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ORIGINAL PAPER

Shikonin changes the lipopolysaccharide-induced expression of inflammation-related genes in macrophages

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Abstract We aimed to find candidate molecules possibly involved in the anti-inflammatory activity of shikonin (active compound of "Shikon") by analyzing its effects on gene expression of lipopolysaccharide (LPS)-treated THP-1 macrophages. Polysome-associated mRNAs (those expected to be under translation: translatome) from cells treated with LPS alone (LPS: 5 μg/mL), shikonin alone (S: 100 nM), or LPS plus shikonin (LPS&S) for 3 h were analyzed by DNA microarray followed by detection of enriched pathways/gene ontologies using the tools of the STRING database. Candidate genes in enriched pathways in the comparison of LPS&S cells vs. LPS cells were analyzed by reverse-transcription quantitative real-time PCR (RT-qPCR; 1, 2, and 3 h). DNA microarray showed shikonin significantly influences gene expression. Gene expression changes between LPS&S cells and LPS cells were compared to detect relevant proteins and/or mRNAs underlying its anti-inflammatory effects: shikonin down-regulated pathways which were upregulated in LPS cells, for example, 'innate immune response'. Within changed pathways, three genes were selected for RT-qPCR analyses as key candidates influencing inflammatory responses: CYBA (component of the superoxide-generating Nox2 enzyme), GSK3B (controller of cell responses after toll-like receptor stimulation), and EIF4E (a key factor of the eukaryotic translation initiation factor 4F complex that regulates abundance of other proteins involved in immune functions). All three mRNAs were decreased at 2 h, and CYBA continued low at 3 h relative to LPS cells. Given that shikonin decreased the expression of CYBA gene of Nox2, in addition to the direct inhibition of the Nox2 activity that we have previously shown, it is suggested that one of its anti-inflammatory mechanisms could be attenuation of oxidative stress.

Keywords Shikonin · Naphthoquinone · Translatome · DNA microarray · THP-1 macrophage · Gene ontology

Abbreviations

DMSO	Dimethylsulfoxide
FDR	False discovery rate
GO	Gene ontology
IL	Interleukin
LPS	Lipopolysaccharide
LPS cells	Cells treated with LPS alone
LPS&S cells	Cells treated with LPS and shikonin
S cells	Cells treated with shikonin alone
MAPK	Mitogen-activated protein kinases
NF-κB	Nuclear factor kappa B
NOS	Nitric oxide synthase
PPI	Protein–protein interaction
PMA	Phorbol myristate acetate
RT-qPCR	Reverse-transcription quantitative real-time PCR
ROS	Reactive oxygen species
TNF	Tumor necrosis factor

Lucia Satiko Yoshida and Tomohito Kakegawa contributed equally to this work.

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Introduction

Shikonin is one of the active naphthoquinone substances of the herbal medicine "Shikon" (*Lithospermum erythrorhizon*). Shikonin has anti-inflammatory, anti-tumor, antibacterial, and wound-healing effects in vitro and in vivo [1–3]. It chemically scavenges reactive oxygen species (ROS) such as superoxide anion (O_2^-) [4–6] and hydroxyl radical [5, 7] which can exacerbate inflammation. Biochemically, it inhibits the activities of several enzymes, including the phagocyte-type NADPH oxidase (Nox2, a major producer of O_2^-) [8, 9], nitric oxide synthases (NOS) [10], and kinases such as spleen tyrosine kinase [11], mitogen-activated protein kinases (MAPKs: p38, MAP2K1, ERK1), protein kinase A [10], and insulin-like growth factor 1 receptor [12]. Other enzyme activities affected by shikonins (shikonin and its derivatives: acetylshikonin and others) are: DNA topoisomerases [13–15], phospholipase C [16], PTEN (phosphatase and tensin homolog deleted on chromosome 10), protein tyrosine phosphatase non-receptor type 1 [17], cyclooxygenases (COX1 and 2) [18, 19], 5-lipoxygenase [18], and pyruvate kinase-M2 [20]. In addition, shikonin inhibits cellular calcium fluxes [16, 21], suggesting Ca^{2+} channels as possible targets as well.

Besides the inhibition of activities of the above enzymes, shikonins have also been reported to influence cellular gene/protein expression of inflammation-related factors: inducible NOS [19, 22, 23], tumor necrosis factor (TNF)-α [23–26], COX2, prostaglandin E_2 [19, 22, 23], interleukins (lowered IL-6 and IL-12 vs. increased IL-4 and IL-10; [26]), etc. Some of the inflammation-related factors were suggested to have their expressions lowered due to influence of shikonin on the nuclear factor (NF)-κB transcription factor [19, 22, 27]. Past gene-profiling studies have shown candidate genes with expressions influenced by shikonins: heat shock proteins (e.g., Hsp70) in U937 cells [28]; genes for chemotaxis, cell migration and cytokines (e.g., TNF-α, IL-1β, IL-4, C–C motif chemokine ligands CCL4 and CCL8) in lipopolysaccharide (LPS)-stimulated THP-1 monocytes [25]; and members of the nuclear orphan receptor 4a family in mast cells [29]. Despite the increasing amount of information about the possible pathways involved in the shikonin-elicited effects in various cells, it is still difficult to delineate the whole picture of its anti-inflammatory mechanisms.

Here, we aimed to elucidate the effects of shikonin on the cellular inflammatory response to LPS with focus on changes in gene expression and possible pathways involved in its anti-inflammatory activity. Presently, we compared the translatomes (i.e., the population of RNAs loaded to polysomes) in THP-1 macrophages treated with LPS in the presence or absence of shikonin by examining gene expression changes by a global DNA microarray approach. Genes with annotations related to inflammation responses were selected and examined by reverse transcription real time-PCR (RT-qPCR).

Materials and methods

Reagents

Shikonin and sterile-filtered, cell-culture grade dimethylsulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phorbol myristate acetate (PMA), *E. coli* serotype O-111 lipopolysaccharide (LPS), RPMI 1640 medium, fetal bovine serum, and other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), otherwise mentioned. All reagents were of the highest grade available. Shikonin stock solution of 30 mM in DMSO was prepared and kept frozen as described previously [11, 21].

Cell culture, differentiation into macrophages, and RNA isolation from polysomes

Human monocytic THP-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured in RPMI 1640 medium with 10% (v/v) fetal bovine serum (Sigma), 5 μg/mL amphotericin B, and 10 μg/mL gentamicin, as described previously [30]. THP-1 monocytes were differentiated into macrophages by 24-h incubation with 100 nM PMA in RPMI medium before use. Macrophages were incubated with LPS alone (5 μg/mL: LPS cells), shikonin alone (100 nM: S cells), or both LPS and shikonin (LPS&S cells) for 3 h before washing of cells and preparation of cytoplasmic extract for ribosomal fractionation, as described previously [30, 31]. RNA extraction from sedimented polysome fractions (i.e., mRNAs with more than three loaded ribosomes) for use in posterior differential gene expression analysis by DNA microarray was performed as follows: after treatment with proteinase K (200 μg/mL, 0.1% SDS, at 37 °C for 0.5–2.0 h), samples (fractions of sucrose gradient-sedimentation) were extracted using saturated (250 mM sodium acetate, pH 4.8–5.2) acidic phenol:$CHCl_3$ (1:1) washes and precipitation with ethanol. The RNAs were dissolved in RNAse-free H_2O before DNA microarray analysis.

Microarray analysis, data mining, and detection of enriched pathways

The DNA microarray analysis was done as a trust analysis service at Filgen, Inc. (Nagoya, Japan). After RNA quality

check (Agilent 2100 Bioanalyzer; Agilent Technologies Inc., Santa Clara, CA, USA), aliquots of 250 ng of RNA were used to prepare fragmented and labeled cRNA, using the GeneChip 3' IVT PLUS reagent kit according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). Then, fragmented and labeled cRNA probes were hybridized to Affymetrix GeneChip® Human Genome U133 Plus 2.0 Arrays (54,675 probe sets corresponding to 38,500 identified genes; Affymetrix, Inc., Santa Clara, CA, USA) for 16 h at 45 °C. Arrays were washed, stained, and read by a GeneChip® Scanner 3000 7G (Affymetrix, Inc.) according to standard protocols of the manufacturer. Resulting gene expression data were analyzed using Affymetrix® Expression Console™ software (Affymetrix, Inc.), and normalized by robust multi-array average method. Then, Microarray Data Analysis Tool version 3.2 software (Filgen, Inc.; called Filgen software here) was used to select probe sets according to appropriate filters to remove technical backgrounds and negative control levels data. Expression fold-changes of $\geq$2.0 and $\leq$0.5 were first set as filtering cutoff values to select differentially expressed probe sets. Since the cutoff value of $\leq$0.5 detected too few genes (Table 1), a cutoff value of $\leq$0.71 was used, and, in consequence, the value $\geq$1.408 was used for selection of the upregulation side. Otherwise stated, these cutoff values were used in the comparison of different treatments vs. DMSO controls, or between data of the treatments indicated (LPS&S vs. S, or LPS&S vs. LPS). When mentioned, cutoff values of $\geq$1.29 and $\leq$0.77 were also used. Selection of probe sets that were upregulated or downregulated within the samples in comparison, as well as subtractions between determined sets of probe sets, were also done with the software. Differentially expressed probe sets obtained by a given filtration criterion are presented after exclusion of those without IDs (which appear in the array lists annotated as 'null' or without explicit gene name/IDs). The resulting probe sets were used for subsequent pathway enrichment and gene ontology (GO) analyses as described below. When mentioned, pathway network visualization was performed using the web accessible tools provided by STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, v. 10.0, available at http://string-db.org/) [32], that shows functional relationship between proteins (protein–protein interaction, PPI) based on multiple sources. Queries consisted of upregulated or downregulated probe sets obtained by the comparisons of microarray data of LPS&S cells vs. either LPS cells or S cells by Filgen software (see above), which were pasted into STRING multiple proteins search window as gene symbols after exclusion of those probes without explicit gene name/IDs. Homo sapiens was selected as the species for analyses and the minimum required interaction score was customized at a confidence level of 0.500, that is higher than the set default medium level of 0.400.

Selection of genes for RT-qPCR

To detect the effects of shikonin on LPS-induced inflammatory responses, we searched for candidate genes within the upregulated and downregulated probe sets detected in the comparison of LPS&S cells vs. LPS cells, for further analysis by RT-qPCR. The criteria for selection were the followings: (1) are included in GO pathways detected in STRING with false discovery rates (FDR) <0.05; (2) have inflammation-related annotations in the literature, or participate in pathways linked with inflammatory responses as verified by two information sources: first, by clicking the interaction edges of STRING PPI networks and seeing in 'evidence suggesting a functional link' the links indicated by the 'Co-Mentioned in PubMed Abstracts', and second, by checking the descriptions for the gene in question in the NCBI gene database (https://www.ncbi.nlm.nih.gov/gene/); (3) show expression changes in the opposite or in the same direction between LPS&S cells and LPS cells in DNA microarray analysis; and (4) are nodes connected in the PPI network resulting from queries in STRING. Candidate key genes were selected from those fulfilling at least three of the above criteria, and priority was given to genes whose protein products are attained as key players in a known pathway linked with inflammation.

Table 1 Numbers of upregulated and downregulated probe sets in THP-1 cells after treatments with LPS, shikonin (S), or both for 3 h

Expression change (fold)[a]	LPS vs. control	S vs. control	LPS&S vs. control	LPS&S vs. LPS	LPS&S vs. S
Upregulated ($\geq$2.0)	185	382	340	0	12
Downregulated ($\leq$0.5)	1	3	1	0	1
Upregulated ($\geq$1.408)	1371	1986	1774	28	48
Downregulated ($\leq$0.71)	398	361	480	45	33

[a] Expression changes obtained by DNA microarray analysis (see "Materials and methods"). Control: DMSO vehicle. The total number of analyzed probe sets were 54,675; GeneChip® Human Genome U133 Plus 2.0 Array was used and data were analyzed with Filgen's Microarray Analysis Tool Version 3.2 software. Filtering options included cutoff values for negative controls. Numbers show probe sets with changed expressions after exclusion of probe sets without IDs, or with 'null' labels

RNA isolation and RT-qPCR

PMA-differentiated THP-1 macrophages were treated as described in "Cell culture, differentiation into macrophages and RNA isolation from polysomes", and total RNAs were isolated from LPS cells and LPS&S cells after 1, 2, and 3 h in culture using an RNeasy mini kit (Qiagen). All cells were treated with 100 μg/mL cycloheximide for 5 min at 37 °C and washed in phosphate-buffered saline containing cycloheximide before cell lysis for RNA extraction. After RNA quality verification by absorbance check (Agilent 2100 Bioanalyzer; "Microarray analysis, data mining and detection of enriched pathways") and genomic DNA elimination, cDNA synthesis from 3 μg RNA each (final volume, 60 μL) was performed using PrimeScript® RT reagent kit with gDNA Eraser (Perfect Real Time; Takara Bio, Inc., Shiga, Japan). RT-qPCR was performed by the intercalator method using SYBR Premix Ex Taq™ II (Tli RNaseH Plus; Takara Bio Inc.) in a Thermal Cycler Dice® Real Time System II (TP900; Takara Bio, Inc.). Ct values were obtained by the second derivative maximum method. Forward and reverse primers for the following human genes were, respectively: CYBA (NM_000101.3; cytochrome b_{-245}, alpha polypeptide), 5′-gtactttggtgcctactccattgtg-3′, and 5′-acggcggtcatgtacttctgtc-3′; EIF4E (NM_001968.3; eukaryotic translation initiation factor 4E, transcript variant 1), 5′-ggaggttgctaacccagaacacta-3′, and 5′-agtcacagccaggcattaaattac-3′; GSK3B (NM_002093, NM_001146156; glycogen synthase kinase 3 beta), 5′-ggcagcatgaaagttagcaga-3′, and 5′-ggcgaccagttctcctgaatc-3′; and β-actin (NM_001101.3), 5′-tggcacccagcacaatgaa-3′, and 5′-ctaagtcatagtccgcctagaagca-3′. The real-time PCR steps (40 ng total RNA equivalent/reaction; reaction mixture volume: 25 μL; duplicate assays) were as follows: an initial denaturation at 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 30 s, followed by melting curve analysis (60 °C to 95 °C). The standard curves for quantification were obtained using RNA from the LPS cells (2 h). Data were analyzed with the second-derivative-maximum method using Multiplate RQ software (Takara Bio Inc.). The expressions of target genes were shown as relative quantity to the housekeeping gene, β-actin.

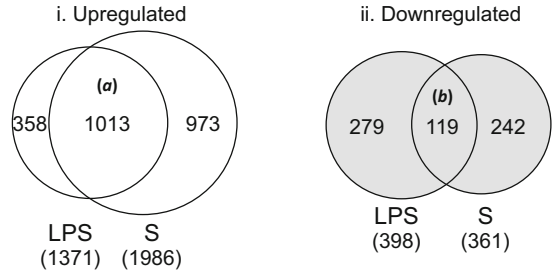
Results

Changes in gene expression of cells treated with LPS alone, shikonin alone, or both

The results showed shikonin has a significant influence on gene expression of many genes both when used alone or concomitantly with LPS. Table 1 shows the results of DNA microarray analyses of THP-1 macrophages treated for 3 h with LPS (LPS), shikonin (S), or both (LPS&S), with cutoff values of ≥2.0/≤0.5 and ≥1.408/≤0.71. Upregulated probe sets outnumbered downregulated ones with both cutoff values when compared with DMSO controls. At cutoff value of ≥1.408, the number of upregulated probe sets in LPS cells, S cells, and LPS&S cells were, respectively, 1371, 1986, and 1774, and greater than those of downregulated probes, which were about 29, 18, and 27% of upregulated ones, respectively. When gene expression changes of LPS&S cells were compared with those of either LPS cells or S cells, at the ≥2.0/≤0.5 cutoffs, none or only few probe sets appeared upregulated or downregulated (Table 1, upper right two columns). At the ≥1.408/≤0.71 cutoffs, the comparison of LPS&S cells vs. S cells showed 48 and 33 probe sets upregulated and downregulated, respectively (Table 1, rightmost column). The comparison of LPS&S cells vs. LPS cells showed 28 and 45 probe sets upregulated and downregulated, respectively (Table 1, second column from right). The respective lists of probe sets are shown in Supplementary Tables S1, S2, S3 and S4).

The relationship of upregulated (≥1.408) or downregulated (≤0.71) probe sets in the LPS cells and S cells is

a



b

Expression change	Treatments compared (no. of probe sets)	Number of probes included in the overlapped regions of LPS and S cells	
		upregulated (*a*) (1013)	downregulated (*b*) (119)
Upregulated (≥1.408)	LPS&S vs. LPS (28)	11	n.a.
	LPS&S vs. S (48)	15	n.a.
Downregulated (≤0.71)	LPS&S vs. LPS (45)	n.a.	0
	LPS&S vs. S (33)	n.a.	0

Fig. 1 Effects of shikonin on the gene expression of cells treated with LPS alone, shikonin (S) alone, or both LPS&S. **a** Venn diagrams of gene expression changes in LPS-treated THP-1 macrophages (LPS: 5 μg/mL, 3 h) and shikonin-treated cells (S: 100 nM, 3 h) show the numbers of upregulated (i: expression ratio ≥1.408 relative to DMSO-treated controls; white circles) and downregulated (ii: expression ratio ≤0.71; grey circles) probe sets. The overlapped regions (*a*) and (*b*) show the number of probe sets which were changed in both of the treatments. **b** Summary of upregulated and downregulated probe sets in the comparison of microarray data of LPS&S cells with those of LPS cells or S cells, respectively, showing the number of probe sets which are included in the overlapping regions (*a*: upregulated) or (*b*: downregulated) shown in **a**. Numbers do not include probes without IDs or nulls in the microarray data, analyzed by Filgen software. For depiction of Venn diagrams, the JavaFX-based free software Venn Diagram Interactive Software (VennDIS v1.0.1) was used (Ignatchenko [42]); n.a.: not applicable

shown in the Venn diagrams (Fig. 1a, i, ii). Based on the expression changes relative to DMSO controls, there were probe sets whose expressions changed both in the treatments of LPS alone and shikonin alone, and thus appeared in the overlapped region of the diagrams: 1013 within upregulated and 119 within downregulated sets [Fig. 1a, i(*a*) and ii(*b*), respectively].

Next, we assessed how many probe sets within those selected by the comparison of LPS&S cells vs. LPS cells or LPS&S cells vs. S cells were included in those of the overlapped regions shown in Fig. 1a, i(*a*) and ii(*b*). This is summarized in Fig. 1b. We verified that a part of the upregulated probe sets were included in those of the overlapped region i(*a*): 11 in the comparison of LPS&S cells vs. LPS cells and 15 in that of LPS&S cells vs. S cells (Fig. 1b). On the other hand, within downregulated probe sets, none was included within those of the overlapped region ii(*b*).

The upregulated and downregulated probe sets in the comparisons of LPS&S cells vs. LPS or LPS&S cells vs. S cells (Fig. 1b, probe sets including those detected in the overlapped regions, and listed in Tables S1, S2, S3, and S4) were further used in queries in STRING to find possible functional interactions and GO annotations, as follows.

GO annotations and pathways enriched in the comparison of microarray data of LPS&S cells vs. S cells: visualization of effects of LPS

The protein–protein interaction (PPI) network obtained by querying in STRING with the 48 upregulated probe sets in the comparison of LPS&S cells vs. S cells gave significant interactions (p value, 1.11×10^{-16}), with many 'edges' and linking 'nodes' as shown in Fig. 2, where the GO pathway 'innate immune response' (GO.0045087; FDR, 1.59×10^{-6}, Supplementary Table S5) is highlighted (*dark grey* or *red*). The PPI showed 40 pathways with GOs for 'biological process' and 1 for 'cellular component' detected at FDR <0.05 (partial list of GOs in Table S5). Other GO examples were: 'response to lipopolysaccharide', 'immune response', 'type I interferon signaling pathway', etc. These GOs indicated the effects of LPS,

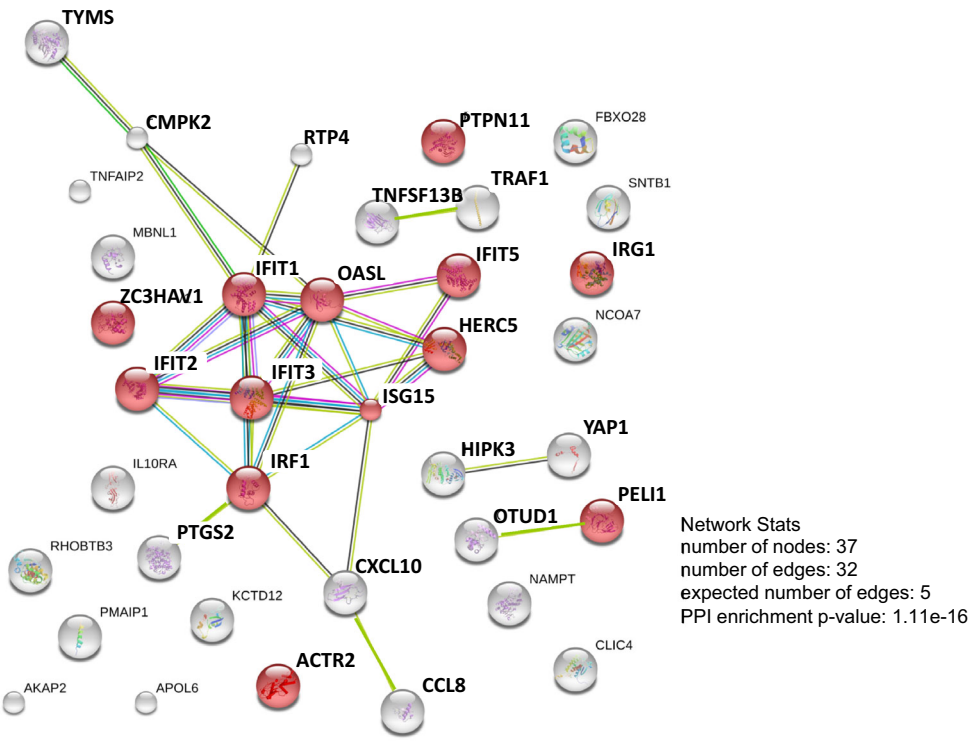


Fig. 2 STRING network view for upregulated genes in the comparison of LPS&S cells vs. S cells (effects of LPS). A PPI network (STRING) was obtained for the 48 upregulated probe sets (Table S1) in the comparison of microarray data of LPS&S cells vs. S cells (expression ratio LPS&S/LPS ≥1.408). Queries were performed as described in "Materials and methods": the minimum required interaction score was set at 0.500 and 'query proteins only' were used as interactors. The GO pathway 'innate immune response' is highlighted (*dark grey* or *red*). The list of other GO pathways is in Table S5. The size of '*nodes*' indicate whether the 3D protein structure is known (*normal size circles*) or not (*small ones*); lines between nodes (*edges*) in different colors (color version only) indicate interaction types such as 'known' (experimentally determined: *pink*; from curated databases: *light blue*), 'predicted interactions' (gene neighborhood, gene fusions, and gene co-occurrence: respectively, *green*, *orange*, and *blue*), and 'others' (text mining such as co-mentioned in PubMed: *light green*; protein homology: *grey*; etc.). Explanations about the layout of network are available in STRING (http://string-db.org/)

Table 2 Functional networks detected for genes upregulated or downregulated in the comparison of LPS&S cells vs. LPS cells according to STRING

	Pathway ID	Pathway description	Observed gene count	FDR	Matching proteins in the network (labels)
Upregulated		Biological process			
	GO.0006334	Nucleosome assembly	5	4.15E−03	HIST1H2BC, HIST1H2BE, HIST1H2BF, HIST1H2BG, HIST1H2BI
	GO.0031497	Chromatin assembly	5	4.15E−03	HIST1H2BC, HIST1H2BE, HIST1H2BF, HIST1H2BG, HIST1H2BI
	GO.0006323	DNA packaging	5	5.42E−03	HIST1H2BC, HIST1H2BE, HIST1H2BF, HIST1H2BG, HIST1H2BI
	GO.0034622	Cellular macromolecular complex assembly	7	1.84E−02	HIST1H2BC, HIST1H2BE, HIST1H2BF, HIST1H2BG, HIST1H2BI, MBNL1, RAC1
		Cellular component			
	GO.0000788	Nuclear nucleosome	5	3.26E−07	HIST1H2BC, HIST1H2BE, HIST1H2BF, HIST1H2BG, HIST1H2BI
	GO.0000790	Nuclear chromatin	5	7.51E−03	HIST1H2BC, HIST1H2BE, HIST1H2BF, HIST1H2BG, HIST1H2BI
	GO.0000228	Nuclear chromosome	5	4.83E−02	HIST1H2BC, HIST1H2BE, HIST1H2BF, HIST1H2BG, HIST1H2BI
Down regulated		Biological process[a]			
	GO.0010543	Regulation of platelet activation	4	3.59E−03	APOE, PDGFA, PLEK, PRKCD
	GO.0008064	Regulation of actin polymerization or depolymerization	5	1.60E−02	CAPG, CORO1A, PLEK, PRKCD, TWF2
	GO.0010544	Negative regulation of platelet activation	3	1.60E−02	APOE, PDGFA, PRKCD
	GO.0048519	Negative regulation of biological process	21	1.60E−02	APOE, BCR, CAPG, CCL5, CYBA, DNAJB5, ENO1, FAM129B, GPS1, IL1RN, JUN, MT1M, PDGFA, PLEK, POLR2E, PRKCD, PRMT1, RRAD, SIPA1, SMARCC1, TWF2
	GO.0009991	Response to extracellular stimulus	7	2.12E−02	APOE, CCL5, CYBA, GSDMD, IL1RN, JUN, SIPA1
	GO.0032535	Regulation of cellular component size	6	2.12E−02	APOE, CAPG, CORO1A, PLEK, PRKCD, TWF2
	GO.0035556	Intracellular signal transduction	13	2.12E−02	APOE, ARF5, BCR, CCL5, CYBA, GPS1, JUN, PDGFA, PLEK, PRKCD, RABL6, RRAD, SIPA1
	GO.0043066	Negative regulation of apoptotic process	9	2.12E−02	APOE, ATF5, CCL5, CORO1A, FAM129B, IL1RN, JUN, PCGF2, PRKCD
	GO.0043254	Regulation of protein complex assembly	6	2.12E−02	CAPG, CORO1A, JUN, PLEK, PRKCD, TWF2
	GO.0043902	Positive regulation of multi-organism process	5	2.12E−02	APOE, CCL5, CYBA, JUN, POLR2E
	GO.0048523	Negative regulation of cellular process	19	2.12E−02	APOE, BCR, CAPG, CCL5, DNAJB5, ENO1, FAM129B, GPS1, IL1RN, JUN, PDGFA, PLEK, POLR2E, PRKCD, PRMT1, RRAD, SIPA1, SMARCC1, TWF2
	GO.0050865	Regulation of cell activation	7	2.12E−02	APOE, BCR, CCL5, CORO1A, PDGFA, PLEK, PRKCD
	GO.0048008	Platelet-derived growth factor receptor signaling pathway	3	3.05E−02	BCR, PDGFA, TXNIP
	GO.0006979	Response to oxidative stress	6	3.12E−02	APOE, CCL5, JUN, PCGF2, PRKCD, TXNIP

Table 2 continued

Pathway ID	Pathway description	Observed gene count	FDR	Matching proteins in the network (labels)
GO.0009607	Response to biotic stimulus	8	3.12E−02	BCR, CCL5, ENO1, HNRNPUL1, IL1RN, JUN, PRKCD, TXNIP
GO.0045087	Innate immune response	9	3.12E−02	CCL5, CORO1A, CYBA, JUN, PDGFA, POLR2E, PRKCD, TXNIP, UBE2M
GO.0048524	Positive regulation of viral process	4	3.12E−02	APOE, CCL5, JUN, POLR2E
GO.0001558	Regulation of cell growth	6	3.28E−02	APOE, CYBA, ENO1, RRAD, SIPA1, TWF2
GO.0007166	Cell surface receptor signaling pathway	13	3.28E−02	BCR, CCL5, CORO1A, CYBA, IL1RN, JUN, PDGFA, PLEK, PRKCD, PRMT1, SMARCC1, TXNIP, UBE2M
GO.0050896	Response to stimulus	25	3.28E−02	APOE, ARF5, CCL5, CYBA, DNAJB5, ENO1, GPS1, GSDMD, HNRNPUL1, IL1RN, JUN, MT1M, PCGF2, PDGFA, PLEK, POLR2E, PRKCD, PRMT1, RABL6, RRAD, SH3BP2, SMARCC1, TWF2, TYMP, UBE2M
	Molecular function			
GO.0005515	Protein binding	23	6.09E−03	APOE, CAPG, CCL5, CORO1A, CYBA, DBN1, DNAJB5, ENO1, HNRNPUL1, IL1RN, JUN, KIF1B, NAP1L4, NRBP1, PDGFA, PLEK, PRKCD, PRMT1, SH3BP2, SMARCC1, TWF2, TXNIP, TYMP

Probe sets detected in the comparison of LPS&S cells vs. LPS cells (upregulated at expression ratio $\geq$1.408, or downregulated at expression ratio $\leq$0.71, listed in supplemental Tables S3 and S4, respectively) were used in queries in STRING. Pathways with false discovery rates (FDR) $<$ 0.05 are listed. The minimum required interaction score was set at a 0.500 confidence level. For details, see "Materials and methods"

[a] The list for 'Biological process' pathways was truncated at the top 20; the full list had 36 pathways (FDR max = 0.049)

since in the comparison of LPS&S cells vs. S cells, the effects by shikonin are included in both treatments. On the other hand, the query in STRING with the 33 downregulated probe sets in the comparison of LPS&S cells vs. S cells showed no significant GO enrichment (data not shown).

We have also verified that a query in STRING with the upregulated probe sets without including those changed in both LPS alone and S alone cells (i.e., 33 probe sets after subtraction of the 15 overlapped probe sets; Fig. 1b) gave similar results as those shown in Fig. 2 and Table S5, as the PPI enrichment *p* value was of the same order and GOs were almost the same as in Table S5 (data not shown).

GO annotations and pathways enriched in the comparison of microarray data of LPS&S cells vs. LPS cells: visualization of effects of shikonin

The upregulated and downregulated probe sets in the comparison of LPS&S cells vs. LPS cells were 28 and 45 (Table 1; lists in Supplementary Tables S3, S4), respectively. The queries in STRING performed with the respective probe sets indicated enrichment of several pathways shown in Table 2, although the PPI enrichment *p* values were not significant (0.59 and 0.176, respectively, for upregulated and downregulated sets; Fig. 3).

The PPI network of upregulated probe sets showed three connected proteins (EIF4E, EIF1, and EIF1AY), but none of them were included in the GO 'cellular macromolecular complex assembly' highlighted *dark grey* or *red* (FDR: 1.84×10^{-2}; Fig. 3a). The lowest FDR was found for GO pathway 'nuclear nucleosome' in the category of 'Cellular component' (Table 2; example of changed gene: HIST1H2BC).

The downregulated probe sets showed more connections than the upregulated ones (Fig. 3b). The genes with decreased expressions pertaining to the GO 'innate immune response' (FDR 3.12×10^{-2}) were highlighted in *dark grey* or *red*. This pathway was also detected in LPS-upregulated genes (Fig. 2), although the highlighted genes in Figs. 2 and 3b were different. The fact that the same GO pathway was detected in Figs. 2 and 3b suggested that shikonin was suppressing the effects of LPS in LPS&S cells. The pathways detected with the downregulated probe sets had inflammation-related annotations: 'response to extracellular stimulus', 'intracellular signal transduction', 'response to oxidative stress', 'regulation of response to

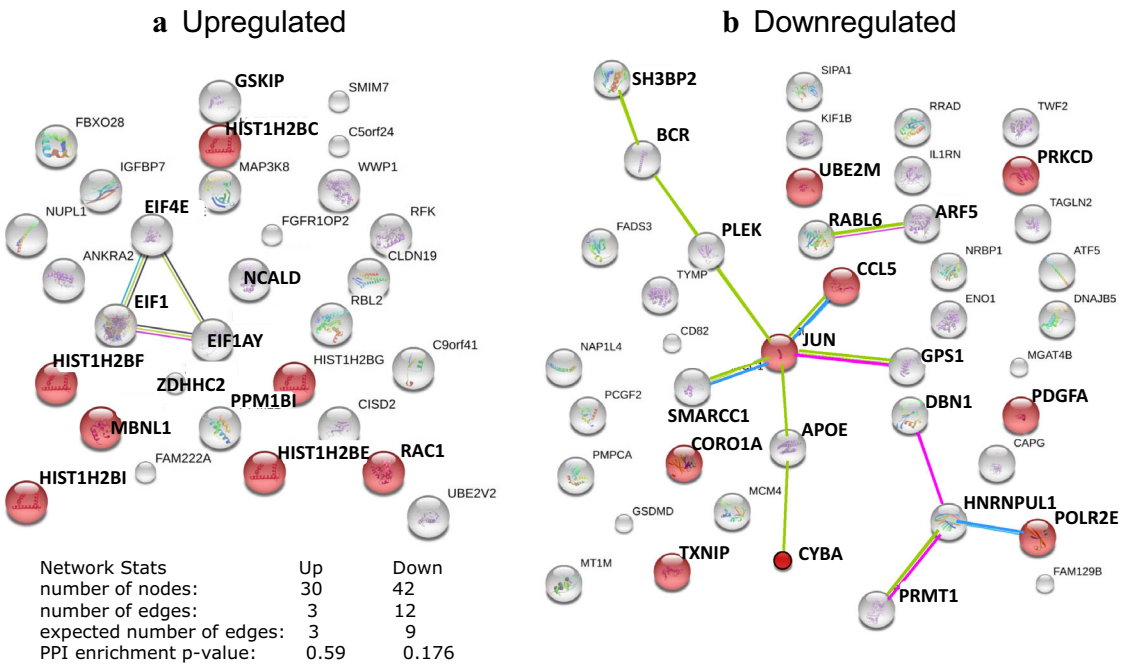


Fig. 3 STRING network views for upregulated and downregulated genes in the comparison of LPS&S cells vs. LPS cells (effects of shikonin). PPI network (STRING) was obtained for upregulated (expression ratio ≥1.408; Table S3) or downregulated (≤0.71; Table S4) probe sets in the comparison of microarray data of LPS&S cells vs. LPS cells. Queries were performed as described in "Materials and methods" (probes without explicit ID were excluded; 'query proteins only' were used as interactors with a customized minimum required interaction score of 0.500). Details of PPI network interactions are the same as described in the legend of Fig. 2. In the PPI network obtained with upregulated probe sets (**a**), the GO pathway 'cellular macromolecular complex assembly' is highlighted (*dark grey* or *red*). Similarly, in the PPI network obtained with downregulated probe sets (**b**), the GO pathway 'innate immune response' is highlighted. The list of other GO pathways is shown in Table 2

wounding', etc. (Table 2 and data not shown; examples of changed genes: APOE and CCL5).

Changes in the expression of inflammation-related genes by RT-qPCR

After verification of enriched GOs as described above, a search for candidate key genes that might underlie the effects of shikonin was done under the criteria described in "Selection of genes for RT-qPCR". From the list of genes fulfilling at least three of the criteria (Table 3), CYBA, EIF4E, and GSK3B were selected for RT-qPCR analyses. CYBA and EIF4E were detected as interacted nodes in the respective STRING networks of downregulated and upregulated probe sets (Fig. 3) and might be involved in inflammation, since CYBA is an essential component of the O_2^--generating enzyme Nox2 [33, 34], and EIF4E is a key in the regulation of immune functions via translational control [35]. GSK3B was selected for its reported role as a modulator of cellular responses to LPS [36], and because its expression showed opposite directions between LPS cells and LPS&S cells (downregulation and upregulation, respectively, Table 3; fold change ≥1.29/≤0.77). Also, in a query with probe sets detected at the ≥1.29 cutoff value, GSK3B is a node in the PPI network (data not shown).

The changes in the expression levels of CYBA, EIF4E, and GSK3B mRNAs in LPS&S cells at time points of 1, 2, and 3 h evaluated by RT-qPCR were shown as ratios relative to LPS cells (Table 4). Roughly, all the three genes showed a tendency to become decreased at 2 h by shikonin as compared to LPS-alone treated cells. At 3 h, only the CYBA mRNA was confirmed to show a matched result with the decreased expression shown by DNA microarray analysis. Complete agreement between the results of DNA microarray analysis and of RT-qPCR is expected to be difficult, considering that the two analyses give gene expression changes based on different methods and that different RNA populations were analyzed. Nevertheless, these results suggested dynamic changes by shikonin in the expression of the selected candidate genes influencing inflammatory responses.

Discussion

The analyses of the effects of shikonin on gene expression of macrophages showed a significant influence, either alone or in combination with LPS (Table 1). The total number of

Table 3 Candidate genes that might be involved in the effects of shikonin, listed according to the criteria described in Materials and methods (section "Selection of genes for RT-qPCR")

Gene Title	Gene symbol	Inflammation-related function/annotation[a]	Expression ratio LPS/control	Expression ratio LPS&S/LPS[b]	Node in PPI network (STRING)[c]
Eukaryotic translation initiation factor 1	EIF1	Upregulated in metabolic syndrome	*0.77*	**1.52**	○
Eukaryotic translation initiation factor 4E	EIF4E	Regulator of immune function via translational control	**1.86**	**1.50**	○
Apolipoprotein E	APOE	Alzheimer's disease, etc.	**1.87**	*0.62*	○
Pleckstrin	PLEK	Overexpressed in chronic inflammatory diseases	**1.85**	*0.64*	○
Cytochrome b_{-245}, alpha polypeptide	CYBA	Essential component of O_2^- -producing NADPH oxidase	**1.55**	*0.62*	○
Protein arginine methyltransferase 1	PRMT1	Increased expression may be involved in many types of cancer	**1.49**	*0.69*	○
Jun proto-oncogene	JUN	Proto-oncogene transcription factor; TLR signaling pathway	**1.39**	*0.71*	○
C–C motif chemokine ligand 5	CCL5	Overexpressed in chronic inflammatory diseases	**1.37**	*0.67*	○
G protein pathway suppressor 1	GPS1	Suppress G-protein and mitogen-activated signal transduction	**1.33**	*0.70*	○
SH3 domain binding protein 2	SH3BP2	Binds to SH3-domains of protein tyrosine kinases (ABL1, SYK)	**1.21**	*0.67*	○
RAB, member RAS oncogene family like 6	RABL6	Overexpression might be involved in breast cancer tumorigenesis	**1.21**	*0.69*	○
Breakpoint cluster region	BCR	GTPase-activating protein for p21rac	**1.21**	*0.71*	○
Drebrin	DBN1	Actin-binding protein; high expression in basal cell carcinoma; possibly involved in the pathogenesis of Alzheimer's disease	**1.09**	*0.68*	○
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1	SMARCC1	Upregulated in prostate cancer	0.99	*0.71*	○
Glycogen synthase kinase 3 beta	GSK3B	Regulator of inflammatory response after TLR4 stimulation	*0.68*	**1.32**	○[d]

Genes that appeared connected in STRING PPI networks (Fig. 3a, b) obtained by the queries of upregulated (Table S3) and downregulated probe sets (Table S4) in the comparison of LPS&S cells vs. LPS cells were checked for the criteria described in "Selection of genes for RT-qPCR". Only those with inflammation-related annotations were listed

[a] Example of inflammation-related annotation

[b] Genes whose expression changes were upregulated (bold) or downregulated (italics) in the comparison of LPS&S vs. LPS cells were listed side-by-side with respective changes observed in LPS alone cells

[c] Circles indicate that the genes are nodes in the PPI networks in Fig. 3a, b. Cutoff values were $\geq$1.408 and $\leq$0.71, except for GS3KB, filtered by cutoff values $\geq$1.29 and $\leq$0.77

[d] GSK3B is a node in the PPI network obtained with a query using probe sets selected with cutoff values $\geq$1.29 (data not shown)

probe sets upregulated by shikonin was around 1.5 times of those upregulated by LPS alone (cutoff value: 1.408). If not considering those probe sets which were upregulated both in S- and LPS-treated cells (overlapped ones in Fig. 1ai), upregulation by shikonin was approximately threefold of that of LPS alone. In contrast, the number of downregulated genes was closer between LPS and S cells, both in total and specific to each of treatments (Table 1; Fig. 1aii). When the changes in gene expression of LPS&S cells were compared with that of LPS cells or S cells, less than 50 probe sets were found in the upregulation and downregulation sides in both of the comparisons (Table 1).

By comparing data from LPS&S cells and S cells, the influence by LPS might become visible (Tables S1, S2, S5). Similarly, the comparison between LPS&S cells and LPS cells might indicate the effects of shikonin (Tables 2, S3, S4). Thus, the GO pathways detected in queries using the respective probe sets mentioned in the above comparisons may indicate which pathways/genes could be relevant in the effects of LPS (Fig. 2) or shikonin (Fig. 3),

Table 4 Changes in the expression of CYBA, EIF4E, and GSK3B mRNA in LPS&S cells as compared with LPS cells

Gene symbol	Expression ratio (LPS&S/LPS cells)			
	DNA microarray	RT-qPCR[a]		
	3 h	1 h	2 h	3 h
CYBA	0.62	1.23	0.70	0.79
EIF4E	1.50	1.23	0.69	0.94
GSK3B	1.32	1.00	0.62	0.99

Values are ratios between LPS&S cells relative to LPS alone cells

[a] Means of two experiments. For comparison, expression fold-changes resulted from DNA microarray analysis at 3 h are also shown

respectively. By this approach, it was shown that: at 3 h, the LPS-induced responses still remain in the LPS&S cells even in the presence of shikonin, as innate immune response-related pathways were significantly enriched (PPI in Fig. 2; Table S5); however, shikonin counteracts some of the pro-inflammatory responses in LPS-treated cells as indicated by downregulation of pathways that were upregulated in LPS cells such as 'innate immune response' in LPS&S cells (Figs. 2 vs. 3b; Table S5 vs. Table 2).

In accordance with this view, shikonin changed the expression of inflammation-related genes such as CYBA, EIF4E, and GSK3B, as examined by DNA microarray analysis (Tables 3, 4) and RT-qPCR (Table 4). It remains to be elucidated whether changes in mRNA expression of these genes are reflected in their protein expression in inflammation responses. The results of DNA microarray and RT-qPCR analyses indicated that one of the possible mechanisms underlying the anti-inflammatory action of shikonin in LPS-stimulated macrophages could be decrease of the expression of Nox2, as shown by the lowered CYBA mRNA expression (Tables 3, 4). CYBA encodes $p22^{phox}$, an essential component of Nox2 enzyme [33]. Such a view was supported by the fact that in addition to CYBA, other components of the Nox2 enzyme such as cytosolic factors NCF1 ($p47^{phox}$), NCF4 ($p40^{phox}$), and RAC2 (rho family, small GTP binding protein Rac2) were also decreased in LPS&S cells relative to LPS cells [by microarray analysis, a 20% decrease for the three genes at 3 h in LPS&S cells (data not shown)]. All of them were upregulated to 1.2–1.5-fold in LPS cells as compared with DMSO controls (data not shown), similarly to CYBA (expression ratio in LPS cells relative to DMSO = 1.55; Table 3). Therefore, regarding Nox2, there is a possibility that shikonin decreases its protein expression, in addition to the previously observed direct inhibition of enzyme activity [8, 37], and also steps essential for enzyme activation such as intracellular calcium ion fluxes [21], all being events for less ROS production.

Concerning EIF4E and GSK3B, it is difficult to layout their involvement in the effects of shikonin by now, as their action in inflammatory responses might vary according to several factors. GSK-3β (i.e., the protein product of GSK3B, which phosphorylates glycogen synthase and other substrates) is known to both positively and negatively regulate a variety of transcription factors including NF-κB and *c*-Myc [38] that are critical in regulation of cytokine production. In addition, GSK-3β and eIF4E (protein product of EIF4E) are reported to interact with each other, as GSK-3β directly phosphorylates translation initiation factor 4E-binding protein 1 (4E-BP1) [39], a negative regulator of eIF4E. The phosphorylation state of 4E-BP1 modulates the availability of free eIF4E to initiate translation. This interplay between eIF4E and GSK-3β is considered of importance in the control of inflammation responses since both participate in the phosphoinositide 3-kinase/phosphatase and tensin homolog (PTEN)/Akt/mammalian target of rapamycin complex 1 (mTORC1) pathway and the Wnt/β-catenin pathway (reviewed in [38]), which are pathways involved in disease formation. The present results indicating that shikonin could change the expressions of EIF4E and GSK3B mRNAs suggested it might interfere with key cellular events, and this should be further investigated. Interestingly, a previous study focusing on the anti-tumor effects of shikonin in leukemia U937 cells [12] has indicated inhibition of the insulin growth factor 1 receptor-Akt-mTOR signaling as one of its targets, and suggested the possibility that shikonin binds with GSK-3β, eIF4E, and other key proteins of this cascade, using a virtual screening method.

Previous transcriptome studies have reported that shikonin decreases the expression of transcription factors such as STAT3 in breast cancer cells [40] and *c*-Myc in U937 cells [41]. Whether these downregulations could be related or not with its influence on EIF4E, which is the key regulator of protein translation events in inflammation [35], remains to be elucidated.

In conclusion, our results suggest that shikonin changes the expression of key gene candidates involved in inflammation, such as the ROS-producing NADPH oxidase and maybe the translational control point consisted of an EIF4E and GSK3B interactive axis.

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Compliance with ethical standards

Conflict of interest The authors have nothing to declare.

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