

Full Paper

Shikonin Directly Inhibits Nitric Oxide Synthases: Possible Targets That Affect Thoracic Aorta Relaxation Response and Nitric Oxide Release From RAW 264.7 MacrophagesLucia S. Yoshida^{1,†}, Tomie Kawada^{1,†}, Kaoru Irie¹, Yasukatsu Yuda¹, Toshiyuki Himi¹, Fumihiko Ikemoto¹, and Hiromi Takano-Ohmuro^{1,*}¹Research Institute of Pharmaceutical Sciences, Musashino University,
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Abstract. Recently, an isomeric mixture of herbal anti-inflammatory naphthoquinones shikonin and alkannin, and their derivatives, have been found to impair cellular responses involving nitric oxide (NO) and NO synthesis, like the acetylcholine-induced relaxation response of rat thoracic aorta and NO release from murine RAW 264.7 macrophages. However, the mechanisms of such effects, including whether NO synthase (NOS) activity is affected, remained unclear. We herein investigate possible targets of shikonin in these NOS-related events. Shikonin by itself dose-dependently inhibited the rat thoracic aorta relaxation in response to acetylcholine (pD₂ value: 6.29). Its optical enantiomer, alkannin, was equally inhibitory in the aorta relaxation–response assay. In RAW 264.7 cells, shikonin inhibited the lipopolysaccharide-induced NO production by 82% at 1 μ M. A cell-free assay to verify direct effects on NOS activity showed that shikonin inhibits all isoforms of NOS (IC₅₀S, 4 – 7 μ M), suggesting NOS as an inhibition target in both the events. Further possible targets of shikonin that might be involved in the inhibitions of the acetylcholine-induced aorta relaxation response and the NO generation by RAW 264.7 cells are also discussed. It is shown for the first time that shikonin inhibits NOS activity.

Keywords: endothelium-dependent vasorelaxation, shikonin, nitric oxide synthase (NOS), thoracic aorta, RAW 264.7 cell

Introduction

Shikonin, a natural naphthoquinone dye from *Lithospermum erythrorhizon* of the medicinal herb “Shikon”, has shown a variety of pharmacological effects in vitro and in vivo (reviews: refs. 1 – 3). Roughly, the majority of the pharmacologic effects of shikonin and its chemically related substances including its *S* enantiomer, alkannin, can be considered as being anti-inflammatory in nature: decrease of capillary permeability (4); inhibition of mast cell degranulation and hind-paw edema (5); inhibition of neutrophil respiratory burst (6); decrease of the expression levels of pro-inflammatory factors like cy-

clooxygenase-2 in transformed human mammary epithelial cells (7); and decrease in tumor necrosis factor- α in mouse skin (8), rat microglial cells (9), and RAW 264.7 murine macrophage cells (10). In these last two cells, shikonin and derivatives such as isobutyryl- and isovaleryl-shikonin also affected nitric oxide (NO) generation by decreasing expression of NO synthase (NOS) (9, 10). Thus, shikonin was suggested to interfere with nuclear factor (NF)- κ B signaling, but the primary targets of the inhibition as well as whether NOS activity is directly affected remain unknown.

On the other hand, the previously observed impairment of endothelium-dependent rat thoracic aorta relaxation in response to acetylcholine, by the use of a mixture of shikonin plus alkannin in a ratio of 17:83 (‘shikonins’: a designation for the enantiomers in mixture) (11), is apparently an effect that has no direct links with inflammation. Since the endothelium-dependent vascular smooth

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muscle relaxation response is dependent on NO from endothelial NOS (eNOS) (12–14), Hu et al. (11) have suggested that ‘shikonins’ might interfere with NO-mediated cellular events. However, which of the enantiomers is responsible and the molecular target(s) in this impairment of the NO-mediated vascular relaxation remained poorly understood. Therefore, ‘shikonins’ were suggested to interfere with NOS-involving events in both the thoracic aorta (11) and RAW 264.7 macrophages (10).

We have recently shown that shikonin inhibits spleen tyrosine kinase activity (Syk; IC_{50} , 7.8 μ M) and suppresses the IgE-mediated histamine release from human basophils (IC_{50} , 2.6 μ M) (15). Syk is known to be expressed in vascular endothelial cells (16) and to participate in activation of the NF- κ B signal cascade upon thrombin stimulation (17), although its role in endothelial function is obscure. Also, Syk may participate in the regulation of inducible NOS (iNOS) expression: in bronchial epithelial cells, inhibition of Syk expression led to down-regulation of iNOS mRNA and NO production (18); and Syk inhibitors like piceatannol were found to prevent lipopolysaccharide (LPS)-induced NO generation in RAW 264.7 cells (19, 20).

We herein investigate the possible targets of shikonin in the NOS-involving events in the rat thoracic aorta and RAW 264.7 cells, including the possible contribution of Syk inhibition in the shikonin effects. In this study, we show for the first time that shikonin directly inhibits all isoforms of NOS.

Materials and Methods

Cells and materials

Shikonin and alkannin were purchased from Wako Pure Chemical Industries (Osaka). Stock solutions were prepared in dimethyl sulfoxide (DMSO; Wako Pure Chemical Ind.), and small aliquots stocked at -20°C . New aliquots were thawed for every experiment. The final DMSO concentration in all the assays was 0.1% (v/v), and had no influence in the reactions. Phenylephrine hydrochloride (Kowa Pharmaceuticals, Tokyo) and acetylcholine chloride (Daiichi Sankyo, Tokyo) were diluted in MiliQ water. [^3H]-L-Arginine monohydrochloride was from GE Healthcare (Tokyo); modified Griess reagent and *E. coli* serotype O2:B6 LPS, from Sigma-Aldrich (St. Louis, MO, USA); Ultima Gold LLT, from Perkin Elmer (Boston, MA, USA); and the Syk inhibitor, 3-(1-methyl-1*H*-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1*H*-indole-5-sulfonamide (OXSI-2), from Calbiochem (Merck Chemicals, Darmstadt, Germany). OXSI-2 was selected herein as a Syk inhibitor instead of the commonly used piceatannol because the later has been re-

ported to inhibit other kinases such as protein kinase C (PKC), myosin light chain kinase, cAMP-dependent kinase α (PKA) catalytic subunit (21), and Src family kinases (22). The murine macrophage cell line RAW 264.7 was purchased from ATCC (Manassas, VA, USA); fetal bovine serum (FBS), from Nichirei Biosciences (Tokyo); Dulbecco's Modified Eagle's Medium (DMEM), from Nissui (Tokyo); and phenol red-free DMEM and Alamar Blue, from Invitrogen (Carlsbad, CA, USA). Sodium nitrite (NO_2^-) standard was from Wako Pure Chemical Ind. Recombinant bovine eNOS, murine iNOS, and rat neuronal NOS (nNOS) were from Cayman Chemical Co. (Ann Arbor, MI, USA) and the recombinant enzymes and reagents for cell-free kinase assays described below, from Carna Biosciences (Kobe). All other reagents were from Nacalai Tesque (Kyoto) or Wako Pure Chemical Ind., otherwise mentioned, and of the purest grade available.

Evaluation of vascular tonus relaxation

The experiments were approved by the Experimental Animal Ethics Review Board at Musashino University and done according to their guidelines. Wistar male rats (Tokyo Laboratory Animals Science, Tokyo) weighing 250–350 g were anesthetized with ether. After sacrifice, the thoracic aortae were quickly isolated and carefully cleaned free of excess tissues and then cut into rings about 2-mm-wide. The aortic rings were mounted on two stainless-steel hooks inserted through the lumen of the ring. The bottom hook in each preparation was fixed, whereas the top hook was attached to a force transducer (UL-10GR; Minebea, Tokyo) mounted on a movable platform, which allowed adjustment of resting tension. Tension changes were recorded via the force-displacement transducer connected to a Power Lab system (AD Instruments, Castle Hill, NSW, Australia). Each ring was suspended in a 5-mL organ bath for recording the isometric contractions. The bathing solution was Krebs-Henseleit solution (pH 7.4) containing 118.4 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 25.0 mM NaHCO_3 , 2.5 mM CaCl_2 , and 11.1 mM glucose. The solution was continuously bubbled with 95% O_2 and 5% CO_2 at $36.0 \pm 0.5^{\circ}\text{C}$. The ring was allowed to equilibrate for 90 min with an optimal resting tension of 1.0 g. Thereafter, the existence of endothelium was confirmed by more than 90% relaxation in response to 1 μ M acetylcholine of rings previously contracted with 0.1 μ M phenylephrine. The concentration–response curves of acetylcholine were made by cumulative additions of acetylcholine (from 0.001 to 10 μ M) to the phenylephrine-precontracted rings. The maximal contraction induced by phenylephrine (0.1 μ M) was taken as the 100%-tension for calculation of relaxation percents of

the various compounds tested. The ring preparations were first evaluated for reproducibility of the relaxation response and found to be useful for two consecutive sets of cumulative additions of acetylcholine. Thus, one ring preparation was assayed for one set following DMSO-treatment (control) and another following one dose of a test compound (shikonin or alkannin: 0.03, 0.1, 0.3, and 1 μ M or OXSI-2: 1 μ M). The preincubation time with the compounds lasted for 15 min before addition of phenylephrine. The inhibitory potency was evaluated upon calculation of pD'_2 values according to Takayanagi (23). The pD'_2 value corresponds to the negative logarithm of the inhibitor concentration causing a 50% inhibition of the maximum relaxation response, which was determined as follows: this value was read on linear plots of inhibitor concentration (abscissa) *versus* relaxation response (ordinate) of two doses of the inhibitor, chosen under the criteria that the interval between the doses includes the dose at which the 50% inhibition occurs; thus, the doses selected for shikonin were 0.3 and 1.0 μ M and those for alkannin, 0.1 and 0.3 μ M, based on the inhibition curves in Fig. 1, panels A and B.

Cell-free NOS activity assay

The NO generation in the presence or absence of shikonin at various concentrations (0.1, 1, 10, and 100 μ M) was assayed with recombinant NOS proteins (iNOS, eNOS, and nNOS) by monitoring the conversion of [3 H]-L-arginine to [3 H]-L-citrulline. The specific activity provided by the supplier (Cayman Chemical) was used as the nominal activity value for adjusting NOS enzyme amount in the assays: one unit (U) of NOS corresponds to the amount of enzyme necessary to produce 1 nanomole of NO per minute at 37°C in 50 mM HEPES, pH 7.4, containing appropriate amounts of arginine and oxyhemoglobin (respectively, 1 mM and 3 μ M for iNOS and 50 μ M and 5 μ M for eNOS and nNOS), 1 mM magnesium acetate, 0.1 mM NADPH, 12 μ M tetrahydrobiopterin, and 120 (iNOS) or 170 (eNOS and nNOS) μ M dithiothreitol. In the case of eNOS and nNOS, additional components were 1 mM $CaCl_2$ and 20 μ g/mL calmodulin. The respective NOS enzyme (final, 8 U/mL in a 50- μ L reaction volume) was preincubated at room temperature in the presence or absence of shikonin for 20 min in a reaction mixture consisting of 25 mM Tris-HCl buffer (pH 7.4), supplemented with 1 μ M each of FAD and FMN, and 3 μ M tetrahydrobiopterin. After this preincubation, 1 mM NADPH and 0.34 mM L-arginine (of which 1/6 of arginine consisted of [3 H]-L-arginine, specific activity 1 μ Ci/ μ L) was added to the reactions, mixed, and further incubated in a water bath for 40 min at 37°C. For eNOS and nNOS assays, 0.6 mM Ca^{2+} plus 0.1 μ M calmodulin were included together with NADPH and

arginine. After the incubation, reactions were stopped by addition of eight volumes of the stop buffer (5 mM EDTA in 50 mM HEPES, pH 5.5). Non-reacted L-arginine was eliminated through absorption to cation exchange resin (Cayman Chemical) followed by centrifugation on spin cups. Radioactivity of L-citrulline in the eluates (0.1-mL aliquots) was measured in 3 mL Ultima Gold LLT by using a liquid scintillation counter (Aloka LSC-6100; Aloka, Tokyo). Appropriate controls without NOS or with addition of the NOS inhibitor L- N^G -nitroarginine (L-NNA, 1 mM) were done simultaneously. The NO-generating activity in the presence of shikonin is expressed as percentages of the controls with DMSO instead of inhibitors.

NO release from RAW 264.7 cells

RAW 264.7 cells (5×10^4 /well) were plated into 96-well plates and cultured in DMEM supplemented with 10% (v/v) FBS, under a humidified atmosphere (5% CO_2 /95% air) at 37°C. When cells were at around 80%–90% confluency, the medium was changed to phenol red-free DMEM containing 1% FBS and then exposed to either shikonin or the Syk inhibitor OXSI-2 at the concentrations of 0.1, 0.3, and 1.0 μ M, for 30 min. After this pretreatment, cells were subjected to LPS treatment (100 ng/mL) for 17 h, in the presence of shikonin or OXSI-2. After induction, the culture supernatant was collected and mixed at a 1:1 ratio with Griess reagent for detection of NO_2^- , the primary stable oxidation product of NO. Shikonin and DMSO (used in controls) had no influence in the assay. The NO_2^- amount was calculated from a concomitantly run standard curve using NO_2^- standard solution. The viability of the cells was checked using Alamar Blue after medium removal for determination of NO_2^- , according to the manufacturer's protocol.

Cell-free inhibition assays of purified kinases

The kinase profiling assays provided by Carna Biosciences were performed in the absence or presence of shikonin (0.01–300 μ M; nine concentration points varying logarithmically at appropriate intervals, in duplicate) and analyzed by off-chip mobility shift assay, unless otherwise mentioned. The following recombinants of human protein kinases were tested: PKA (full-length, PDB ID: NP_002721); Akt1 (catalytic domain, amino acids 104–480 of PDB ID: NP_005154); PKC isoforms α , β_1 , γ , and δ (full-length proteins of respective PDB IDs, NP_002728, NP_997700, NP_002730, and NP_006245); the mitogen-activated protein kinases (MAPKs): mitogen-activated protein kinase 2 (MEK1; full-length, NP_002746), extracellular regulated kinase 1 (ERK1; full-length, NP_002737), and p38 isoform α (amino acids 9–352; NP_620581). Proteins were ex-

pressed as N-terminal glutathione-S-transferase (GST)–fusion proteins using the baculovirus or the *E. coli* expression systems; and they were purified by glutathione Sepharose chromatography, as detailed in the Profiling Book (https://www.carnabio.com/output/pdf/Profiling-ProfilingBook_en2.pdf; Carna Biosciences). Appropriate substrates and assay conditions using ATP in the K_m range for each kinase were carried out in parallel with control assays in which a reference inhibitor was included: staurosporine for Akt-1, MEK1, PKA, and PKCs; 5-iodotubersidin for ERK1; and SB202190 for p38 kinase. The inhibitors were added together with ATP. After incubation for 1 h at room temperature, the reaction was stopped by adding QuickScout Assist MSA (Carna Biosciences) and analyzed by electrophoretic separation of substrate and products (24), using a Caliper LC3000 platform (Caliper Life Sciences, Mountain View, CA, USA). The ratio of phosphorylated product was calculated at each concentration of shikonin and the percent inhibition was expressed relative to the vehicle alone control assays, as previously described (15). Only MEK1 activity was analyzed by an ELISA platform using a mouse monoclonal antibody against phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (E10; Cell Signaling Technology, Danvers, MA, USA) as primary antibody, and horseradish peroxidase–labeled goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) as secondary antibody.

Statistical analyses

Data are expressed as the mean \pm S.E.M. of the indicated *n* determinations. Statistical significance was analyzed by the non-parametric Mann-Whitney's U test or Student's *t*-test, when data distribution was found to be normal upon the F test. All reported *P* values are two-sided, and *P* < 0.05 was considered statistically significant.

Results

Shikonin or alkannin inhibits the acetylcholine-induced relaxation response of vascular smooth muscle

Phenylephrine-precontracted rat thoracic aorta with intact endothelium shows a dose-dependent relaxation response upon addition of increasing amounts of acetylcholine. To differentiate from a previous study in which a mixture (shikonin and alkannin ratio, 17:83) was investigated (11), in the present study, the effects on the vascular smooth muscle relaxation in response to acetylcholine were examined separately for each enantiomer. None of the compounds at any of the concentrations tested, as well as DMSO alone, had any influence on the baseline tension of the thoracic aorta rings equilibrated in the or-

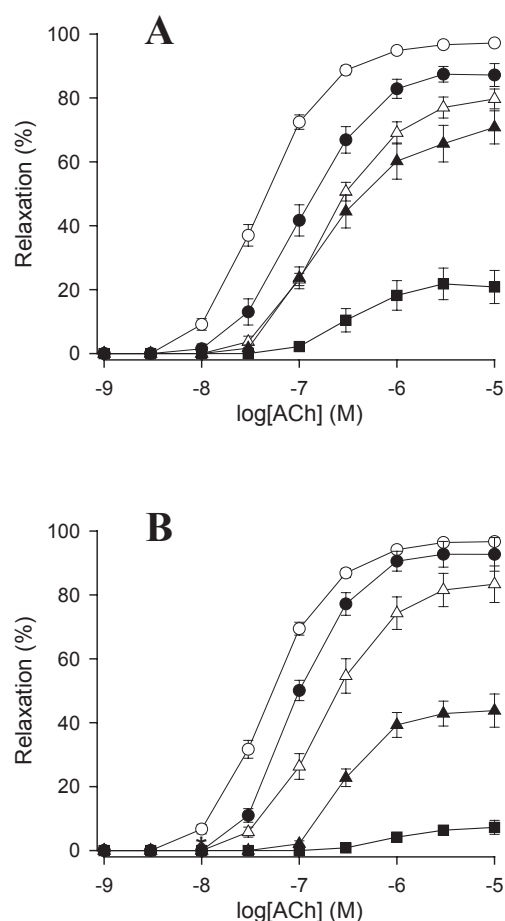


Fig. 1. Effects of shikonin or alkannin on the acetylcholine-induced relaxation response of rat thoracic aorta. Shikonin (panel A) or alkannin (panel B) was allowed to react with equilibrated rat thoracic aorta rings for 15 min previous to addition of phenylephrine (0.1 μ M). Pre-contracted rings were then relaxed upon cumulative additions of acetylcholine (ACh, 0.001 – 10 μ M). The concentrations and number (*n*) of determinations for shikonin (panel A) or alkannin (panel B) were, respectively, as follows: closed circles (0.03 μ M; *n* = 6); open triangles (0.1 μ M; *n* = 6); closed triangles (0.3 μ M; *n* = 5); and closed squares (1.0 μ M; *n* = 5). DMSO vehicle controls in both panels are shown by open circles (each point, mean of 22 determinations). Data are expressed as the mean \pm S.E.M. Relaxation responses in the presence of either shikonin or alkannin were significantly different from DMSO controls for ACh concentrations \geq 0.03 μ M at *P* < 0.01, as analyzed by Student's *t*-test.

gan baths. Adding shikonin or alkannin (0.03, 0.1, 0.3, and 1 μ M), prior to the addition of phenylephrine, significantly inhibited the subsequent acetylcholine-dependent relaxation response as compared to the DMSO controls, causing a shift to the right in the relaxation curves, with a gradual lowering of the maximum vasodilatation (Fig. 1, panels A and B, respectively). In the case of shikonin, the maximum vasodilatation decreased to 22.3% at 1 μ M (Fig. 1A). Shikonin and alkannin showed similar inhibition patterns (Fig. 1, panels A and B). The pD'_2 value,

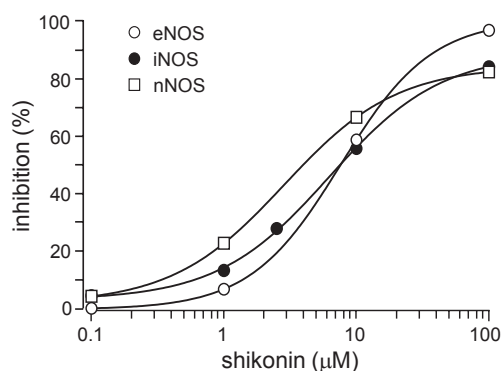


Fig. 2. Direct inhibition of NOS activity by shikonin. The inhibitory effect of shikonin on the cell-free NOS activity assay was evaluated by conversion of [^3H]-L-arginine to [^3H]-L-citrulline, as described in Materials and Methods. Shikonin was preincubated with NOS (8 U/mL, protein concentrations of 0.59–0.69 mg/mL) for 20 min at room temperature before the reaction was started by addition of NADPH and [^3H]-L-arginine (for iNOS) or the former two supplemented with both Ca^{2+} and calmodulin (for eNOS and nNOS), then followed by reaction at 37°C for 40 min. Percent inhibitions are expressed relative to DMSO control assays. Each point is the mean of two separate experiments, in which individual concentrations were assayed at least in duplicate. L-NNA (1 mM) used as a control NOS inhibitor resulted in inhibitions of 98.4%, 80.3%, and 82.4%, respectively, for eNOS, iNOS, and nNOS.

which is the negative logarithm of the inhibitor concentration at which 50% inhibition of maximal relaxation occurs, was 6.29 and 6.59, respectively, for shikonin and alkannin (corresponding to concentrations of 0.51 and 0.26 μM , respectively).

Shikonin directly inhibits NOS activity

In order to settle whether shikonin can directly inhibit NOS activity, the effect of shikonin on eNOS activity was evaluated in cell-free assays. It was found that shikonin directly inhibits the eNOS activity, beginning from 1 μM shikonin (Fig. 2). Inhibition of nearly 60% occurred at 10 μM . The IC_{50} for eNOS activity was 7.1 μM . For comparison, the inhibition of iNOS and nNOS were also examined. Inhibitions of iNOS and nNOS by shikonin occurred in a similar fashion, both being as sensitive as eNOS (Fig. 2). All the isoforms were inhibited by around 60% at 10 μM and by more than 80% at 100 μM shikonin under the assay conditions herein. The IC_{50} s for iNOS and nNOS were 7.0 and 4.0 μM , respectively. Controls were done with DMSO, which had no influence on the reactions.

In contrast to Fig. 2 where shikonin was preincubated with NOS, when shikonin is added to NOS after NADPH plus arginine additions, almost no inhibition could be observed, as verified in assays with 1 and 10 μM shikonin skipping the preincubation step (data not shown).

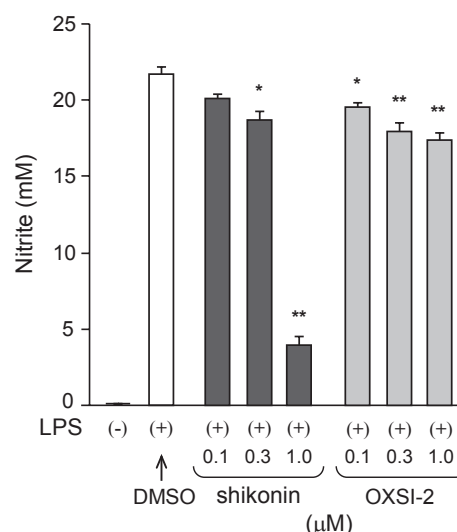


Fig. 3. Effects of shikonin or OXSI-2 on the LPS-induced NO generation by RAW 264.7 cells. Shikonin or OXSI-2 was added to RAW 264.7 cells 30 min before induction with LPS (final, 100 ng/mL). LPS was added to medium with appropriately supplemented shikonin or OXSI-2 to keep their initial concentration unchanged. After LPS-induction in the presence of shikonin or OXSI-2 for 17 h, the NO_2^- accumulated in the culture medium was evaluated photometrically by reaction with Griess reagent. Alamar Blue tests revealed good and consistent cell viabilities in all the treatments presented. Values are each the mean \pm S.E.M. of multiple wells ($n = 6$) of a representative experiment out of two. Significantly different from LPS-induced, DMSO-treated controls at $*P < 0.05$ or $**P < 0.01$ by Mann-Whitney's U test.

Inhibitory effect of shikonin on the NO-generating activity of RAW 264.7 cells

The fact that shikonin inhibits iNOS, as described above, raised the possibility of a new explanation for a previously observed effect of shikonin and derivatives in rat microglial cells and RAW 264.7 cells: inhibition of LPS-induced NO production (9, 10).

We then examined the effect of shikonin on NO production in RAW 264.7 cells. Cells were induced with LPS overnight (17 h) in either the presence or absence of shikonin, before evaluation of their NO-generating ability by means of NO_2^- accumulation in culture supernatants. As shown in Fig. 3, because of induction of iNOS expression, LPS-treated cells showed a remarkable elevation of NO generation when compared to cells grown in the absence of LPS. In cells induced with LPS in the presence of shikonin, the NO generation decreased slightly at 0.1 μM , was significantly inhibited at 0.3 μM , and was markedly inhibited by around 82% at 1 μM (Fig. 3). Neither cell growth nor cell viabilities were affected by DMSO vehicle or shikonin in the concentrations used herein (data not shown).

Table 1. IC₅₀ of shikonin in cell-free kinase assays

Kinase	IC ₅₀ (μM)
MEK1	32.0
ERK1	13.9
p38-MAPK	12.8
PKCγ	95.0
PKCα, β1, δ	>100 ^a
Syk	7.8 ^b
PKA	17.8
Akt	>100

Kinase activities were determined by off-chip mobility shift assay (except for MEK1, which was assayed by an ELISA platform) using appropriate substrates for the respective recombinant kinases. The concentration of shikonin in the assays ranged from 0.01 to 300 μM, and assays were validated by the use of established inhibitors for each kinase (see Materials and Methods). ^a: Percent inhibitions at 100 μM shikonin were 2.0%, 5.3%, and 26.7% for PKCα, PKCβ1, and PKCδ, respectively. Vehicle controls were taken with DMSO, which had no effects on the reactions. ^b: From reference 15.

Other targets of shikonin in the inhibition of NO production by RAW 264.7 cells

The possibility of Syk inhibition being involved in the decreased NO production in shikonin-treated RAW 264.7 cells was checked by testing the effects of the Syk inhibitor OXSI-2. OXSI-2 did not influence cell growth and viability in the experiments. The addition of OXSI-2 at 0.1, 0.3, and 1 μM showed weak inhibitions of the NO generation, respectively, of 11%, 18%, and approximately 20% (Fig. 3), suggesting that Syk inhibition by OXSI-2 mimics to some extent that of shikonin but has a limited effect on the overall NO production triggered by LPS-induction.

In order to verify other possible targets of shikonin that might synergistically amplify the inhibition of NO production in RAW 264.7 cells, we next tested the possibility that other kinases operating downstream of LPS-activation of toll-like receptor 4 were affected by shikonin. Direct effects on kinase activity were evaluated in cell-free assays for MAPKs such as ERK, p38-MAPK, and some major PKCs. The IC₅₀s for the kinases tested are shown in Table 1. PKCs α, β1, γ, and δ were not affected (IC₅₀ of 95 μM for PKCδ and >100 μM for other isoforms), while considerable inhibitions were found for MEK1 (32.0 μM), which is the upstream kinase of ERKs, ERK1 (13.9 μM), and p38-MAPK (12.8 μM).

Other possible targets of shikonin in the thoracic aorta relaxation response

Given the discrepancy between the IC₅₀s of shikonin for the thoracic aorta relaxation response (Fig. 1A) and the cell-free eNOS enzymatic activity (Fig. 2), we further examined possible involvement of additional targets such

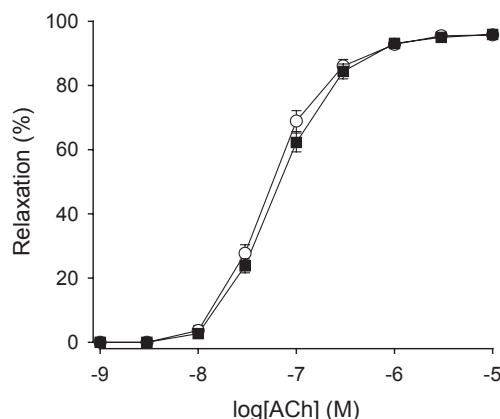


Fig. 4. Effects of OXSI-2 on the acetylcholine-induced relaxation response of rat thoracic aorta. The endothelium-dependent relaxation of precontracted rat thoracic aorta in response to acetylcholine was tested in the presence of OXSI-2 (added 15 min previous to phenylephrine), as described in the legend of Fig. 1. Precontracted rings were then relaxed upon cumulative additions of acetylcholine (ACh, 0.001–10 μM). DMSO (open circles, n = 5) or OXSI-2 (closed squares, 1 μM, n = 5). Data are expressed as the mean ± S.E.M.

as Syk (by testing the effects of OXSI-2) and kinases that activate eNOS (by cell-free assays).

OXSI-2 was allowed to react with thoracic aorta previous to addition of phenylephrine, and then the relaxation response upon acetylcholine additions was examined. No influence was noted even at a high concentration of 1 μM OXSI-2 (Fig. 4), which is higher than its reported IC₅₀ for the basophil degranulation response, 0.31 μM (25).

Next, it was verified by cell-free assays if shikonin is able to inhibit relevant kinases upstream of eNOS, namely, PKA and Akt that phosphorylate eNOS and are known to activate endothelial NO production (26, 27). Although Akt was not influenced, PKA was inhibited (IC₅₀, 17.8 μM; Table 1), suggesting that PKA might be another target of shikonin in the thoracic aorta relaxation response.

Discussion

The major findings of this study can be summarized as follows: The inhibition of thoracic aorta relaxation in response to acetylcholine was concluded herein to be caused by either of the enantiomers, shikonin (pD'₂, 6.29) or alkannin (pD'₂, 6.59). In cell-free assays, shikonin was able to inhibit the enzymatic activity of eNOS, iNOS, and nNOS, with IC₅₀s of 4–7 μM. Shikonin inhibited the NO generation of LPS-induced RAW 264.7 cells with an IC₅₀ <1 μM. Cell-free assays showed that shikonin also inhibits PKA and MAPKs such as MEK, ERK, and p38.

Previous studies have suggested that shikonin and

derivatives are able to interfere in NOS-involved events, such as the endothelium (i.e., eNOS)-dependent relaxation response of thoracic aorta (by 'shikonins', a 17:83 mixture of shikonin and alkannin) (11), and NO generation (i.e., iNOS-dependent) in cells like RAW 264.7 macrophages (10) and microglial cells (9). However, the primary targets of such effects, including whether 'shikonins' and derivatives are able to directly inhibit enzymatic activity of NOS, remained unknown.

In this regard, it was found herein that shikonin *per se* inhibits NOS activity, which provided an additional new explanation for the mechanism of altered NO production in the above previously reported events. To our knowledge, this is the first time that shikonin is recognized to inhibit NOS activity. Similar inhibition potencies were found for eNOS, iNOS, and nNOS isoforms (Fig. 2). It was noted that when shikonin is added to NOS enzymes after NADPH and arginine, inhibition became negligible (data not shown). This fact might be an indication that shikonin may attack NOS sites that are accessible before binding of L-arginine and/or NADPH to the enzyme molecule. In addition, alkannin was found to be equally able to suppress NOS activity, as verified by reaction with iNOS (inhibitions of 11.9%, 13.3%, 60.1%, and 84.5%, respectively for 0.1, 1.0, 10, and 100 μM ; IC_{50} , 6.0 μM) (data not shown).

The direct suppression of eNOS activity by shikonin suggested that the depletion of NO might be one of the causes of the inhibition of the acetylcholine-induced relaxation response of thoracic aorta (Fig. 1). It was found that shikonin or alkannin alone provokes strong inhibition of the aorta relaxation response. However, as the inhibitory concentration for the vascular relaxation response (Fig. 1A: pD'_2 , 6.29, corresponding to 0.51 μM) was roughly ten times lower than that in the cell-free eNOS assay (Fig. 2: 7.1 μM), the existence of other targets of shikonin in the thoracic aorta response was suggested. The vascular relaxation response follows the L-arginine-NO-cyclic GMP pathway (12–14, 27), where NO activates guanylate cyclase to increase cGMP levels. According to Hu et al. (11), 'shikonins' in mixture affected only aortic rings with intact endothelium (IC_{50} , approximately 0.24 μM) and had no influence on the relaxation elicited by sodium nitroprusside, a guanylate cyclase activator. Their study has denied guanylate cyclase and downstream pathways, including protein kinase G, as targets of 'shikonins' and pointed to reactions upstream of eNOS as inhibition sites. Herein, an examination of the effects of shikonin on upstream kinases that activate eNOS activity indicated PKA as one possible target (IC_{50} , 17.8 μM ; Table 1). If inhibition of PKA occurs simultaneously, a stronger inhibition of the vascular relaxation response may appear than that caused by tar-

geting of eNOS alone, since PKA is regarded as being involved in eNOS-mediated vasorelaxation (28). On the other hand, the negligible effects of OXSI-2 (IC_{50} s for Syk cell-free assay and basophil degranulation response of 14 nM and 0.31 μM , respectively) (25) indicated that Syk is not a major factor regulating aorta relaxation responses to acetylcholine (Fig. 4). The role of Syk in the regulation of vascular contraction/relaxation is not well defined, but our result seems to indicate that the effect of shikonin in the thoracic aorta relaxation response might not be due to Syk inhibition. In addition, it remains to be elucidated whether other molecules are also targeted by shikonin in the suppression of vasorelaxation responses. Another possibility could be that shikonin or alkannin *per se* is able to scavenge NO radical. Superoxide anion (IC_{50} s of 7.2 and 5.5 μM , respectively, for shikonin and alkannin) (29) and hydroxyl radical (IC_{50} of 40 μM for either enantiomer) (30) are reported as being scavenged, but there is no data concerning NO. This possibility remains to be elucidated.

On the other hand, the shikonin inhibition of NO production in RAW 264.7 cells, occurring with an IC_{50} <1 μM (Fig. 3), which was lower than that observed for the direct iNOS assay (Fig. 2: IC_{50} , 7.0 μM), suggested the involvement of other inhibition targets in addition to iNOS. Cheng et al. (10) have shown that 'shikonins' in mixture provoke downregulation of iNOS protein expression. Thus, certainly the inhibition seen in Fig. 3 (82% loss of NO at 1 μM shikonin) includes decreased iNOS expression as a cause, a view in agreement with the inhibitions of MAPKs (MEK, ERK, and p38; Table 1) participating in the activation of the NF- κ B pathway for induction of iNOS expression. Regarding inhibition of iNOS expression, PKA might be another additional target of shikonin (Table 1), as inhibition of PKA has been reported to decrease iNOS expression/NO production of LPS-induced RAW 264.7 cells (31). In addition to the decreased expression of iNOS as a cause of the markedly low NO release in shikonin-treated cells, a possible contribution by direct inhibition of iNOS activity was suggested from our observations that shikonin is able to decrease NO release from RAW 264.7 cells when added after LPS induction (approximately 20% upon 2-h incubation with 1 μM , data not shown). Thus, the decrease in NO production in Fig. 3 might involve decrease of iNOS expression and, to some extent, direct inhibition of iNOS activity.

Although a direct mechanistic analogy is not possible between the inhibition of the NO-mediated thoracic aorta relaxation response (Fig. 1) and that of the NO production by LPS-induced RAW 264.7 cells (Fig. 3), it is noteworthy that MEK and ERK are reported to participate in a new pathway for acetylcholine-induced aorta

relaxation (32). According to Zecchin et al. (32), MEK and ERK are intermediary kinases involved in activation of eNOS. Thus, these MAPKs might also be included in the list of additional targets of shikonin in the vascular relaxation response, a matter that needs further investigations.

Taken together, our present findings support the view that shikonin may target NOS, with possible involvement of additional targets, in the inhibitions of the thoracic aorta relaxation response to acetylcholine and the LPS-induced NO generation in RAW 264.7 cells. It was demonstrated for the first time that shikonin inhibits NOS activity.

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References

- Papageorgiou VP, Assimopoulou AN, Culadourous EA, Hepworth D, Nicolaou KC. The chemistry and biology of alkannin, shikonin and related naphthazarin natural products. *Angew Chem Int Ed*. 1999;38:270-300.
- Chen X, Yang L, Oppenheim JJ, Howard MZ. Cellular pharmacology studies of shikonin derivatives. *Phytother Res*. 2002;16:199-209.
- Papageorgiou VP, Assimopoulou AN, Ballis AC. Alkannins and shikonins: a new class of wound healing agents. *Curr Med Chem*. 2008;15:3248-3267.
- Tanaka S, Tajima M, Tsukada M, Tabata M. A comparative study on anti-inflammatory activities of the enantiomers, shikonin and alkannin. *J Nat Prod*. 1986;49:466-469.
- Wang JP, Raung SL, Chang LC, Kuo SC. Inhibition of hind-paw edema and cutaneous vascular plasma extravasation in mice by acetylshikonin. *Eur J Pharmacol*. 1995;272:87-95.
- Kawakami N, Koyama Y, Tanaka J, Ohara A, Hayakawa T, Fujimoto S. Inhibitory effect of acetylshikonin on the activation of NADPH oxidase in polymorphonuclear leukocytes in both whole cell and cell-free systems. *Biol Pharm Bull*. 1996;19:1266-1270.
- Subbaramaiah K, Bulic P, Lin Y, Dannenberg AJ, Pasco DS. Development and use of a gene promoter-based screen to identify novel inhibitors of cyclooxygenase-2 transcription. *J Biomol Screen*. 2001;6:101-110.
- Staniforth V, Wang SY, Shyr LF, Yang NS. Shikonins, phyto-compounds from *Lithospermum erythrorhizon*, inhibit the transcriptional activation of human tumor necrosis factor alpha promoter in vivo. *J Biol Chem*. 2004;279:5877-5885.
- Nam KN, Son MS, Park JH, Lee EH. Shikonins attenuate microglial inflammatory responses by inhibition of ERK, Akt, and NF-kappaB: neuroprotective implications. *Neuropharmacology*. 2008;55:819-825.
- Cheng YW, Chang CY, Lin KL, Hu CM, Lin CH, Kang JJ. Shikonin derivatives inhibited LPS-induced NOS in RAW 264.7 cells via downregulation of MAPK/NF-kappaB signaling. *J Ethnopharmacol*. 2008;120:264-271.
- Hu CM, Cheng YW, Cheng HW, Kang JJ. Impairment of vascular function of rat thoracic aorta in an endothelium-dependent manner by shikonin/alkannin and derivatives isolated from roots of *Macrotomia euchroma*. *Planta Med*. 2004;70:23-28.
- Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980;288:373-376.
- Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, et al. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*. 1995;377:239-242.
- Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*. 1987;327:524-526.
- Takano-Ohmuro H, Yoshida LS, Yuda Y, Morioka K, Kitani S. Shikonin inhibits IgE-mediated histamine release by human basophils and Syk kinase activity. *Inflamm Res*. 2008;57:484-488.
- Yanagi S, Inatome R, Ding J, Kitaguchi H, Tybulewicz VL, Yamamura H. Syk expression in endothelial cells and their morphologic defects in embryonic Syk-deficient mice. *Blood*. 2001;98:2869-2871.
- Bijli KM, Fazal F, Minhajuddin M, Rahman A. Activation of Syk by protein kinase C-delta regulates thrombin-induced intercellular adhesion molecule-1 expression in endothelial cells via tyrosine phosphorylation of RelA/p65. *J Biol Chem*. 2008;283:14674-14684.
- Ulanova M, Marcet-Palacios M, Munoz S, Asfaha S, Kim MK, Schreiber AD, et al. Involvement of Syk kinase in TNF-induced nitric oxide production by airway epithelial cells. *Biochem Biophys Res Commun*. 2006;351:431-437.
- Islam S, Hassan F, Mu MM, Ito H, Koide N, Mori I, et al. Piceatannol prevents lipopolysaccharide (LPS)-induced nitric oxide (NO) production and nuclear factor (NF)-kappaB activation by inhibiting IkappaB kinase (IKK). *Microbiol Immunol*. 2004;48:729-736.
- Lee YG, Chain BM, Cho JY. Distinct role of spleen tyrosine kinase in the early phosphorylation of inhibitor of kappaB alpha via activation of the phosphoinositide-3-kinase and Akt pathways. *Int J Biochem Cell Biol*. 2009;41:811-821.
- Wang BH, Lu ZX, Polya GM. Inhibition of eukaryote serine/threonine-specific protein kinases by piceatannol. *Planta medica*. 1998;64:195-199.
- Law DA, Nannizzi-Alaimo L, Ministri K, Hughes PE, Forsyth J, Turner M, et al. Genetic and pharmacological analyses of Syk function in alphaIIb beta3 signaling in platelets. *Blood*. 1999;93:2645-2652.
- Takayanagi I. [Methods and problems in screening tests - Pharmacological experiments with Magnus apparatus (on the smooth muscle preparations).] *Pharmacometrics*. 1968;2:131-142. (in Japanese)
- Gouda M. Kinase assay development: a strategy for comprehensive kinase profiling for drug discovery. *Screening*. 2008;2:15-17.
- Lai JY, Cox PJ, Patel R, Sadiq S, Aldous DJ, Thurairatnam S, et al. Potent small molecule inhibitors of spleen tyrosine kinase

- (Syk). *Bioorg Med Chem Lett*. 2003;13:3111–3114.
- 26 Boo YC, Sorescu G, Boyd N, Shiojima I, Walsh K, Du J, et al. Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A. *J Biol Chem*. 2002;277:3388–3396.
- 27 Fleming I, Busse R. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am J Physiol*. 2003;284:R1–R12.
- 28 Toyoshima H, Nasa Y, Hashizume Y, Koseki Y, Isayama Y, Kohsaka Y, et al. Modulation of cAMP-mediated vasorelaxation by endothelial nitric oxide and basal cGMP in vascular smooth muscle. *J Cardiovasc Pharmacol*. 1998;32:543–551.
- 29 Sekine T, Masumizu T, Maitani Y, Nagai T. Evaluation of superoxide anion radical scavenging activity of shikonin by electron spin resonance. *Int J Pharmaceutics*. 1998;174:133–139.
- 30 Sekine T, Masumizu T, Maitani Y, Takayama K, Kohno M, Nagai T. [Effect of shikonin and alkannin on hydroxyl radical generation system concerned with iron ion.] *Yakugaku Zasshi*. 1998;118:609–615. (text in Japanese with English abstract)
- 31 Chen CC, Chiu KT, Sun YT, Chen WC. Role of the cyclic AMP-protein kinase A pathway in lipopolysaccharide-induced nitric oxide synthase expression in RAW 264.7 macrophages. Involvement of cyclooxygenase-2. *J Biol Chem*. 1999;274:31559–31564.
- 32 Zecchin HG, Priviero FB, Souza CT, Zecchin KG, Prada PO, Carnevali JB, et al. Defective insulin and acetylcholine induction of endothelial cell-nitric oxide synthase through insulin receptor substrate/Akt signaling pathway in aorta of obese rats. *Diabetes*. 2007;56:1014–1024.