Bitter Gourd Suppresses Lipopolysaccharide-Induced Inflammatory Responses

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Bitter gourd (*Momordica charantia* L.) is a popular tropical vegetable in Asian countries. Previously it was shown that bitter gourd placenta extract suppressed lipopolysaccharide (LPS)-induced TNFα production in RAW 264.7 macrophage-like cells. Here it is shown that the butanol-soluble fraction of bitter gourd placenta extract strongly suppresses LPS-induced TNFα production in RAW 264.7 cells. Gene expression analysis using a fibrous DNA microarray showed that the bitter gourd butanol fraction suppressed expression of various LPS-induced inflammatory genes, such as those for TNF, IL1α, IL1β, G1p2, and Ccl5. The butanol fraction significantly suppressed NFκB DNA binding activity and phosphorylation of p38, JNK, and ERK MAPKs. Components in the active fraction from bitter gourd were identified as 1-α-linolenoyl-lysophosphatidylcholine (LPC), 2-α-linolenoyl-LPC, 1-lynoleoyl-LPC, and 2-linoleoyl-LPC. Purified 1-α-linolenoyl-LPC and 1-linoleoyl-LPC suppressed the LPS-induced TNFα production of RAW 264.7 cells at a concentration of 10 μg/mL.

KEYWORDS: Bitter gourd; inflammation; RAW 264.7 cells; NFκB; MAPKs; DNA microarray; linolenoyl-lysophosphatidylcholine; linoleoyl-lysophosphatidylcholine

INTRODUCTION

Bitter gourd is a common vegetable in tropical areas of Asia and Africa. It has been traditionally eaten in Okinawa prefecture and has recently become popular throughout Japan. The unripe fruit, characterized by a typically bitter taste, is expected to contribute to maintenance of health. Bitter gourd also has been traditionally used as a bitter stomachic and an antidiabetic.

Bitter gourd extract or juice was reported to reduce the blood glucose level in streptozotocin- or alloxan-induced diabetic rats (*1–3*). Recently, some cucurbitane triterpenoids from bitter gourd were shown to have a hypoglycemic effect on induced diabetes in mice (*4*). However, although the results of some clinical studies suggest that bitter gourd reduces blood glucose levels of patients with type 1 or type 2 diabetes mellitus, its efficacy has not yet been fully demonstrated (*5–7*). Bitter gourd was also shown to have a liver triglyceride-lowering effect and a suppressive effect on adipocyte hypertrophy in rats (*8–10*). Bitter gourd extract and the cucurbitane triterpenoids were reported to suppress skin carcinogenesis in mice (*11, 12*). Spontaneous tumorigenesis in SHN mice was suppressed by bitter melon water extract (*13*).

We previously screened the suppressive effect of vegetable ethanol extracts on cancer cell growth. Among the vegetables that we studied, bitter gourd was the most effective in inhibiting the growth of HL60 human leukemia cells (*14*). All of the pericarp, placenta, and seed extracts of unripe fruits of bitter gourd cultivars that we examined suppressed growth and induced apoptosis in HL60 cells (*15*). Suppression of inflammatory response can be expected to reduce development of cancer and inflammatory diseases. In our study on the preventive effect of bitter gourd on cancer and other lifestyle-related diseases, we found that bitter gourd placenta extract suppressed lipopolysaccharide (LPS)-induced TNFα production in RAW

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264.7 macrophage-like cells (*15*). Here we show that butanol extract from the bitter gourd placenta suppressed TNFα and other inflammatory gene expressions in RAW 264.7 cells. We identified the active components in bitter gourd placenta extract to be linoleoyl- and linolenoyl-lysophosphatidylcholines (LPCs). In this paper, we have first demonstrated the suppressive effect of linoleoyl- and linolenoyl-LPCs on inflammatory responses in macrophages.

MATERIALS AND METHODS

Materials. Bitter gourd (*Momordica charantia* L.) cultivar ('Sadowara 3') grown in Miyazaki, Japan, was kindly provided by the Miyazaki Agricultural Experiment Station. Bitter gourd juice was kindly provided from Miyazaki Nokyo Kajyu Co., Ltd.

Cells and Cell Culture. RAW 264.7 mouse macrophage-like cells (ATCC TIB-71) were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). RAW 264.7 cells were maintained in RPM1640 medium (Invitrogen Japan KK, Tokyo, Japan). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air in medium supplemented with 10% heat-inactivated fetal calf serum (FCS; ICN Biomedicals, Inc., Aurora, OH).

Extraction of Bitter Gourd. Placentas of unripened bitter gourd fruits were lyophilized and extracted with 80% ethanol. Ethanol extract of bitter gourd placenta was concentrated by an evaporator and lyophilized and then was dissolved in distilled water and partitioned with ethyl acetate. The water-soluble fraction was then partitioned with butanol. After removal of the solvent, the water-soluble and butanol fractions were dissolved in dimethyl sulfoxide just before being added to a culture medium of RAW 264.7 cells.

Determination of LPS-Induced TNFα Production in RAW 264.7 Cells. RAW 264.7 cells were seeded at 1×10^5 cells/mL on a 96-well plate, incubated for 24 h, and then treated with the bitter gourd fraction or purified LPC and 2 ng/mL LPS (from *Escherichia coli* serotype 0111: B4; Sigma-Aldrich Co., St. Louis, MO) for 6 h, after which we determined TNFα production and cell viability. The TNFα levels in the culture medium were determined by ELISA according to the manufacturer's instructions (eBioscience, Inc., San Diego, CA). Cell viability was spectrophotometrically determined using WST-1 reagent (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

DNA Microarray. DNA oligonucleotide probes were synthesized for detection of 204 genes related to the immune response using ProbeQuest software (Dynacom Co., Ltd., Chiba, Japan). Synthetic DNA oligonucleotide probes were installed as probes onto Genopal (Mitsubishi Rayon, Tokyo, Japan), which is composed of plastic hollow fibers. With this system oligonucleotide DNA probes are attached to a gel within the three-dimensional space of each hollow fiber (*16*).

RNA Isolation and DNA Chip Analysis. Total RNA was extracted from RAW 264.7 cells using the RNeasy Mini Kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions. RNA was then amplified using the MessageAmpII biotin-enhanced amplification kit (Applied Biosystems Japan, Tokyo, Japan), according to the manufacturer's instructions, and column purified. Biotinylated aRNA (5 μg) was fragmented using fragmentation reagents (Applied Biosystems Japan) and then incubated at 70 °C for 7.5 min. The fragmentation reaction was terminated by the addition of stop solution. Hybridization was carried out with a DNA microarray (Genopal) in 150 μL of hybridization buffer [0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20] and 5 μg of fragmented biotinylated aRNA at 65 °C overnight. After hybridization, the DNA microarray was washed twice in 0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20 at 65 °C for 20 min followed by washing in 0.12 M Tris-HCl/0.12 M NaCl for 10 min. The DNA microarray was then labeled with streptavidin-Cy5 (GE Healthcare Bio-Science KK, Tokyo, Japan). The fluorescent labeled-DNA microarray was washed for 5 min four times in 0.12 M Tris-HCl/0.12 M NaCl/0.05% Tween-20 at room temperature. Hybridization signal acquisition was performed using a DNA microarray reader adopting multibeam excitation technology (Yokogawa Electric Co., Tokyo, Japan) (*17*). The
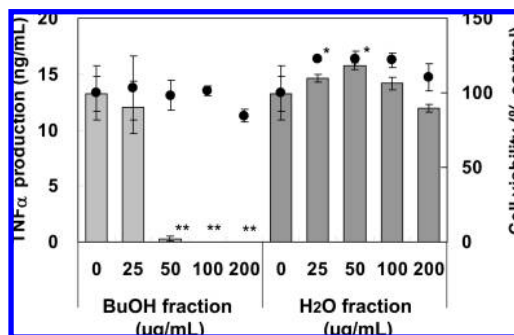

Figure 1. Bitter gourd butanol fraction suppressed LPS-induced TNFα production in RAW 264.7 macrophage-like cells. Cells were incubated overnight and then treated with LPS (2 ng/mL) and bitter gourd butanol extract for 6 h. The amount of TNFα (■) in the medium was then determined by ELISA and cell viability (●) determined using WST-1 reagent. All assays were performed in triplicate, and data are expressed as means ± SD. ∗, $P < 0.05$; ∗∗, $P < 0.01$ (two-sided); significantly different from the group treated with LPS alone by Bonferroni-type multiple *t* test.

DNA microarrays were scanned at multiple exposure times ranging from 0 to 10 s. Then the intensity values with the best exposure condition for each spot were selected.

Quantitative RT-PCR Analysis. Total RNA was extracted from RAW 264.7 cells using an RNeasy Midi Kit (Qiagen KK) according to the manufacturer's instructions. Quantitative real-time PCR (RT-PCR) was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems Japan, Ltd.) using SYBR Green Realtime PCR Master Mix (Toyobo Co. Osaka, Japan), according to the manufacturer's protocol. Sequences of primers used for quantitative RT-PCR were as follows: IL1α, 5′-attcggatcagcacct-3′ and 5′-ctcctc-ccgacgagta-3′; IL1β, 5′-caaccaacaagtgatattctccatg-3′ and 5′-gatcca-cactcctccagctgca-3′; IL18, 5′-aagatgattagcacacatgcg-3′ and 5′-tctataaat-catgcagcctcgg-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-cctggagaaacctgccaagtatg-3′ and 5′-agagtgggagttgctgttgaagtc-3′. The relative amount of each transcript was normalized to the amount of GAPDH transcript in the same cDNA.

Determination of NFκB and C/EBPβ DNA Binding Activity. RAW 264.7 cells (1×10^5 cells/mL) were treated with the bitter gourd butanol fraction for 6 h and with 2 ng/mL LPS for 30 min. Nuclear extract from the cells was then prepared by a Nuclear Extraction Kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). The protein concentration of the extract was determined by a Bradford-based assay (Bio-Rad protein assay, Bio-Rad Laboratories, Richmond, CA). DNA binding activity of NFκB in 0.5 μg of protein of each nuclear extract was determined using a TransAM NFκB Chemi kit (Active Motif). NFκB p65 bound to the immobilized oligonucleotide containing a p65-binding site was detected by ELISA with a chemiluminescent reagent according to the manufacturer's instructions. DNA binding activity of C/EBPβ using 2 μg of protein from each nuclear extract was determined using a TransAM C/EBPβ kit (Active Motif). C/EBPβ bound to the immobilized oligonucleotide containing the C/EBP-binding site was spectrophotometrically detected by ELISA according to the manufacturer's instructions. All assays were performed in triplicate, and data are expressed as means ± SD.

Western Blotting. RAW 264.7 cells (1×10^5 cells/mL) were treated with the bitter gourd butanol fraction for 6 h and with LPS for 30 min. Cells were lysed in a loading buffer (10 mM Tris-HCl, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, and 10% glycerol). Whole cell lysates were separated by 12% SDS−polyacrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane (GE Healthcare U.K. Ltd., Buckinghamshire, U.K.). MAP kinases (MAPKs) and phospho-MAPKs were detected using antibodies [rabbit anti-MAPKs (p38, JNK and ERK(p44/42) (Cell Signaling Technology, Danvers, MA)] and antiphopho-MAPKs [phospho-p38, phospho-JNK, phospho-ERK(p44/42) (Cell Signaling Technology)] and ECL Plus detection system (GE Healthcare).

Table 1. Bitter Gourd Butanol Fraction Suppressed the LPS-Induced Gene Expressions in RAW 264.7 Cells[a]

GenBank accession no.	gene symbol	gene name	signal of control cells	-fold change in LPS-treated cells		
				LPS alone	50 μg/mL bitter gourd	100 μg/mL bitter gourd
NM_015783	G1p2	interferon, α-inducible protein	429.9 ± 46.8	256.5 ± 19.23	5.07 ± 0.96	2.57 ± 0.58
NM_008361	Il1b	interleukin 1, β	325.4 ± 16.9	255.7 ± 12.07	2.29 ± 0.56	1.08 ± 0.26
NM_013653	Ccl5	chemokine (C−C motif) ligand 5	411.9 ± 61.5	193.0 ± 13.40	1.51 ± 0.36	1.04 ± 0.27
NM_010554	IL1a	interleukin 1, α	134.8 ± 49.5	168.0 ± 35.79	2.60 ± 1.17	1.27 ± 0.26
NM_007707	Socs3	suppressor of cytokine signaling 3	126.5 ± 24.2	68.3 ± 28.60	1.76 ± 0.61	1.26 ± 0.18
NM_008330	Ifi47	interferon γ inducible protein 47	332.3 ± 35.9	49.0 ± 12.64	0.53 ± 0.43	0.43 ± 0.08
NM_009971	Csf3	colony-stimulating factor 3 (granulocyte)	413.2 ± 14.2	42.3 ± 3.85	0.78 ± 0.29	0.76 ± 0.16
NM_011198	Ptgs2	prostaglandin-endoperoxide synthase 2	2629.8 ± 166.4	30.1 ± 21.47	0.18 ± 0.02	0.11 ± 0.01
NM_018734	Gbp3	guanylate nucleotide binding protein 3	268.7 ± 12.5	29.7 ± 11.39	0.69 ± 0.55	0.75 ± 0.11
NM_018738	Igtp	interferon γ induced GTPase	2606.3 ± 306.0	29.6 ± 4.89	0.57 ± 0.39	0.37 ± 0.07
NM_013652	Ccl4	chemokine (C−C motif) ligand 4	204.4 ± 24.4	25.1 ± 3.28	0.38 ± 0.09	0.22 ± 0.04
NM_013693	Tnf	tumor necrosis factor	340.1 ± 17.1	20.1 ± 3.69	2.57 ± 0.72	2.17 ± 0.11
NM_010807	Marcksl1	MARCKS-like 1	3442.2 ± 449.5	20.0 ± 1.73	0.51 ± 0.11	0.36 ± 0.12
NM_013683	Tap1	transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	1453.7 ± 92.8	17.4 ± 3.19	0.86 ± 0.02	0.27 ± 0.16
NM_009140	Cxcl2	chemokine (C−X−C motif) ligand 2	3643.8 ± 53.3	16.5 ± 1.96	0.64 ± 0.03	0.54 ± 0.04
NM_009283	Stat1	signal transducer and activator of transcription 1	315.1 ± 34.0	16.3 ± 4.65	0.13 ± 0.13	0.03 ± 0.03
NM_010260	Gbp2	guanylate nucleotide binding protein 2	383.9 ± 20.2	15.9 ± 2.90	0.60 ± 0.41	0.84 ± 0.15
NM_011333	Ccl2	chemokine (C−C motif) ligand 2	9994.0 ± 630.3	14.9 ± 1.93	0.55 ± 0.09	0.36 ± 0.06
NM_008357	Il15	interleukin 15	347.3 ± 25.3	14.3 ± 2.01	0.95 ± 0.20	0.71 ± 0.19
NM_009742	Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a	7327.6 ± 249.9	12.9 ± 1.65	0.87 ± 0.14	0.46 ± 0.11
NM_007609	Casp11	caspase 11, apoptosis-related cysteine peptidase	913.2 ± 106.1	12.6 ± 2.35	1.83 ± 0.25	1.28 ± 0.17
NM_007534	Bcl2a1b	B-cell leukemia/lymphoma 2 related protein A1b	6924.0 ± 688.4	12.4 ± 0.96	0.68 ± 0.10	0.37 ± 0.09
NM_008360	Il18	interleukin 18	1991.9 ± 130.6	12.1 ± 2.98	0.79 ± 0.22	0.52 ± 0.10
NM_013654	Ccl7	chemokine (C−C motif) ligand 7	906.5 ± 69.5	12.0 ± 3.05	0.82 ± 0.57	0.42 ± 0.18
NM_027320	Ifi35	interferon-induced protein 35	1953.5 ± 218.1	11.9 ± 1.94	1.70 ± 0.30	1.06 ± 0.08
NM_010395	H2-T10	histocompatibility 2, T region locus 10	1002.8 ± 24.0	11.6 ± 2.14	0.91 ± 0.20	0.39 ± 0.06
NM_011593	Timp1	tissue inhibitor of metalloproteinase 1	343.1 ± 25.7	11.1 ± 1.98	0.88 ± 0.33	0.92 ± 0.21
NM_009421	Traf1	Tnf receptor-associated factor 1	951.1 ± 87.1	10.6 ± 2.88	1.04 ± 0.16	0.74 ± 0.14

[a] RAW 264.7 cells were treated with 2 ng/mL LPS and 0, 50, or 100 μg/mL bitter gourd butanol fraction (bitter gourd) for 6 h and subjected to customized DNA microarray (Genopal; Mitsubishi Rayon Co., Ltd.). The degree of change (-fold) was calculated as the normalized intensity in LPS-treated vs control cells. Values of gene expression are the means (av) and SE of triplicate cultures.

Identification of Components in Bitter Gourd Butanol Fraction. The butanol fraction of bitter gourd placenta was applied to a silica gel column (Wakogel C-200, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and eluted with chloroform/methanol/water (v/v/v) 15:3:1 (fraction 1), 10:3:1 (fraction 2), 65:35:10 (fraction 3), 6:4:1 (fraction 4), and 100% methanol (fraction 5) (18). Because fraction 3 but not the other fractions had a suppressive effect on LPS-induced TNFα production by RAW 264.7 cells, fraction 3 (125 μL) was applied on an HPLC column (Inertsil ODS-3 column, 250 mm × 20 mm i.d., 5 μm, GL Sciences, Inc. Tokyo, Japan) and eluted with 25% methanol (fractions 3-1−3-5) and 100% methanol (fractions 3-6 and 3-7) at a flow rate 5 mL/min for 55 min. The constituents were detected at 210 nm using a diode array detector (SPD-M10AVP, Shimadzu Corp., Kyoto, Japan).

Five microliters of the active fraction 3-6, which suppressed LPS-induced TNFα production in RAW 264.7 cells, was applied on an Inertsil ODS-3 column (150 mm × 2.1 mm, 3 μm, GL Sciences) and eluted with 0−100% CH$_3$CN containing 0.1% FA (linear gradient) at a flow rate 0.2 mL/min for more than 15 min at 40 °C. The eluate was introduced into an LCQclassic mass spectrometer (Thermo Fisher Scientific, Waltham, MA) and ionized by electrospray ionization (ESI). The spectra were recorded in the positive-ion mode. NMR spectra of fraction 3-6, including one-dimensional ^{1}H NMR, one-dimensional ^{13}C NMR, and two-dimensional NMR spectra such as DQF-COSY, TOCSY, HSQC, and HMBC, were recorded on an Avance 800 spectrometer with a CryoProbe (Bruker Biospin, Karlsruhe, Germany) in methanol-d_4 (Wako Pure Chemical Industries) at 298 K. The one-dimensional ^{31}P NMR spectrum and ^{1}H−^{31}P HMBC spectrum were measured using a BBO-Multi Probe on an Avance 800 spectrometer at 298 K.

Purification of 1-Linoleoyl- and 1-α-Linolenoyl-lysophosphatidylcholines from Standard Soybean LPC. L-α-LPC from *Glycine max* (soybean) (Sigma) was dissolved in 50% acetonitrile, applied to an Inertsil ODS-3 column (150 mm × 2.1 mm, 3 μm, GL Sciences), and eluted with 70% acetonitrile. After confirmation of the mass spectra,

peaks corresponding to 1-linoleoyl-LPC and 1-α-linolenoyl-LPC were collected as purified standards.

RESULTS

Bitter Gourd Butanol Fraction Suppresses Bacterial LPS-Induced Inflammatory Responses in RAW 264.7 Macrophage-like Cells. We previously showed that the ethanol extract of bitter gourd placenta suppressed LPS-induced TNFα production in RAW 264.7 macrophage-like cells (15). The placenta ethanol extract was fractionated with butanol-soluble and water-soluble fractions. **Figure 1** shows the effect of bitter gourd fractions on LPS-induced TNFα production in RAW 264.7 cells. RAW 264.7 cells were incubated with LPS and a bitter gourd fraction for 6 h. The butanol fraction strongly suppressed TNFα production but not the viability of RAW 264.7 cells. On the other hand, the water-soluble fraction did not suppress TNFα production in RAW 264.7 cells. We then determined the effect of the bitter gourd butanol fraction on the expression of TNFα and other genes related to inflammatory responses. We developed a fibrous DNA microarray carrying 204 genes that were related to inflammation, immunity, and housekeeping. **Table 1** shows the genes for which expression was induced >10-fold that of control cells by 6 h of LPS treatment. All gene expressions, including TNF expression, were strongly suppressed by 50 μg/mL of the bitter gourd butanol fraction (**Table 1**). Expression of 65 of the 204 genes carried on the DNA microarray was induced by LPS, and LPS-induced gene expression was reduced by 50 μg/mL of the bitter gourd butanol fraction (data not shown). We also performed quantitative RT-PCR analysis of the expression of the representative inflammatory genes IL1α, IL1β, and IL18. Results showed that the
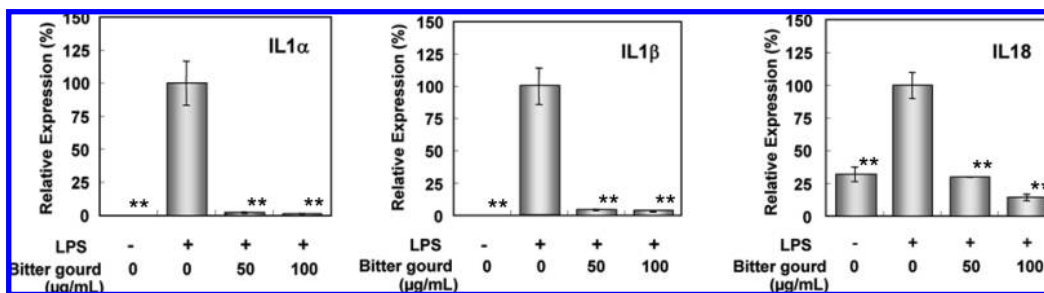
Figure 2. Bitter gourd butanol fraction (bitter gourd) suppressed LPS-induced gene expression in RAW 264.7 cells. Cells were treated with LPS and bitter gourd butanol extract for 6 h. Expression levels determined by RT-PCR were normalized to GAPDH and plotted relative to those in cells treated with LPS (control). All assays were performed in triplicate, and data are expressed as means ± SD. ∗∗, $P < 0.01$ (two-sided); significantly different from the group treated with LPS alone by Bonferroni-type multiple t test.
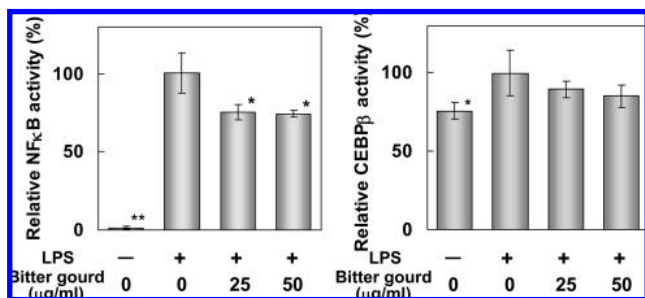
Figure 3. Bitter gourd butanol fraction (bitter gourd) suppressed NFκB DNA binding activity in RAW 264.7 cells. Cells were treated with bitter gourd butanol fraction for 6 h and LPS for 30 min. DNA binding activity in the nuclear extract was determined using TransAM NFκB and C/EBPβ kits (active motif). Relative NFκB or C/EBPβ binding activity is expressed as a percentage of that of the group treated with LPS alone (control). All assays were performed in triplicate. Data are expressed as means ± SD. ∗, $P < 0.05$; ∗∗, $P < 0.01$ (two-sided); significantly different from the group treated with LPS alone by Bonferroni-type multiple t test.
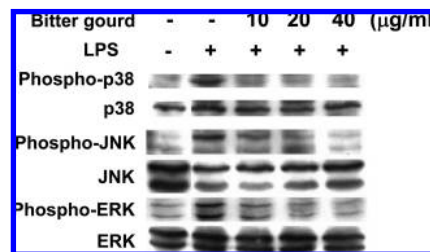

Figure 4. Bitter gourd butanol fraction (bitter gourd) suppressed the phosphorylation of MAPKs in RAW 264.7 cells. Cells were treated with bitter gourd butanol extract for 6 h and LPS (2 ng/mL) for 30 min. Protein levels of MAPKs and phosphorylated MAPKs were examined by Western blot analysis.

bitter gourd butanol fraction significantly suppressed expression of IL1α, IL1β, and IL18 in RAW 264.7 cells at a concentration of 50 μg/mL (**Figure 2**).

Bitter Gourd Butanol Fraction Suppresses Activities of Transcriptional Factor NFκB and MAP Kinases (MAPKs). LPS induced inflammatory gene expression through induction of NFκB and C/EBP transcriptional activities. To elucidate the mechanism of the suppressive effect of the bitter gourd butanol fraction, we determined its effect on LPS-induced NFκB and C/EBP DNA binding activities in RAW 264.7 cells. Cells were first treated with bitter gourd butanol extract for 6 h and then treated with LPS for 15 min. The NFκB p65 and C/EBPβ promoter binding activities in the nuclear extracts were determined by monitoring their affinity to immobilized oligonucleotides containing NFκB p65 and C/EBPβ consensus binding sites, respectively, and detected by ELISA. The bitter gourd butanol fraction significantly suppressed the DNA binding activity of NFκB p65 but not that of C/EBPβ (**Figure 3**). LPS-induced inflammatory responses in macrophages are also known to be through the activation of MAPKs. We treated cells with the bitter gourd butanol fraction for 6 h and then with LPS for 15 min and determined the phosphorylation of p38, JNK, and ERK MAPKs in the cells. The bitter gourd butanol fraction suppressed the LPS-induced phosphorylation of p38, JNK, and ERK MAPKs in a dose-dependent manner (**Figure 4**).

Bitter Gourd Juice Suppresses the Anticollagen Antibody and LPS-Induced Arthritis of Balb/c Mice. To determine the suppressive effect of bitter gourd on LPS-induced inflammation in vivo, we orally administered 100% bitter gourd juice to Balb/c

mice with arthritis that was induced by anticollagen antibody and LPS. Bitter gourd juice (200 μL/mouse/day) was orally administered daily after induction of arthritis. Thickness of the hind feet was significantly reduced after an 8-day administration of bitter gourd juice, but not after a 12-day administration of control water containing 3% glucose (**Figure 5**).

Identification of Linoleoyl- and Linolenoyl-LPCs As Active Components in the Bitter Gourd Butanol Fraction. The bitter gourd butanol fraction was further fractionated into five fractions (fractions 1−5) by silica gel column chromatography. Fraction 3 but not other fractions suppressed LPS-induced TNFα production in RAW 264.7 cells (**Figure 6**). Fraction 3 was then applied on HPLC and eluted with 25% methanol (fractions 3-1−3-5) and 100% methanol (fractions 3-6 and 3-7). Fraction 3-6 was the only active faction that suppressed the LPS-induced TNFα production in RAW 264.7 cells after HPLC. Because four peaks were detected for fraction 3-6 at 210 nm, we analyzed the constituents by mass spectrometry and NMR spectroscopy. Among the four peaks, the first and second peaks were detected at m/z 518 and the other two peaks, at m/z 520 in LC/ESI-MS. The ion at m/z 518 gave product ions at m/z 500 ($[M - H_2O]^+$) and 184, and the ion at m/z 520 gave product ions at m/z 502 ($[M - H_2O]^+$) and 184 in the MS/MS experiments (**Figure 7**). These dehydrated product ions lost 59 and gave ions at m/z 441 and 443, respectively, by MS^3 experiments (**Figure 7**).

The ^{1}H NMR spectrum of fraction 3-6 suggested the presence of double bonds by signals at 5.27−5.39 ppm in addition to methylene at 1.25−1.38 ppm and methyl signals at 0.88−0.98 ppm. The signals of ^{1}H at the double bond coupled with signals at 2.07 and 2.80 ppm, which correspond to ^{1}H at the allyl position of a double bond and ^{1}H at the allyl position between two double bonds, respectively. ^{13}C NMR signals at 175.0 and 175.3 ppm revealed the presence of carbonyls. These carbonyls had a correlation with ^{1}H at 2.35 ppm in HMBC, and this ^{1}H signal coupled with a signal at 1.61 ppm, which showed
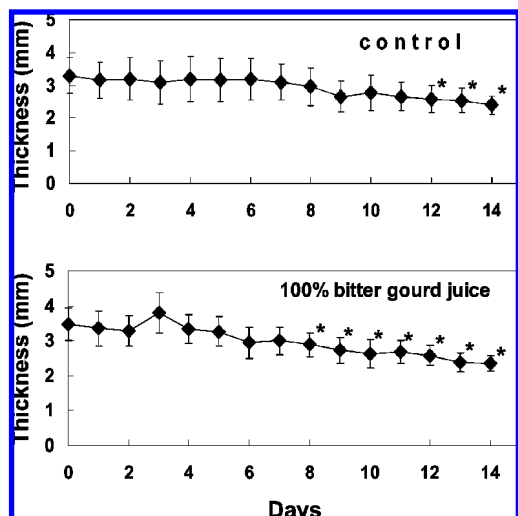
Figure 5. Effect of bitter gourd juice on anticollagen antibody and LPS-induced arthritis in Balb/c mice. Arthritis was induced by anticollagen antibody and LPS in Balb/c mice. Then the mice were given 200 μL of 100% bitter gourd juice or 3% glucose in water daily. Hind-foot thickness was determined by a slide caliper. Values are expressed as means $\pm$ SE of 10 mice in each group. $*$, $P < 0.05$ (two-sided); significantly different from thickness on day 0 by a Bonferroni-type multiple *t* test.

coupling with methylene protons. This set of signals indicated the presence of an unsaturated fatty acid moiety in the molecule. In addition to these signals, the ^{1}H NMR of fraction 3-6 exhibited geminal proton signals at 4.10 (1H) and 4.16 (1H) ppm on a spin system with signals at 3.84−3.92 (2H) and 3.96 (1H) ppm, which suggested the presence of glycerol. On ^{1}H NMR, a singlet at 3.21 (13.5H) ppm and broad multiplets at 3.63 (3H) and 4.28 (3H) ppm were also observed. Between the two multiplets strong coupling was observed. The ^{1}H chemical shift of 3.21 ppm and its bound ^{13}C chemical shift of 54.6 ppm suggested methyl groups on a positively charged nitrogen. HMBC correlation between ^{13}C at 54.6 ppm and ^{1}H at 3.63 ppm indicated the presence of a choline moiety.

A signal of phosphate was detected in the ^{31}P NMR spectrum of fraction 3-6. Connection of the phosphorylcholine moiety to the 3-position of glycerol was confirmed by decoupling of the phosphate ^{31}P, which simplified or sharpened the multiplets at 3.63, 3.84−3.92, and 4.28 ppm. Besides, correlations between phosphate ^{31}P and [1]Hs at 3.63, 3.84−3.92, and 4.28 ppm were clearly detected in the [31]P−^{1}H HMBC experiment.

The major carbonyl signal at 175.3 ppm had HMBC correlation with the geminal [1]Hs at 4.10 and 4.16 ppm, which suggested that the 1-position of glycerol was esterified with unsaturated fatty acid. On the other hand, ^{1}H on the 2-position of glycerol at 3.96 ppm had no correlation with carbonyl, and the ^{1}H chemical shift and ^{13}C chemical shifts at 69.8 ppm indicated this position to be free. Thus, the major compound in this fraction was identified as 1-acyl-LPC. In addition of these signals, minor signals of glycerol were detected in the ^{1}H NMR spectrum at 3.67 (0.5H), 3.71 (0.5H), 3.99 (1H), and 4.99 (0.5H) ppm at about a half-magnitude of the major ones. HMBC correlation between the minor carbonyl at 175.0 ppm and ^{1}H of the 2-position of glycerol at 4.99 ppm proved that the minor component was 2-acyl-LPC.

As for the fatty acid composition of the LPCs, chemical shifts of major ^{13}C NMR signals in the olefinic region coincided with those of α-linolenic acid (*19*). Signals of the 18:2 type fatty acid, for example, linoleic acid, were also detected as a minor fatty acid component. These results of NMR analysis revealed that the compounds in fraction 3-6 with a molecular weight of 518 were 1-α-linolenoyl-LPC (**Figure 8**, **1**) and 2-α-linolenoyl-LPC (**Figure 8**, **2**). Components with a molecular weight of 520 were estimated to be 1-linoleoyl-LPC (**Figure 8**, **3**) and 2-linoleoyl-LPC (**Figure 8**, **4**). Cochromatography of bitter gourd fraction 3-6 with soybean 1-acyl-LPCs confirmed that the second peak of fraction 3-6 was 1-α-linolenoyl-LPC and the fourth peak, 1-linoleoyl-LPC.

To confirm the suppressive effect of linoleoyl- and α-linolenoyl-LPCs on LPS-induced inflammatory responses in RAW 264.7 cells, we used 1-linoleoyl-LPC and 1-α-linolenoyl-LPC purified from commercially available soybean LPC for the assay. The 1-linoleoyl-LPC derived from soybean LPC significantly suppressed TNFα production in RAW 264.7 cells without suppressing cell viability at a concentration of 10 μg/mL (**Figure 9**). The purified 1-α-linolenoyl-LPC also strongly suppressed TNFα production of RAW 264.7 cells and slightly suppressed cell viability at a concentration of 10 μg/mL (**Figure 9**). On the other hand, the active fraction 3-6 purified from the bitter gourd butanol fraction significantly suppressed TNFα production in RAW 264.7 cells at a concentration of 10 μg/mL (**Figure 9**).

DISCUSSION

The butanol fraction of bitter gourd placenta significantly suppressed LPS-induced TNFα production in RAW 264.7
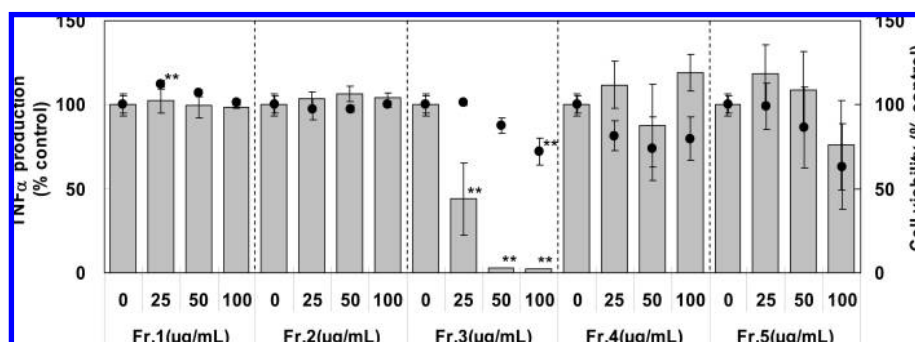

Figure 6. Fraction 3 after silica gel column chromatography suppressed the LPS-induced TNFα production in RAW 264.7 cells. Bitter gourd butanol fraction was applied to a silica gel column and eluted with chloroform/methanol/water (v/v/v) 15:3:1 (fraction 1), 10:3:1 (fraction 2), 65:35:10 (fraction 3), 6:4:1 (fraction 4), and 100% methanol (fraction 5). Cells were treated with LPS (2 ng/mL) and bitter gourd fractions for 6 h. The amount of TNFα (gray bars) in the medium was then determined by ELISA and cell viability ($\bullet$) determined using WST-1 reagent. All assays were performed in triplicate, and data are expressed as means $\pm$ SD. $**$, $P < 0.01$ (two-sided); significantly different from the group treated with LPS alone by Bonferroni-type multiple *t* test.

Bitter Gourd Suppresses LPS-Induced Inflammatory Responses

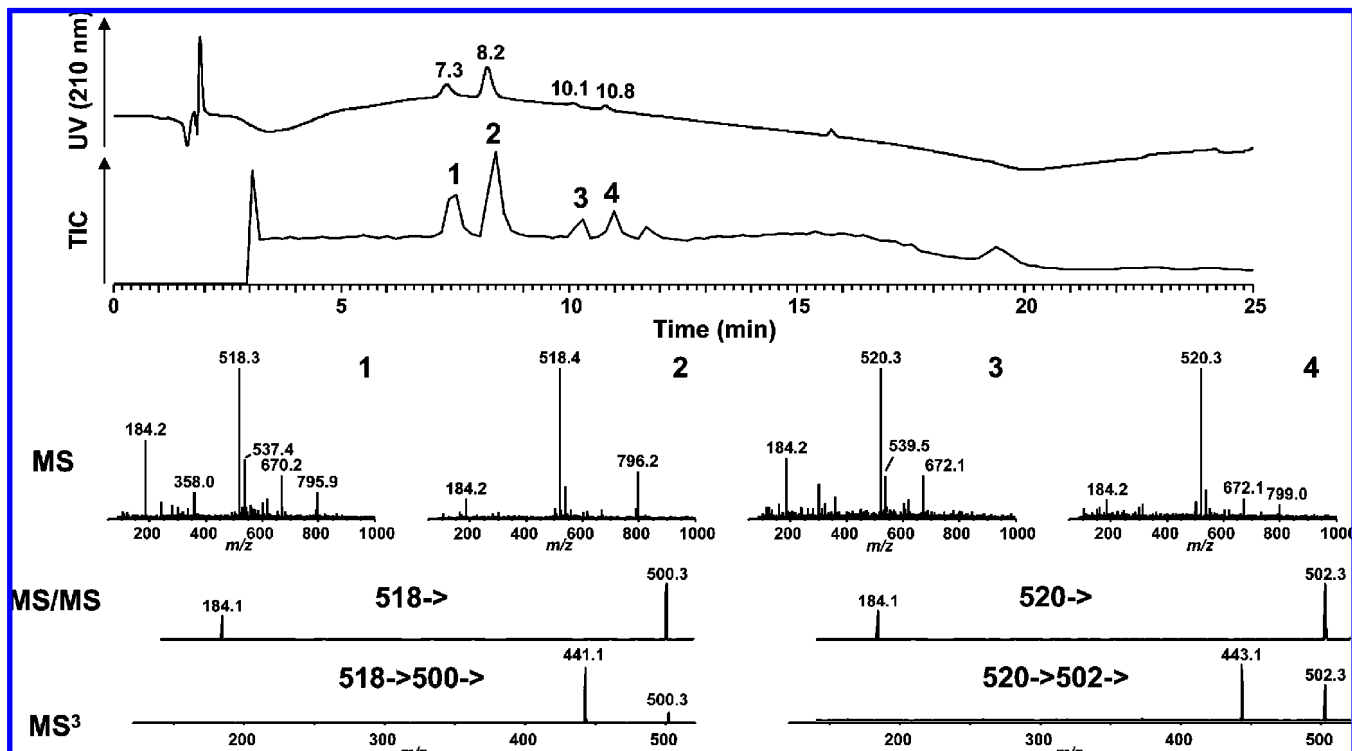*J. Agric. Food Chem.*, Vol. 56, No. 11, 2008 **4009**

Figure 7. LC-MS/MS analyses of fraction 3-6 of bitter ground placenta extract. Fraction 3-6 was applied on an Inertsil ODS-3 column (150 × 2.1 mm, 3 μm) at 40 °C. The gradient elution by 0.1% FA and CH_3CN containing 0.1% FA at 0.2 mL/min over 15 min gave four peaks. Those MS, MS/MS, and MS^3 spectra were analyzed. The MS/MS and MS^3 spectra were from the results of direct infusion of the sample.
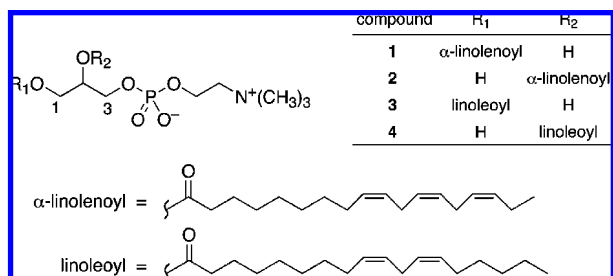

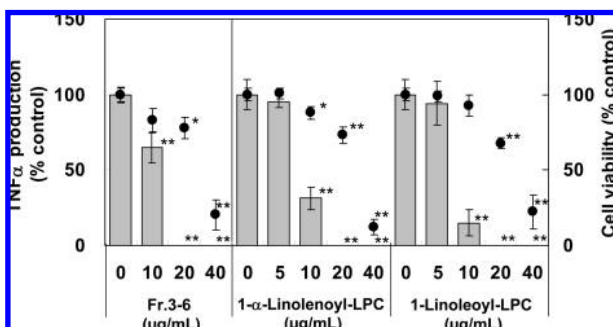**Figure 8.** Four components in active fraction 3-6.

Figure 9. Bitter gourd fraction 3-6 and purified lysophosphatidylcholines (LPCs) suppressed LPS-induced TNFα production in RAW 264.7 cells. Cells were incubated overnight and then treated with LPS (2 ng/mL) and bitter gourd fraction 3-6, 1-α-linolenoyl-LPC, or 1-linoleoyl-LPC for 6 h. The amount of TNFα (■) in the medium was then determined by ELISA and cell viability determined (●) using WST-1 reagent. All assays were performed in triplicate, and data are expressed as means ± SD. *, $P <$ 0.05; **, $P < 0.01$ (two sided); significantly different from the group treated with LPS alone by Bonferroni-type multiple *t*-test.

macrophage-like cells without inhibition of cell viability. To elucidate the anti-inflammatory effect of bitter gourd, we prepared a DNA microarray carrying genes related to inflam-

mation. We carefully selected 204 genes related to inflammation and immunity and housekeeping to be carried on a fibrous DNA microarray. IL1-IL7, IL9-IL13, IL15, IL16, IL18, Ccl1-Ccl9, CCR1-CCR9, and Csf1-Csf3 were included among these 204 genes. The DNA microarray was useful to detect not only an anti-inflammatory effect but also antiallergic effects of food components. LPS induced expression of 65 genes >3-fold that in control cells. The bitter gourd butanol fraction reduced LPS-induced gene expression almost to levels in control cells.

LPS induces inflammatory gene expression through the activation of MAPKs and transcriptional factors NFκB and C/EBP in macrophages (*20, 21*). The components that suppress inflammatory gene expression may inhibit some pathways. We previously isolated ergosterol and ergosterol peroxide from an edible mushroom and showed that the mushroom components suppressed the phosphorylation of MAPKs and DNA binding activities of NFκB and C/EBPβ at concentrations of 30 and 60 μM (*22*). The bitter gourd butanol fraction suppressed the activation of p38, JNK, and ERK MAPKs and NFκB but not C/EBPβ DNA binding activity.

We examined the effect of bitter gourd juice, which is available on the market, on LPS-induced arthritis in mice. Although the average thickness of the hind feet was not significantly different between groups of mice receiving either control glucose water or bitter gourd juice, arthritis improved earlier in those receiving the bitter gourd juice. Our result suggests that orally administered bitter gourd juice has an anti-inflammatory effect in vivo. Further study is necessary to confirm the anti-inflammatory effect of bitter gourd juice in vivo.

In our previous study we showed that bitter gourd pericarp, placenta, and seed extracts induced apoptosis in HL60 human leukemia cells (*15*). Several fractions from silica gel chromatography of bitter gourd ethyl acetate extract induced apoptosis in HL60 cells (unpublished data). On the other hand, only one fraction, which suppressed LPS-induced TNFα production in

RAW 264.7 cells, was found after silica gel column chromatography and HPLC of the bitter gourd placenta butanol fraction. Two of the four peaks detected in the isolated active fraction were identified as acyl position isomers of α-linolenoyl-LPC. The other two peaks were identified as isomers of linoleoyl-LPC. We confirmed that purified 1-α-linolenoyl- and 1-linoleoyl-LPCs from soybean equally suppressed LPS-induced TNFα production in RAW 264.7 cells. Thus, linolenoyl and linoleoyl types of LPCs in bitter gourd had a suppressive effect on LPS-induced inflammatory responses in RAW 264.7 cells. The effective doses of 1-α-linolenoyl-LPC and 1-linoleoyl-LPC were about the same as that of the active fraction, fraction 3-6, purified from the bitter gourd butanol fraction. 2-α-Linolenoyl-LPC and 2-linoleoyl-LPC probably possess a suppressive effect on LPS-induced inflammatory responses in RAW 264.7 cells as well as 1-α-linolenoyl-LPC and 1-linoleoyl-LPC.

Recently, α-linolenic acid was reported to suppress LPS-induced iNOS, COX-2, and TNFα expression in RAW 264.7 cells (*23*). We examined the effects of linoleic acid and α-linolenic acid on LPS-induced TNFα production, but the fatty acids did not inhibit LPS-induced TNFα production in RAW 264.7 cells (data not shown). LPC is a constituent of animal cell membrane. Linoleoyl- and linolenoyl-LPCs, which are easily incorporated into cells, are probably more effective in inhibiting the inflammatory gene expressions induced by LPS in RAW 264.7 cells.

Bitter gourd seeds are known to contain the 9c,11t,13t-conjugated linolenic acid, α-eleostearic acid (*24–26*). Conjugated linolenic acid of bitter gourd was reported to inhibit azoxymethane-induced colon carcinogenesis in rat and induce apoptosis in colon cancer cells (*24–26*). α-Eleostearic acid is scarcely contained in the placenta of bitter gourd unripe fruits (data not shown). However, α-eleostearic acid did not inhibit the LPS-induced TNFα production in RAW 264.7 cells (data not shown).

Thus, LPCs of bitter gourd were shown to suppress LPS-induced inflammatory responses in RAW 264.7 macrophage-like cells. The 1-α-linolenoyl-, 2-α-linolenoyl-, 1-linoleoyl-, and 2-linoleoyl-LPCs probably equally contribute to the suppressive effect of bitter gourd on LPS-induced inflammatory responses by inhibiting the activation of MAPKs and NFκB DNA binding activity. Our results suggest that bitter gourd suppresses LPS-induced inflammatory responses in vitro and in vivo.

ABBREVIATIONS USED

LPS, lipopolysaccharide; LPC, lysophosphatidylcholine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPKs, MAP kinases.

ACKNOWLEDGMENT

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