Original Article

Inhibition of neutrophil superoxide generation by shikonin is associated with suppression of cellular Ca^{2+} fluxes

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Shikonin, an anti-inflammatory compound of "Shikon", inhibits the neutrophil superoxide ($O_2^{\bullet-}$) generation by NADPH oxidase 2 (Nox2); however, the mechanisms of how shikonin affects Nox2 activity remained unclear. We aimed to elucidate the relationship between the inhibition of Nox2 activity and influences on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by shikonin. For this purpose, we used a simultaneous monitoring system for detecting changes in $[Ca^{2+}]_i$ (by fluorescence) and $O_2^{\bullet-}$ generation (by chemiluminescence) and evaluated the effects of shikonin on neutrophil-like HL-60 cells stimulated with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). Since fMLP activates Nox2 by elevation in $[Ca^{2+}]_i$ via fluxes such as inositol 1,4,5-trisphosphate-induced Ca^{2+} release (IICR) and store-operated Ca^{2+} entry (SOCE), we also evaluated the effects of shikonin on IICR and SOCE. Shikonin dose-dependently inhibited the fMLP-induced elevation in $[Ca^{2+}]_i$ and $O_2^{\bullet-}$ generation (IC_{50} values of 1.45 and 1.12 μM, respectively) in a synchronized manner. Analyses of specific Ca^{2+} fluxes showed that shikonin inhibits IICR and IICR-linked $O_2^{\bullet-}$ generation (IC_{50} values: 0.28 and 0.31 μM for $[Ca^{2+}]_i$ and $O_2^{\bullet-}$, respectively), as well as SOCE and SOCE-linked $O_2^{\bullet-}$ generation (IC_{50} values: 0.39 and 0.25 μM for $[Ca^{2+}]_i$ and $O_2^{\bullet-}$, respectively). These results suggested that shikonin inhibits the $O_2^{\bullet-}$ generation by Nox2 in fMLP-stimulated neutrophils by targeting Ca^{2+} fluxes such as IICR and SOCE.

***Key Words*:** **simultaneous detection, intracellular calcium, superoxide, NADPH oxidase, shikonin**

Anti-inflammatory compounds can act by suppressing cellular pro-inflammatory responses and oxidative stress. In the latter case, the anti-inflammatory compound can be an antioxidant by itself or an inhibitor of reactive oxygen species (ROS)-producing enzymes by inhibiting enzymatic activity directly or upstream events needed for enzymatic activation such as Ca^{2+} fluxes. We have previously developed a simultaneous monitoring system for intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and superoxide anion ($O_2^{\bullet-}$) generation by measuring changes in fluorescence and chemiluminescence, respectively,[1–3] using *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-stimulated neutrophils as a tool to evaluate anti-inflammatory properties of test compounds. The fMLP-stimulated $O_2^{\bullet-}$ generation by NADPH oxidase 2 (Nox2) in cells depends on Ca^{2+}-regulated pathways;[4–10] therefore, impairment of $O_2^{\bullet-}$ generation in parallel with altered $[Ca^{2+}]_i$ profiles will indicate that Ca^{2+} fluxes are likely target candidates. We have previously found that ibuprofen is an anti-inflammatory compound showing impaired $O_2^{\bullet-}$ generation due to altered Ca^{2+} fluxes in neutrophil-like cells.[3] In contrast, if $O_2^{\bullet-}$ scavenging occurs without alterations in $[Ca^{2+}]_i$ profiles as in the case of ascorbic acid,[3] one can recognize it as an antioxidant (scavenger of $O_2^{\bullet-}$) not affecting upstream pathways for Nox2 activation, including Ca^{2+} fluxes. Therefore, the simultaneous monitoring system is especially useful in elucidating such different anti-inflammatory mechanisms of test compounds.

We have also been investigating the anti-inflammatory effects of shikonin, the major active substance of the herbal medicine "Shikon" (*Lithospermum erythrorhizon*),[11–13] for its therapeutic potential in inflammatory diseases. Shikonin scavenges ROS such as $O_2^{\bullet-}$,[14–16] hydroxyl radical,[15,17] singlet oxygen,[15] and alkyl-oxy radical[16]. Moreover, shikonin also inhibits the activities of enzymes that produce ROS such as Nox2[16,18,19] and nitric oxide synthase[20] by mechanisms likely independent of ROS-scavenging effects. The inhibition of Nox2 by shikonin (IC_{50} range, 0.4~2 μM) was reported to occur before activation of the enzyme,[16,18,19] a process that requires assembly of cytosolic and membrane components to form a complex with $O_2^{\bullet-}$-generating activity.[21] Although the inhibition targets are believed to be processes and/or molecules relevant to enzyme activation, such targets remain unknown and might differ depending on the stimulant used for activation of Nox2.

Here, we aimed to elucidate the effects of shikonin on the fMLP-stimulated $O_2^{\bullet-}$ generation by Nox2 linked with Ca^{2+} fluxes in neutrophils. Ca^{2+} entry into neutrophils results from the stimulation of cells by agonists such as fMLP.[4,22] Agonist interaction with G-protein linked receptors on the cell membrane causes the formation of phospholipase C (PLC)-mediated inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG).[4,22] IP_3 activates its receptor on the endoplasmic reticulum (ER), causing the release of stored Ca^{2+} from the ER to the cytosol, which is termed IP_3-induced calcium release (IICR). The emptying of the ER via IICR is then followed by an influx of external Ca^{2+} across the plasma membrane, through capacitative or store-operated calcium entry (SOCE).[4,23–25] Another route for Ca^{2+} entry through plasma membranes is via PLC-linked receptor occupation by agonists, but is store-independent (i.e., non-SOCE); it is called receptor-operated calcium entry (ROCE).[4,24,26]

Presently, the effects of shikonin on the fMLP-induced $O_2^{\bullet-}$ generation and Ca^{2+} fluxes in neutrophil-like HL-60 cells were

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investigated. We first verified that the direct scavenging of $O_2^{\bullet-}$ by shikonin can be considered negligible under the assay conditions herein. Cells pretreated with shikonin had synchronized changes in $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation in response to fMLP. The inhibition of $O_2^{\bullet-}$ generation by shikonin was linked to the inhibition of the cellular Ca^{2+} fluxes, IICR and SOCE.

Material and Methods

Reagents. Shikonin and sterile-filtered, cell-culture grade DMSO were purchased from Wako Pure Chem. Ind., Ltd. (Osaka, Japan). fMLP, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA: a chelating agent with high specificity for Ca^{2+}), hypoxanthine, xanthine oxidase from bovine milk, *N*-{4-[3,5-bis (trifluoromethyl)-1H-pyrazol-1-yl]phenyl}-4-methyl-1,2,3-thiazole-5-carboxamide (BTP2: an inhibitor of SOCE)[27,28] and thapsigargin (TG: an irreversible inhibitor of sarco/endoplasmic reticulum membrane Ca^{2+}-ATPase (SERCA) that pumps Ca^{2+} into the stores; besides inhibiting SERCA, TG passively depletes Ca^{2+} stores thus allowing SOCE upon Ca^{2+} addition)[29–31] were purchased from Sigma-Aldrich (St. Louis, MO). The following reagents were from the sources in parentheses: 1-[2-amino-5-(2,7-dichloro-6-acetoxymethoxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetra (acetoxymethyl) ester (fluo-3 AM; Dojindo Laboratories Kumamoto, Japan) and 2-methyl-6-phenyl-3,7-dihydroimidazo [1, 2-*a*] pyrazin-3-one (CLA; Tokyo Kasei, Tokyo, Japan).

Stock solutions were prepared in the solvents indicated and stored at –20°C: BTP2 (10 mM, DMSO); CLA (50 μM, MilliQ water); EGTA (100 mM; MilliQ water, pH adjusted to 8.1); fMLP (1 mM, DMSO); TG (1 mM, DMSO); and shikonin (30 mM, DMSO). The stock solutions of fMLP and TG were diluted 10 times in 1:3 (*v*/*v*) DMSO and Ringer-Hepes buffer (RH buffer: 154 mM NaCl, 5.6 mM KCl, and 10 mM Hepes, pH 7.4) before use. The stock solution of shikonin was diluted with DMSO to 3.3, 1.0, 0.33, 0.1, and 0.033 mM solutions, of which aliquots of 1/1,000 of the assay volume were used. In assays with BTP2, a 1/1,000 assay volume of the stock solution was used. The final concentration of DMSO in the assays depended on the number of lipophilic reagents added, but did not exceed 0.3% (*v*/*v*), a level that was verified to have no influence on cellular fluorescence and chemiluminescence responses.

Analytical apparatus. The analyses were carried out using a simultaneous monitoring system for fluorescence and chemiluminescence, the CFL-C2000 (Hamamatsu Photonics K.K., Hamamatsu, Japan). The system used is the same as previously described[3] with the exception of a more sensitive photomultiplier tube (H10682-210, Hamamatsu Photonics K.K.) as a detector. This detector enables measurements with higher sensitivity than the previous model. In brief, by repeatedly turning on and off the LED excitation light at high speed, two kinds of light signals, fluorescence (from fluo-3 AM preloaded within cells; excitation at 500 nm and emission at 523 nm) and chemiluminescence (from the reaction of CLA with $O_2^{\bullet-}$ generated upon fMLP-stimulation of cells) can be detected by a single detector.

Chemical scavenging of $O_2^{\bullet-}$ by shikonin. The $O_2^{\bullet-}$-scavenging ability of shikonin was tested in the previously described hypoxanthine-xanthine oxidase assay,[3] using the CFL-C2000 system. The difference was that CLA was used as the $O_2^{\bullet-}$-sensitive reagent because its chemiluminescence is detected at 380 nm, which corresponds to a region of low absorption in the visible region of the shikonin spectrum (the absorbance increases above 450 nm and peaks at 520 nm). Briefly, the reaction mixture (0.1 μM hypoxanthine, 1 μM CLA, shikonin or DMSO alone in RH buffer; assay volume: 1.5 ml) was placed in a disposable polymethylmethacrylate cuvette (1-cm light path) and pre-incubated for 5 min at 37°C in a dedicated incubator (PI6100-prototype, Hamamatsu Photonics K.K.) with mild stirring at approximately 150 rotations/min, with a cross-head magnetic stirrer (9-mm diameter, 6-mm height) placed in the bottom of the cuvette. Then, the cuvette containing the mixture was transferred to the sample holder of the CFL-C2000 under the same temperature and stirring conditions as in the pre-incubation step. After baseline acquisition for 50 s, xanthine oxidase (final, 2.4×10^{-3} U/ml) was added and chemiluminescence monitoring continued. The $O_2^{\bullet-}$-scavenging ability was determined by decreases in the peak area under the curve (AUC) of the responses as previously described,[3,32] and expressed as ratios relative to control without shikonin.

Preparation of neutrophil-like cells from HL-60 cells. The human HL-60 acute promyelocytic leukemia cell line was obtained from the American Type Culture Collection (Manassas, VA), and cultured in GIT® medium (Wako Pure Chem. Ind., Ltd.) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced with fresh GIT medium containing 1.3% (*v*/*v*) DMSO to induce differentiation of the HL-60 cells into neutrophil-like cells, as previously described.[3] After culture for 96 h, the resulting neutrophil-like cells were used in all cell assays. The neutrophil-like cells were washed with RH buffer and loaded with fresh GIT medium containing 3 μM fluo-3 AM for 45 min at 37°C and 5% CO_2-atmosphere. The cells were then washed twice with RH buffer, suspended, and maintained in RH buffer on ice. Cell count was performed in Trypan Blue.

Assessment of $O_2^{\bullet-}$ generation and $[Ca^{2+}]_i$ levels associated with full cellular responses to fMLP. The effects of shikonin on $O_2^{\bullet-}$ generation and $[Ca^{2+}]_i$ levels of fMLP-stimulated cells were verified using the CFL-C2000 (*Analytical apparatus*), as follows. Assays contained 1×10^5 fluo-3 AM loaded cells/ml (*Preparation of neutrophil-like cells from HL-60 cells*; total volume: 1.5 ml throughout) in RH buffer with 1 μM CLA supplemented with 1 mM $CaCl_2$, unless otherwise stated. Cuvettes and apparatus for pre-incubation were the same as described in *Chemical scavenging of $O_2^{\bullet-}$ by shikonin*. Briefly, shikonin or DMSO as a vehicle was added to the cells suspended in RH buffer with CLA and Ca^{2+}, and incubated for 7 min at 37°C with stirring before transferring the cuvette containing the mixture to the CFL-C2000. After acquisition of chemiluminescence ($O_2^{\bullet-}$) and fluorescence ($[Ca^{2+}]_i$) baselines for 50-s, the cells were stimulated by injection of fMLP (final, 1 μM), and data were acquired under the same temperature and stirring conditions as in the pre-incubation step. The order and timing for when the test compounds were added are detailed in the legend of Fig. 2.

The chemiluminescence and fluorescence intensities were monitored and recorded as time courses of the cellular responses. These responses were quantified by AUCs with appropriate settings made in the software of the detection system. Data were expressed as a function of shikonin concentration after calculation of ratios relative to control assays without shikonin.

Assessment of Ca^{2+} fluxes: separate assays for IICR and SOCE. For verification of the effects of shikonin on IICR, fluo-3 AM loaded cells (1×10^5 cells/ml; described in *Preparation of neutrophil-like cells from HL-60 cells*) were maintained for 1 min in 1 mM $CaCl_2$-containing RH buffer to replenish Ca^{2+} stores depleted during cell isolation and washings, and then assayed in the presence of 5 mM EGTA. EGTA chelates extracellular Ca^{2+}, preventing both SOCE and ROCE; therefore, stimulation of cells with fMLP in the presence of EGTA allows only IICR-mediated elevation of $[Ca^{2+}]_i$ (i.e., due to release of Ca^{2+} from intracellular stores). Both $O_2^{\bullet-}$ generation and changes in $[Ca^{2+}]_i$ due to IICR were monitored using the CFL-C2000 as described in the section before this one. The incubation periods, additions and periods of data acquisition are detailed in the legend of Fig. 3. Addition of EGTA did not change the pH of the reaction, which remained constant at pH 7.4.

To evaluate the effect of shikonin on Ca^{2+} flux from the extracellular environment via SOCE, Ca^{2+} entry after the addition of 1 mM Ca^{2+} was verified in cells pretreated with TG under Ca^{2+}-

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free conditions (RH buffer with 0.1 mM EGTA and 1 μM CLA). TG has been used to investigate SOCE because it selectively interferes with intracellular Ca^{2+} pools by depleting IP_3-sensitive Ca^{2+} stores without generation of IP_3.[29,30] The depletion of Ca^{2+} stores then provokes the entry of Ca^{2+} via SOCE if Ca^{2+} is added to the extracellular buffer. The effects of shikonin on SOCE were verified by monitoring $O_2^{\bullet-}$ generation and $[Ca^{2+}]_i$ levels as described in the legend of Fig. 4. BTP2 was used as a standard inhibitor of SOCE.

Statistical analysis. Experiments were repeated at least 3 times and measurements for each concentration of test compound were done in triplicate or more. Data are expressed as means ± SD, as indicated in the legends. Effects of different shikonin concentrations were analyzed using one-way analysis of variance (ANOVA). When ANOVA showed significant differences, post hoc analysis was performed with the Dunnett's test. Statistical significance was set at a confidence level of 1%, and refers to a two-sided probability.

Results

Shikonin is a chemical scavenger of $O_2^{\bullet-}$. As we[16] and others[14,15] have previously reported, shikonin scavenges $O_2^{\bullet-}$, with IC_{50} values of 2.9~17 μM depending on the source, assay conditions and $O_2^{\bullet-}$ detection system used. To know the contribution of the chemical scavenging of $O_2^{\bullet-}$ by shikonin in RH buffer with 1 μM CLA used in assays with cells, we first investigated the quenching of $O_2^{\bullet-}$-produced chemiluminescence of the hypoxanthine-xanthine oxidase reaction[3] in the CFL-C2000 (Fig. 1). The scavenging activity was not noted until a concentration of 3.3 μM shikonin, but it significantly increased at higher concentrations: the IC_{50} was 24.6 ± 3.7 μM (Fig. 1: mean ± SD, $n = 3$). These results show that the chemical scavenging of $O_2^{\bullet-}$ can be considered negligible below 3.3 μM in the buffer conditions of the following assays with cells, where the effects of shikonin appear at concentrations lower than 1.0 μM (Fig. 2–4).

Shikonin elicits synchronized changes in $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation of cells. fMLP induced a biphasic elevation of $[Ca^{2+}]_i$ as indicated by the fluo-3 AM signal within cells (Fig. 2A, control: 0 μM shikonin). The rapid transient elevation of $[Ca^{2+}]_i$ at 6 s after fMLP stimulation reflects the release of Ca^{2+} from intracellular Ca^{2+}-stores (IICR). This initial response was followed by a second peak of elevation of $[Ca^{2+}]_i$ at around 25 s of fMLP stimulation due to an overlapped influx of extracellular Ca^{2+} via SOCE and ROCE. This influx of extracellular Ca^{2+} decreased in approximately 4~5 min after fMLP stimulation to a level higher than the initial baseline (Fig. 2A top, grey chart). We assigned the two peaks as IICR, and SOCE plus ROCE, respectively, based on comparison of the fMLP-stimulation chart profiles in the presence and absence of EGTA (e.g., Fig. 3A subpanel a; single-peaked chart with EGTA vs two-peaked chart without EGTA). As such, the second peak corresponding to SOCE plus ROCE disappears when extracellular Ca^{2+} is chelated. Shikonin-induced inhibition of $[Ca^{2+}]_i$ elevation differed depending on the concentration used: at lower concentrations (0.033 to 0.33 μM), it gradually decreased the peak intensities, especially of the second peak. However, the two-peaked appearance of the responses was kept similar to the DMSO-control assay (Fig. 2A: 0.1 μM, and 2B). In contrast, high concentrations of shikonin (greater than 1.0 μM) caused severe inhibition of Ca^{2+} fluxes with a different pattern. Specifically, the response appeared as a single, low and broad peak that decayed slowly (Fig. 2A and B, 1.0 μM). It was also noted that the baseline of fluorescence increased at concentrations of shikonin greater than 1.0 μM. The increase in baseline fluorescence was seen in the absence of cells, indicating that it could be originated from shikonin itself. Indeed, shikonin has an intrinsic fluorescent emission that increases in the range of 570–700 nm upon excitation at 500 nm (data not shown), which is the excitation wavelength used in the assay.

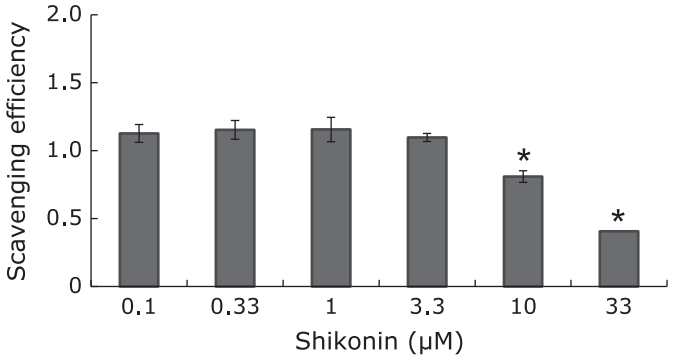

Fig. 1. Direct scavenging of $O_2^{\bullet-}$ by shikonin. Shikonin (final, 0.1–33.0 μM) was added to cuvettes containing 0.1 μM hypoxanthine, 1 μM CLA and 1 mM $CaCl_2$ in RH buffer and then preincubated at 37°C for 5 min before setting in CFL-C2000. The chemiluminescence baseline was monitored for 50 s before addition of xanthine oxidase (final, 2.4×10^{-3} U/ml) to produce $O_2^{\bullet-}$. Scavenging efficiencies were expressed as decreases in the chemiluminescence response (ratios of the areas under the curve, AUCs, of assays with shikonin relative to DMSO-control assays) and shown as a function of shikonin concentration. Values are means ± SD ($n = 3$). Statistically significant difference between all treatments was determined by one-way ANOVA followed by Dunnett's test: *p<0.01 vs DMSO-controls.

Coincident with the above changes in $[Ca^{2+}]_i$ responses, the chemiluminescence charts also show a two-peaked response, indicating two detectable waves of $O_2^{\bullet-}$ generation (Fig. 2A and C: 0 μM) with maximum points delayed by 6.5 s from those of the $[Ca^{2+}]_i$ peaks. These results support the fact that Nox2 activation depends on $[Ca^{2+}]_i$-mediated responses. Owing to the lack of specific granules in neutrophil-like cells originated from HL-60 cells,[33] the $O_2^{\bullet-}$ generation detected in the assay could be attributed to Nox2 assembled at the plasma membranes (i.e., to $O_2^{\bullet-}$ produced in the extracellular space).

The generation of $O_2^{\bullet-}$ was gradually inhibited in the presence of shikonin. At shikonin concentrations of up to 0.1 μM, inhibition of the second peak due to reduced $O_2^{\bullet-}$ generation was more pronounced than the inhibition seen for the first peak, as judged by peak heights (Fig. 2C). At 1.0 μM shikonin, the shape of the $O_2^{\bullet-}$ generation peak changed to a single, low and broad one owing to disappearance of the first peak (Fig. 2A and C); the activity also decayed slowly. Peaks became broader when 0.1 μM or more shikonin was used and at 3.3 μM, the generation of $O_2^{\bullet-}$ was completely inhibited (Fig. 2C).

The graphs showing $O_2^{\bullet-}$ generation and $[Ca^{2+}]_i$ responses (Fig. 2D), expressed as AUC ratios relative to DMSO-control assays, were very coincident, suggesting that the effects of shikonin on fMLP-induced Nox2 activity are based on changes in $[Ca^{2+}]_i$.

Unexpectedly, in assays with shikonin concentrations up to 0.33 μM, the AUC ratios relative to DMSO-controls showed a consistent elevation for both $[Ca^{2+}]_i$ and $O_2^{\bullet-}$ generation (Fig. 2D). The increase was likely caused by slower decays of $[Ca^{2+}]_i$; although peaks became shorter in height, the responses were prolonged and thus gave greater areas than DMSO-controls. In contrast, at shikonin concentrations of 1.0 μM or higher, pretreatment with shikonin caused a significant decrease in the AUC ratios of $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation (Fig. 2D). The decreases in $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation were paralleled until a concentration of 1.0 μM shikonin; at 3.3 μM shikonin, $O_2^{\bullet-}$ generation was completely lost although $[Ca^{2+}]_i$ still showed a small response (charts in Fig. 2B and C). The IC_{50} values for the $[Ca^{2+}]_i$ response and $O_2^{\bullet-}$ generation were 1.45 ± 0.06 ($n = 6$) and 1.12 ± 0.03 μM ($n = 6$), respectively.

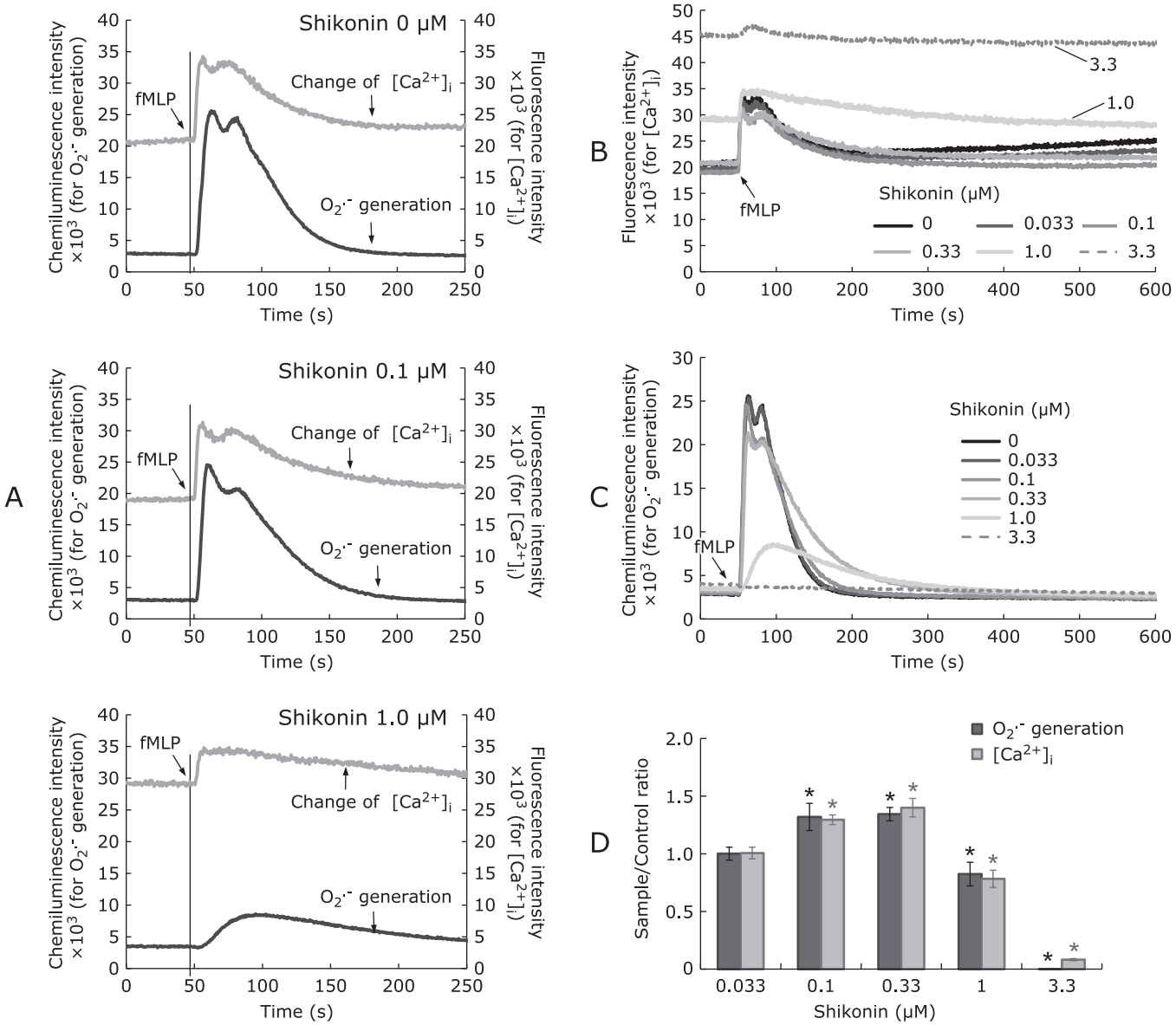

Fig. 2. Synchronized inhibition of fMLP-elicited changes in $[Ca^{2+}]_i$ and $O_2^{\bullet-}$ generation by shikonin. Fluo-3 AM-preloaded neutrophil-like cells (1×10^5/ml) were incubated at 37°C in the absence (DMSO) or presence of shikonin (0.033–3.3 μM) under low-rotation stirring in a dedicated incubator for 7 min before transferring of the assay cuvette to CFL-C2000. The assay buffer was RH buffer with 1 μM CLA and 1 mM $CaCl_2$ without EGTA added. After 50-s baselines of fluorescence and chemiluminescence were obtained, cells were stimulated with fMLP (1 μM), as indicated (panels A, B and C). Representative assays of 0, 0.1 and 1.0 μM shikonin focused on the initial 250-s periods (panel A), and superimposed charts for comparison of changes in $[Ca^{2+}]_i$ (panel B) and $O_2^{\bullet-}$ generation (panel C) are shown. The responses (AUCs) of shikonin-treated cells were shown as ratios relative to DMSO-control assays (panel D). Values are means ± SD ($n = 6$). Statistically significant difference between all treatments was determined by one-way ANOVA followed by Dunnett's test: *p<0.01 vs DMSO-controls.

In order to elucidate the inhibition of individual pathways involved in the shikonin-induced changes in $[Ca^{2+}]_i$ and $O_2^{\bullet-}$ generation, we performed experiments under conditions that allowed evaluation of distinct Ca^{2+} fluxes such as IICR and SOCE.

Shikonin inhibits IICR. The effects of shikonin on IICR were evaluated in the presence of EGTA (Fig. 3). In these assays, changes in $[Ca^{2+}]_i$ following fMLP stimulation occur only due to IICR. The IICR under such conditions corresponded to approximately 8.5% (based on peak AUCs; Fig. 3A, grey chart in subpanel a) of the total elevation in $[Ca^{2+}]_i$ seen in the controls in the presence of Ca^{2+} (i.e., fMLP stimulation of cells in RH with 0.1 mM EGTA, 1 mM $CaCl_2$). The IICR is followed by a peak of $O_2^{\bullet-}$ generation accounting for approximately 50.2% of the activity measured in the control assays with Ca^{2+} (Fig. 3A, grey chart in subpanel b).

Shikonin affected the IICR-mediated responses, as determined by the gradual decreases in both peaks of $[Ca^{2+}]_i$ and $O_2^{\bullet-}$ generation (Fig. 3A, subpanels c and d). The respective AUCs relative to DMSO-controls showed roughly coincident inhibition of $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation until 0.33 μM shikonin (Fig. 3B). Above this concentration, both of the AUC ratios continued to decrease. However, the AUC ratio of $O_2^{\bullet-}$ generation decreased more dramatically than that of the $[Ca^{2+}]_i$ levels. At concentrations above 0.33 μM, shikonin likely impairs Nox2 activity either by direct suppression or by inhibiting events involved in enzyme activation besides events related to IICR.

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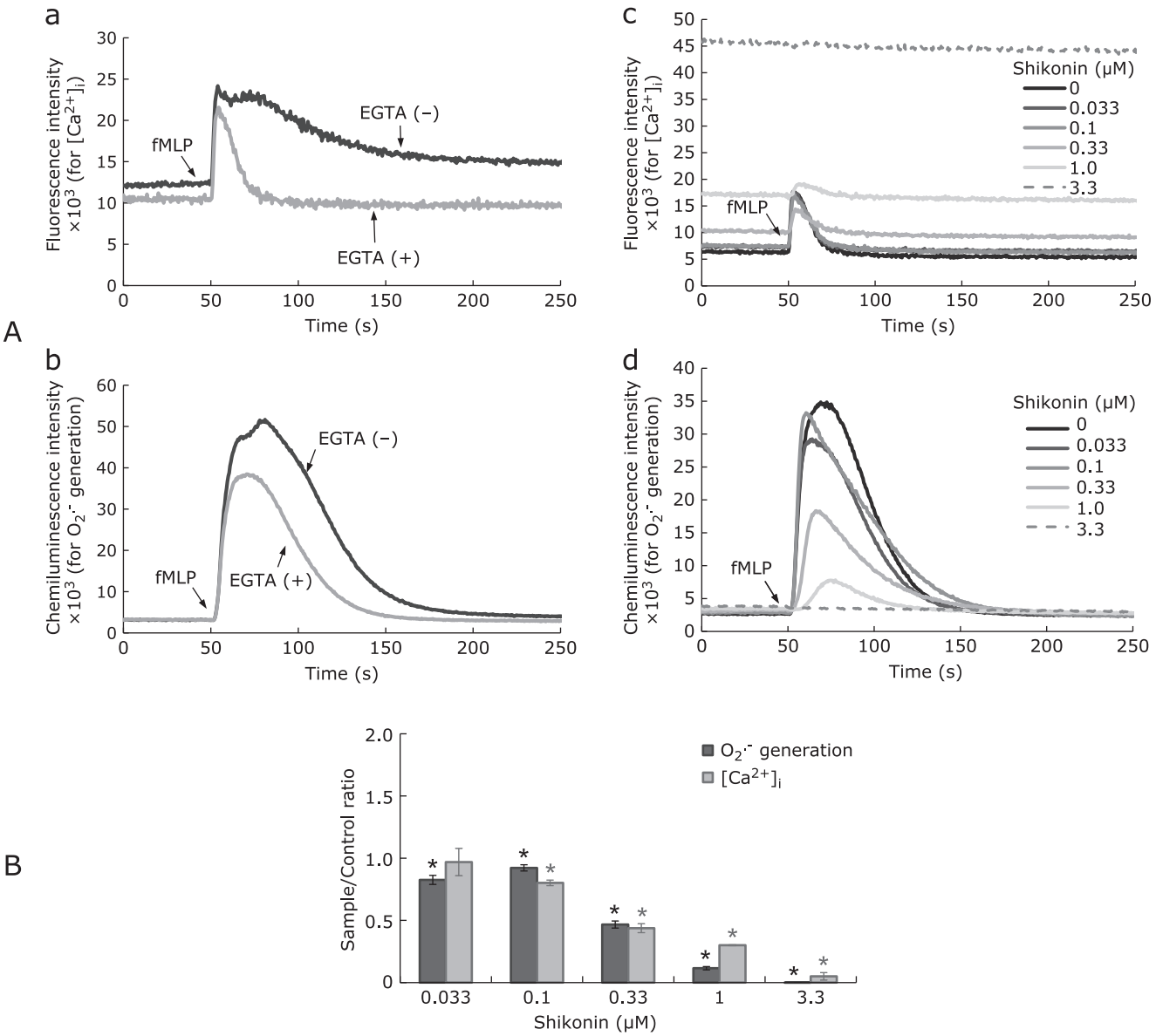

Fig. 3. Inhibition of IICR and subsequent $O_2^{\bullet-}$ generation by shikonin. Fluo-3 AM-preloaded neutrophil-like cells (1×10^5/ml RH buffer, 1 μM CLA) were incubated with 1 mM $CaCl_2$ for 1 min in a 37°C-preincubator before addition of 5 mM EGTA. Shikonin (0.033–3.3 μM) was added 1 min after EGTA, and pre-incubation was continued for a further 5 min before transferring of the assay cuvette to the CFL-C2000 to start simultaneous monitoring of chemiluminescence and fluorescence. Stimulation with fMLP (1 μM) was done after 50 s, and changes in $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation were further monitored. Panel A: Charts of changes in $[Ca^{2+}]_i$ (a) and $O_2^{\bullet-}$ generation (b) of control assays without shikonin in the presence (grey lines) or absence (black lines) of EGTA are shown, with focus on the initial 250-s periods. The effects of increasing concentrations of shikonin on IICR-originated changes in $[Ca^{2+}]_i$ (c) and $O_2^{\bullet-}$ generation (d) are shown superimposed for comparison. Panel B: Shikonin effects were shown as ratios of the responses (AUC) relative to DMSO-control assays. The AUCs were calculated by integrated signals in the period between the addition of fMLP until complete baseline decay is observed. Periods were the followings, for assays with and without EGTA, respectively: 110 and 700 s (fluorescence); 267 and 773 s (chemiluminescence). Values are means ± SD (n = 3). Statistically significant difference between all treatments was determined by one-way ANOVA followed by Dunnett's test: *p<0.01 vs DMSO-controls.

Shikonin inhibits SOCE. The effects of shikonin on SOCE that occurs when external Ca^{2+} is added to cells pretreated with TG under Ca^{2+}-free conditions are shown in Fig. 4. As described in *Reagents*, TG allows observation of Ca^{2+} entry via SOCE upon addition of Ca^{2+} to the reaction mixture.[29–31]

Treatment of cells with TG (1 μM) under Ca^{2+}-free conditions causes a transient elevation of $[Ca^{2+}]_i$ at around 20 s after the addition of TG (Fig. 4A), reflecting the release of stored Ca^{2+}. After that elevation, the $[Ca^{2+}]_i$ levels return to baseline by around 280 s (Fig. 4A). After Ca^{2+} stores are depleted, addition of Ca^{2+} to the extracellular buffer results in a rapid entry of Ca^{2+} through the plasma membrane. This is demonstrated by a sudden jump in the intensity of fluorescent signals followed by a peak that decays in ~120 s to a stable but higher $[Ca^{2+}]_i$ level compared to the Ca^{2+}-free conditions (Fig. 4A). This Ca^{2+} entry occurs because empty stores activate SOCE through the plasma membrane using the same channels that work for Ca^{2+} entry following fMLP stimulation.[31]

The depletion of Ca^{2+} stores by TG was accompanied by $O_2^{\bullet-}$ generation both before and after the addition of Ca^{2+} to the reaction

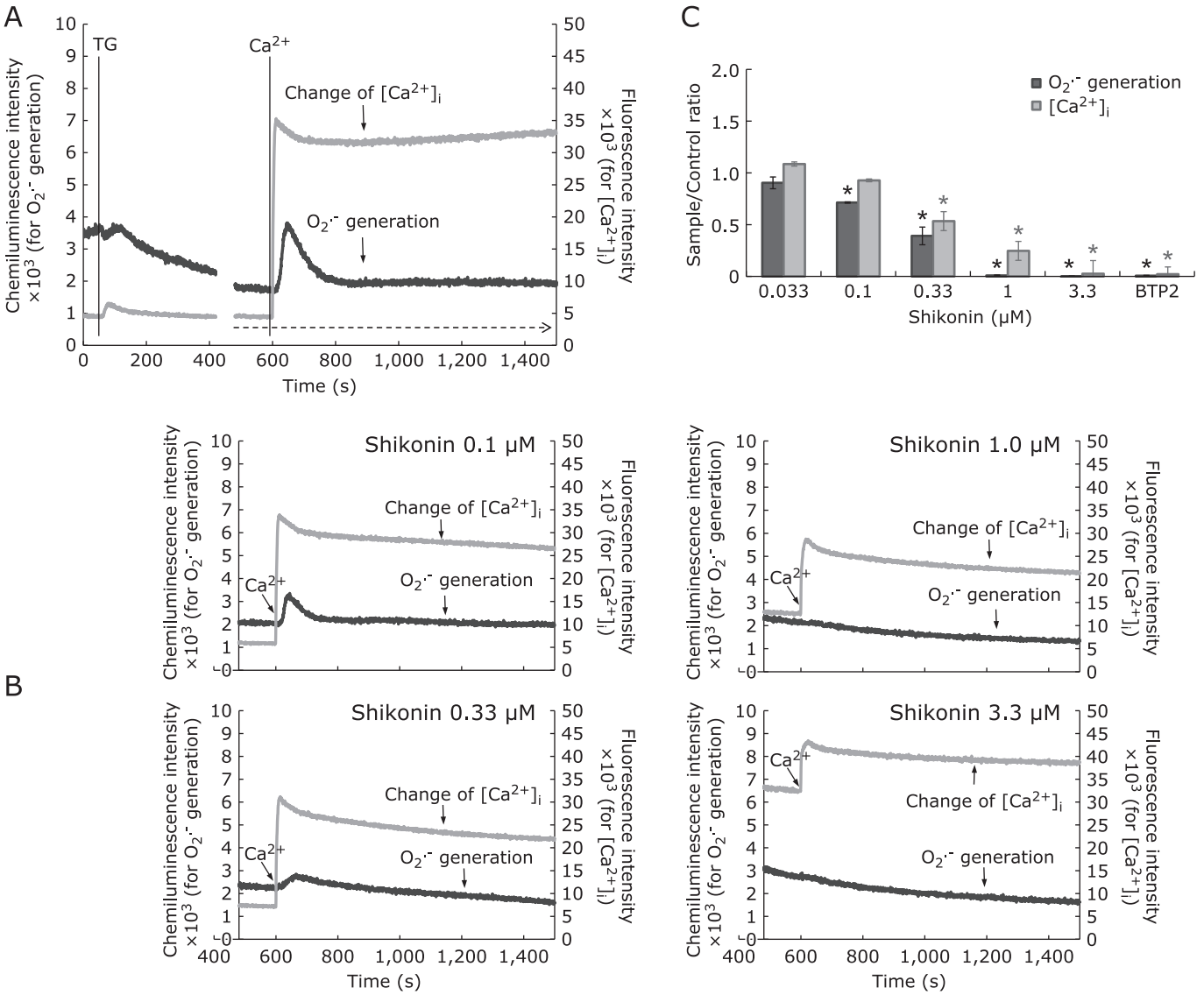

Fig. 4. Inhibition of SOCE by shikonin. Panel A: SOCE triggered by TG was checked in the absence of shikonin, as follows. Neutrophil-like cells (1×10^5/ml), preloaded with fluo-3 AM and washed as described in Materials and Methods, were suspended in Ca^{2+}-free buffer (RH buffer, 0.1 mM EGTA, 1 μM CLA), warmed for 3 min in a 37°C-preincubator, and then set in CFL-C2000 for monitoring of chemiluminescence and fluorescence. Addition of TG 1 min later (final 1 μM; left vertical line) causes a transient elevation of $[Ca^{2+}]_i$ due to release of Ca^{2+} from stores. After the release of Ca^{2+} from stores is ended, addition of external Ca^{2+} (1 mM; vertical line near 600 s) causes SOCE, detected as a sharp jump in the fluorescence signal followed by a peak, and finally, stabilization of $[Ca^{2+}]_i$ at a relatively high level (grey lines). $O_2^{\bullet-}$ generation following such changes in $[Ca^{2+}]_i$ is shown by simultaneously monitored chemiluminescence (black lines). Panel B: Charts of TG and shikonin added cells show the responses before and after addition of external Ca^{2+}; only responses corresponding to the latter part of the reaction in Panel A (i.e., part indicated by the dotted line arrow) are shown. The order of additions and incubation times were as follows: cells, pre-incubated for 3 min as in Panel A and kept in the pre-incubator, received additions of TG (1 μM) at 4 min, and shikonin (0.1–3.3 μM), BTP2 (10 μM) or DMSO at 6 min, and were further incubated for 5 min before transferring of the assay cuvette to the CFL-2000. After 2 min of monitoring of fluorescence and chemiluminescence, Ca^{2+} (final 1 mM) was added to the reaction mixture. Panel C: Inhibition of $O_2^{\bullet-}$ generation associated with SOCE ($[Ca^{2+}]_i$) is shown as a function of shikonin concentration by AUC ratios relative to DMSO control assays. The AUCs corresponding to SOCE were calculated from integrated signal responses in the period of 900 s after Ca^{2+} addition, from assays exemplified in panel B, as follows: parallel controls without TG were performed for each shikonin concentration, and these backgrounds were subtracted from respective assays with TG. Data are means ± SD (n = 3). Statistically significant difference between all treatments was determined by one-way ANOVA followed by Dunnett's test: *p<0.01 vs DMSO-controls.

mixture (Fig. 4A). The amount of $O_2^{\bullet-}$ produced by the release of Ca^{2+} from stores before addition of external Ca^{2+} was around 1/11 of that produced after the addition of Ca^{2+} (i.e., that of SOCE-associated response). The TG-induced $O_2^{\bullet-}$ generating activities including the SOCE-associated responses corresponded to approximately 3.4% of the full-reaction with fMLP stimulation in Ca^{2+}-containing buffer. It was previously unknown that a small $O_2^{\bullet-}$ generation response occurs upon TG-induced release of Ca^{2+} stores, even under Ca^{2+}-free conditions;[5,34] the disagreement in study results could be owing to the high sensitivity of the present system. However, the entry of extracellular Ca^{2+} to cells through SOCE resulting in a much higher generation of $O_2^{\bullet-}$ after TG-treatment is in agreement with previous reports.[5,34,35]

The reaction described above was used to investigate whether shikonin inhibits SOCE. Shikonin was added 2 min after TG, i.e., at a time when the TG-induced release of Ca^{2+} stores is

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ended (Fig. 4A), and cells were further incubated for 5 min in the presence of both compounds; the responses were monitored with the CFL-C2000. BTP2 was used as a positive control for SOCE inhibition.[28] Shikonin inhibited SOCE as shown by the gradually diminishing peak heights and AUCs of $[Ca^{2+}]_i$ in the charts of Fig. 4B (0.1 to 3.3 μM) and the bar graph in panel C. After correction for backgrounds in the absence of TG ($[Ca^{2+}]_i$ and $O_2^{\bullet-}$), it was noted that the inhibition of SOCE was roughly parallel with the decrease in $O_2^{\bullet-}$ generation until a shikonin concentration of 0.33 μM (Fig. 4C); however, with 1.0 μM or more shikonin, the $O_2^{\bullet-}$ generation zeroed whereas SOCE still remained observable. These results suggested the possibility that shikonin as high as 1.0 μM or above could directly affect Nox2 enzyme or its activation, in addition to effects on SOCE. At 3.3 μM shikonin, both $O_2^{\bullet-}$ generation and SOCE were completely inhibited, similar to the inhibition seen with BTP2.

These results show that the inhibition of SOCE by shikonin is also involved in the suppression of $O_2^{\bullet-}$ generation.

Discussion

Based on the knowledge that shikonin suppresses $O_2^{\bullet-}$ generation in neutrophils, the present study aimed to elucidate the effects of shikonin on the cellular Ca^{2+} fluxes that lead to the generation of $O_2^{\bullet-}$. For this purpose, we investigated whether inhibition of $O_2^{\bullet-}$ generation by shikonin is attributed to changes in $[Ca^{2+}]_i$, by measuring $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation with the CFL-C2000 system, in fMLP-stimulated neutrophil-like cells. This system simultaneously monitors both $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation within the same cell sample, and thus is highly applicable for screening the effects of compounds on cellular $[Ca^{2+}]_i$ associated with $O_2^{\bullet-}$ generation.

Previous studies have indicated that shikonin chemically scavenges ROS such as $O_2^{\bullet-}$[14–16] and hydroxyl radical,[15,17] and biologically behaves as an anti-inflammatory compound by directly targeting these oxidative molecules. The effects of shikonin on gene expression of key molecules involved in the inflammatory response have also been described;[11,13] however, the mechanisms of how shikonin suppresses $O_2^{\bullet-}$ generation of cells remained unclear. Under the assay conditions described here, it is likely that the $O_2^{\bullet-}$-scavenging activity of shikonin has no significant role in the inhibition of the respiratory burst from fMLP-stimulated cells, since the IC_{50} values differed by more than 20-fold (Results, *Shikonin is a chemical scavenger of* $O_2^{\bullet-}$ and *Shikonin elicits synchronized changes in* $[Ca^{2+}]_i$ *levels and* $O_2^{\bullet-}$ *generation of cells*: 24.6 μM for chemical scavenging vs 1.12 μM for $O_2^{\bullet-}$ generation; Fig. 1 and 2D, respectively). This difference in the IC_{50} values indicated that the inhibition mechanisms of shikonin include more sensitive intracellular targets than the direct scavenging of $O_2^{\bullet-}$. We herein found a likely target candidate to be the cellular Ca^{2+} fluxes.

Our data indicate that shikonin inhibits the fMLP-stimulated $O_2^{\bullet-}$ generation through inhibition of cellular Ca^{2+} fluxes (Fig. 2), such as IICR (Fig. 3) and SOCE (Fig. 4). The apparent IC_{50} values for the fMLP-stimulated responses in Ca^{2+}-containing buffer for $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation are 1.45 and 1.12 μM, respectively (Fig. 2D). In contrast, the IC_{50} values for IICR and SOCE were one order of magnitude lower (for $[Ca^{2+}]_i$ and $O_2^{\bullet-}$: 0.28 and 0.31 μM for IICR in Fig. 3B; and 0.39 and 0.25 μM for SOCE, estimated from Fig. 4C, respectively), indicating that very sensitive molecule(s) or reactions in the IICR and SOCE are targeted by shikonin. Therefore, the gradual inhibition of the two-peaked responses of both $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation seen in the fMLP-stimulated cells (Fig. 2B and C: peak height decreases by shikonin in the range of 0.1–0.33 μM) might be owned to the influences of shikonin on IICR and SOCE.

The target of shikonin when inhibiting IICR might be on IP_3 formation, since it has been reported that a shikonin derivative, acetylshikonin, inhibits IP_3 formation via impairment of PLC activity in fMLP-stimulated rat neutrophils.[36] This observation was concomitant with inhibition of Ca^{2+} release from internal stores (IC_{50}: ~5 μM, in the presence of 1 mM EDTA),[36] suggesting that Ca^{2+}-related events could be involved in the inhibition of Nox2 activity in fMLP-stimulated cells. Here, we obtained direct proof for this link because our monitoring system simultaneously examines changes in $[Ca^{2+}]_i$ levels and $O_2^{\bullet-}$ generation: with increasing shikonin, both chart profiles had similar shapes and were almost synchronous (Fig. 2).

It is still difficult to discuss about possible components of SOCE that are targeted by shikonin because all molecules involved in phagocytic Ca^{2+} fluxes have yet to be determined. However, molecules known to be involved in SOCE such as the $[Ca^{2+}]_i$-sensor protein stromal interacting molecule 1 (STIM1)[37] expressed in ER membranes, the plasma membrane-located calcium release-activated Ca^{2+} channel protein 1 (Orai1), and members of the transient receptor potential channels (TRPC)[4,23,25,38] might be considered as targets of shikonin. Among these molecules, STIM1 and Orai proteins might be potential targets because these proteins are thought to be regulated by critical cysteine modifications in a redox-dependent manner,[39] and shikonin reacts with thiols.[40] Knockdown of STIM1 has been shown to abrogate SOCE and Nox2 activity in murine neutrophils.[10] In addition, Nox2 itself requires cysteines for its activity, providing a likely target for shikonin.[41]

Our findings indicate that the inhibition of Nox2 activity by shikonin in fMLP-stimulated cells can be explained in great part by suppression of IICR and SOCE. Former studies have shown the importance of extracellular Ca^{2+} entry for Nox2 activation (reviews[4,25,38]). We did not directly evaluate whether shikonin has any influence on ROCE. This possibility exists and remains to be elucidated. The existence and importance of ROCE in neutrophils have been suggested,[42–44] however, current molecular and mechanistic understanding cannot delineate its role in Nox2 activation. It has been reported that $O_2^{\bullet-}$ generation by Nox2 at plasma membranes (i.e., $O_2^{\bullet-}$ release to the extracellular space) requires Ca^{2+} entry from outside the cell since studies have shown that Nox2 activity decreases in the presence of EGTA[4,5,45] or depends on extracellular Ca^{2+} concentrations.[10]

Our results show a slight $O_2^{\bullet-}$ generation followed by TG-induced depletion of intracellular stores under Ca^{2+}-free conditions that is enhanced to around 11-fold after the addition of Ca^{2+} (Fig. 4A). These results are in agreement with previous neutrophil studies[6,35] showing $O_2^{\bullet-}$ generation with 1 μM TG in Ca^{2+}-containing buffer detected by the superoxide dismutase-sensitive reduction of cytochrome *c*. In our study, the total TG-induced response (before and after the addition of Ca^{2+}; Fig. 4A) accounted for 3.4% of the usual activity with fMLP stimulation in the presence of Ca^{2+}. When fMLP is used as a stimulator, the activation signal spreads from the G-protein coupled receptors linked to heterotrimeric G-proteins into multiple pathways including the interaction of Gα and Gβγ with not only PLC but also phosphoinositide-3-kinase and p21-activated kinase, whose downstream effects lead to Nox2 activation.[22] This might explain the difference between TG- and fMLP-stimulated $O_2^{\bullet-}$ generation. These results support the view that both intracellular and extracellular Ca^{2+} are required for fMLP-stimulated $O_2^{\bullet-}$ generation.[46]

The present results suggested that the relevance of targets of shikonin other than Ca^{2+} fluxes to the inhibition of Nox2 activity appeared only at concentrations above 1.0 μM (Fig. 3B and 4C). These results do not exclude, however, the existence of inhibition sites that directly affect Nox2 enzyme activity or its assembly steps. Previous studies with acetylshikonin reported an impaired translocation of the Nox2 cytosolic component $p47^{phox}$ to membranes with concentrations above 3.0 μM.[19]

In summary, we show that shikonin affects the fMLP-elicited $O_2^{\bullet-}$ generation of neutrophil-like cells by targeting Ca^{2+} fluxes

such as SOCE and IICR. The use of a simultaneous monitoring system and proper selection of assay conditions discriminating for specific Ca^{2+} fluxes is a valuable strategy for elucidating the role of Ca^{2+} fluxes in the ROS-generating activity of cells.

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Abbreviations

AUC	area under the curve
BTP2	*N*-{4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl}-4-methyl-1,2,3-thiazole-5-carboxamide
$[Ca^{2+}]_i$	cytosolic, intracellular Ca^{2+} concentration
CLA	2-methyl-6-phenyl-3,7-dihydroimidazo [1, 2-*a*] pyrazin-3-one
DAG	diacylglycerol
DMSO	dimethylsulfoxide
ER	endoplasmic reticulum
EGTA	ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
fluo-3 AM	1-[2-amino-5-(2,7-dichloro-6-acetoxymethoxy-3-oxo-9-xanthenyl) phenoxy]-2-(2-amino-5-methylphenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetra (acetoxymethyl) ester
fMLP	*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine
IC_{50}	50% inhibitory concentration
IICR	inositol 1,4,5-trisphosphate-induced calcium release
IP_3	inositol 1,4,5-trisphosphate
Nox2	NADPH oxidase 2
$O_2^{\bullet-}$	superoxide anion
RH	Ringer-Hepes buffer
ROCE	receptor-operated calcium entry
ROS	reactive oxygen species
SERCA	sarco/endoplasmic reticulum membrane Ca^{2+}-ATPase
SOCE	store-operated calcium entry
STIM1	$[Ca^{2+}]_i$-sensor protein stromal interacting molecule 1
TG	thapsigargin
TRPC	transient receptor potential channel

Conflict of Interest

No potential conflicts of interest were disclosed.

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