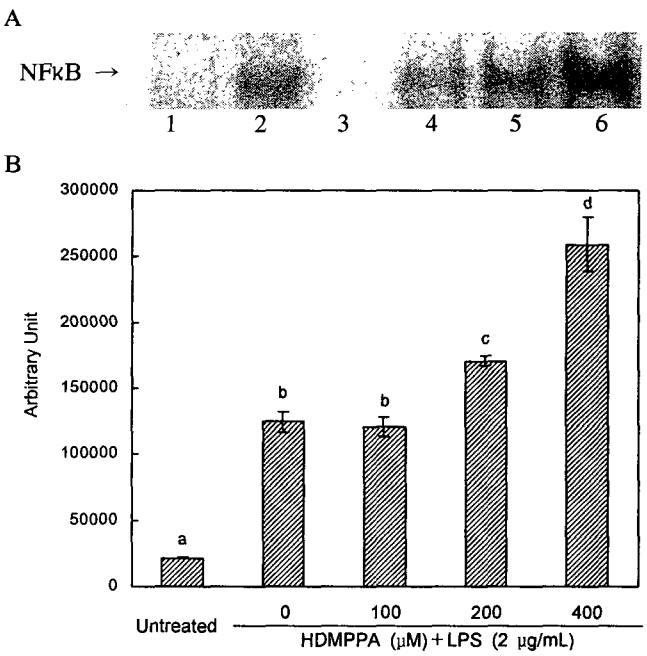

Fig. 5. Effect of HDMPPA on NFκB activity in LPS-stimulated RAW 264.7 cells. Cells (5×10^6 cells) in 10 mm dishes were first incubated with and without indicated concentrations of HDMPPA for 2 hr, and then incubated with LPS (2 μg/mL) for 20 hr. Untreated is negative control without LPS treatment. (A) DNA binding activity of NFκB was performed by EMSA. (B) Values are expressed as relative intensity of radioactivity. Data represent the means±SD of triplicate experiments. Values sharing the same superscript are not significantly different at $p<0.05$.

DNA binding activity of NFκB

Early works implicated ROS in LPS-stimulated NFκB activation, which was largely based on the suppression of both oxidative stress and NFκB activation by antioxidants (13-15). Specific DNA binding of NFκB using EMSA showed that LPS treatment of RAW 264.7 cells enhanced NFκB activation (Fig. 5A, lane 2). The band representing the nuclear extract was competitively inhibited by 100-fold excess of unlabelled NFκB oligonucleotide (Fig. 5A, lane 3). Pre-incubation of cells with 100~400 μM concentrations of HDMPPA up-regulated NFκB activity in a dose-dependent manner (Fig 5B). The concentrations of HDMPPA used in this study did not affect cell viability. We used 5 g of nuclear protein extracted from macrophages treated with different levels

of HDMPPA and LPS. Thus, the differences in NFκB activities among treatments were not due to differences in cell number per treatment. Based on the observations made in this study, the up-regulation of LPS NFκB activation by HDMPPA in RAW 264.7 cells appears to be a different behavior from that of antioxidants. It has been reported that carnosol (13), an antioxidant in rosemary, and Se (15) can specifically prevent activation of NFκB by LPS in RAW 264.7 cells. Our previous studies using rats and rabbits confirmed strong antioxidative activity of HDMPPA present in *kimchi*. However, chemically synthesized HDMPPA in this *in vitro* study showed very weak antioxidative activity, which might be due to differences in the conformational form between native and synthesized HDMPPA. In summary, HDMPPA may attenuate intracellular oxidative stress by directly scavenging NO and delaying the consumption of cellular GSH. Further studies to synthesize the derivatives of HDMPPA in *kimchi* with strong antioxidative and anti-inflammatory activity are needed.

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