Inhibition of Tumor Necrosis Factor-α through Selective Blockade of Pre-mRNA Splicing by Shikonin

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ABSTRACT

We previously developed a gene-gun-based in vivo screening system and identified shikonin as a potent suppressor of tumor necrosis factor-α (TNF-α) gene expression. Here, we show that shikonin selectively inhibits the expression of TNF-α at the RNA splicing level. Treatment of lipopolysaccharide-stimulated human primary monocytes and THP-1 cells with shikonin resulted in normal transcriptional induction of TNF-α, but unspliced pre-mRNA accumulated at the expense of functional mRNA. This effect occurred with noncytotoxic doses of shikonin and was highly specific, because mRNA production of neither a housekeeping gene nor another inflammatory cytokine gene, interleukin-8 (IL-8), was affected. Moreover, cotreatment with lipopolysaccharide (LPS) and shikonin increased the endpoint protein production of IL-8, accompanied by suppressed activation of the double-stranded RNA-activated protein kinase (PKR) pathway. Because PKR inactivation has been shown to down-regulate the splicing process of TNF-α RNA and interfere with translation, our findings suggest that shikonin may achieve differential modulation of cytokine protein expression through inactivation of the PKR pathway and reveal that regulation of TNF-α pre-mRNA splicing may constitute a promising target for future anti-inflammatory application.

Normal immune function is maintained by an elaborate balance between the activities of pro- and anti-inflammatory cytokines. One of the most important pro-inflammatory cytokines, TNF-α, plays a pivotal role in immune and inflammatory responses (Aggarwal et al., 2001; Locksley et al., 2001). Inappropriate expression or overexpression of TNF-α can lead to the progression of inflammatory and autoimmune diseases (Locksley et al., 2001; Schottelius et al., 2004; Wood et al., 2006), and recent studies suggest that inhibition of TNF-α production is an appealing target for the development of novel anti-inflammatory drugs (Palladino et al., 2003).

Expression of the "immediate-early" TNF-α gene is tightly controlled at several levels to ensure its silence in the absence of stimulation (Kontoyiannis et al., 1999) and enable a rapid response to noxious stimuli. Activator protein 1 and nuclear factor-κB, the two major transcription factors regulating TNF-α, are currently the primary targets for anti-

TNF-α drug discovery (Aggarwal et al., 2001; Tsukagoshi et al., 2001), and they are also important in regulating the expression of many other immune-related genes.

Shikonin (Fig. 1A), from the herb *Lithospermum erythrorhizon*, has been shown to possess numerous pharmacological properties, including anti-inflammatory and antitumor properties (Chen et al., 2002; Nakaya and Miyasaka, 2003). Shikonin has also been shown to have insulin-like actions through inhibition of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and tyrosine phosphatases (Nigorikawa et al., 2006). In our previous report, we identified shikonin to be a potent suppressor of TNF-α production (Staniforth et al., 2004). However, the mechanism through which shikonin achieves TNF-α-specific regulation remained unclear.

Evidence has emerged from additional post-transcriptional regulation of TNF-α pre-mRNA present in resting T-cells (Yang et al., 1998) and B-cells (Li et al., 2006), whereby T- or B-cell receptor activation induces splicing and leads to massive and rapid expression of TNF-α mRNA. Another study shows that a *cis*-acting element regulates the splicing of TNF-α pre-mRNA in response to external stimuli, via acti-

ABBREVIATIONS: TNF-α, tumor necrosis factor-α; PKR, double-stranded RNA-activated protein kinase; 2-AP, 2-aminopurine; CHX, cycloheximide; LPS, lipopolysaccharide; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; PBST, phosphate-buffered saline/Tween 20; IL, interleukin; 2APRE, 2-AP responsive element; eIF2α, eukaryotic initiation factor-2α.

vation of the double-stranded RNA-activated protein kinase (PKR) pathway (Osman et al., 1999). Thus, maturation of TNF-α mRNA precursor proceeds efficiently in response to external stimuli in early inflammation and is subject to regulation.

To study the mechanism of inhibition of TNF-α expression by shikonin, we used two types of LPS-induced monocytic cell cultures to monitor the effect of shikonin on TNF-α production. We report here that shikonin achieves TNF-α-specific inhibition through impairment of pre-mRNA splicing process. Moreover, we found that shikonin exerts its effect through suppression of PKR signaling. These results constitute the first detailed explanation for the anti-TNF-α activity of shikonin and reveal that the regulation of TNF-α premRNA splicing may be a promising target for future antiinflammatory studies. It may also provide novel insights for the exploration of specific phytocompounds with potential pharmaceutical applications.

Materials and Methods

Chemicals and Antibodies. 2-Aminopurine (2-AP), cycloheximide (CHX), dexamethasone, and LPS were obtained from Sigma (St. Louis, MO). Isohelenin and SB203580 were from Calbiochem (San Diego, CA). U0126 was obtained from Cell Signaling Technol-

Fig. 1. Effect of shikonin on TNF-α protein expression in monocytic cells at nontoxic doses. A, structure of the naphthoquinone, shikonin. B, the effect of shikonin on cell viability. THP-1 and primary monocyte cells were cultured in the presence of shikonin for 24 h, and the proportion of viable cells was determined by trypan blue exclusion assay. C, dose-dependent inhibitory effect of shikonin on TNF-α protein expression. THP-1 cells were treated with 100 ng/ml LPS and different dosages of shikonin for 3 h. The culture medium was collected and analyzed by ELISA.

ogy (Danvers, MA). Shikonin was prepared as described previously (Staniforth et al., 2004).

Cell Culture and Monocyte Preparation. THP-1 cell cultures (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Invitrogen, Carlsbad, CA) at 37°C in 5% CO$_2$.

Peripheral blood mononuclear cells from heparinized blood of healthy volunteers (Taipei Blood Center, The Chinese Blood Services Foundation) underwent density gradient centrifugation on Ficoll-Paque (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Human primary monocytes were obtained by magnetic cell separation (Miltenyi Biotech, Bergisch Gladbach, Germany). Purity of isolated monocytes was 95% (by flow cytometry using CD14-PE antibody; Immunotech; Beckman Coulter, Fullerton, CA).

Primer Design and Reverse Transcriptase-PCR Condition. Extraction of total RNA was followed the manufacturer's instructions of TRIzol reagent (Invitrogen). RT-PCR reactions were conducted with the AccessQuick RT-PCR system (Promega, Madison, WI), according to the manufacturer's instructions, at cycle numbers that yielded products within the linear range as determined for each pair of primers. Analysis was performed with BIO-1D (Vilber-Lourmat, Marne-La-Vallée, France) image software. The primers used were: human TNF-α: sense 1, 5′CTCTTCTCCTTCCTGATCGTG-GCA; sense 2, 5′GAAAGCATGATCCGGGACGTGGA; antisense, 5′-GTTGGATGTTCGTCCTCCTCACA; human GAPDH: sense, 5′CAT-CACTGCCACCCAGAAGACTGTGGA; antisense, 5′TACTCCTTGG-AGGCCATGTAGGCCATG; human IL8: sense, 5′CTTGGCAGCCT-TCCTGATTT; antisense 1, 5′CTCAGCCCTCTTCAAAAACT; antisense 2, 5′CTTGGATACCACAGAGAATG.

Quantitative Real-Time Polymerase Chain Reaction. cDNA was produced from 2 μg of total RNA samples using Oligo(dT)$_{20}$ and SuperScript II (Invitrogen). The primers for real-time PCR were: TNF-α, sense 5′CCCAGGGACCTCTCTCTAATCA; antisense, 5′AG-CTGCCCCTCAGCTTGAG; IL-8: sense, 5′AGGTGCAGTTTTGCCA-AGGA; antisense, 5′TTTCTGTGTTGGCGCAGTGT; GAPDH: sense, 5′-CCCCTTCATTGACCTCAACTAC; antisense, 5′GATGACAAGCT-TCCCGTTCTC. LightCycler PCR was performed with DNA SYBR Green I according to the manufacturer's instructions (Roche Diagnostics). Gel electrophoresis and melt curve analysis were used to verify the formation of specific products. mRNA levels of analyzed genes were normalized to GAPDH mRNA to determine relative expression ratios.

Br-UTP Incorporation. The amount of newly synthesized RNA in THP-1 cells was evaluated by incorporating bromo-UTP (Sigma) into de novo-synthesized RNA transcripts on flow cytometry analysis according to the protocol previously reported (Jensen et al., 1993). Anti-Bromo-dUTP conjugated with fluorescein isothiocyanate antibodies were obtained from Roche (Indianapolis, IN). To rule out the interference of DNA synthesis, propidium iodide (Sigma) was used to gate cells in the G$_1$ phase from the bromo-UTP-labeled THP-1 cells.

ELISA. Culture media were harvested at the times indicated and underwent ELISA [HU TNF-α CYTOSET (Invitrogen, Carlsbad, CA) antibody for TNF-α and HU IL-8 CYTOSET (Invitrogen) antibody for IL-8; Invitrogen] following the manufacturer's instructions. Measurement involved use of an Autoreader and analysis with KCjunior software (both from Bio-Tek Instruments, Winooski, VT).

Western Blot Analysis. For isolation of total protein, treated THP-1 cells were centrifuged at 300*g* for 3 min, and cell pellets were lysed in 2 ml of ice-cold lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.4, 20 mM EGTA, 1 mM dithiothreitol, 1 mM Na$_3$VO$_4$, and protease inhibitor cocktail tablets) for 10 min. Lysates were centrifuged at 12,000*g* for 20 min, and supernatant containing 30 μg proteins was boiled in SDS sample loading buffer for 10 min before undergoing electrophoresis on 12% NuPAGE Bris-Tris gels (Invitrogen). After electrophoresis for 2 h, proteins in gel were transferred to a polyvinylidene difluoride membrane (Pall Inc., East Hills, NY), and the blots were blocked with 5% nonfat dry
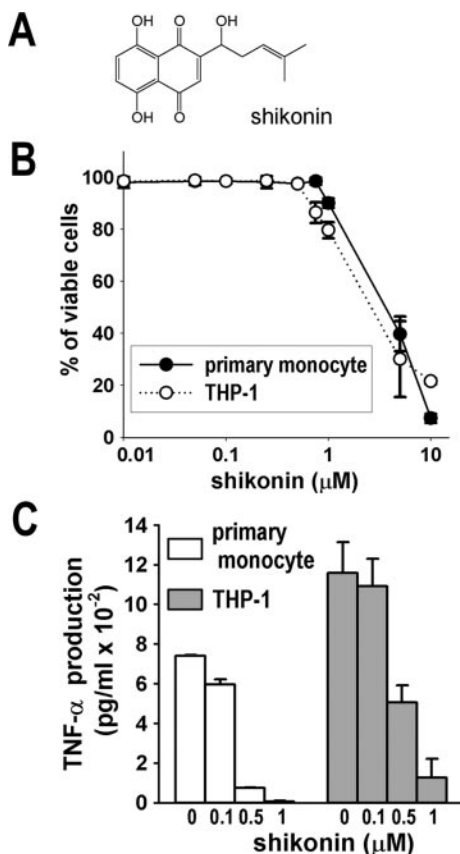
milk-phosphate-buffered saline containing 0.1% Tween 20 (PBST) for 60 min at room temperature. The membranes were incubated overnight at 4°C with 1:1000 dilutions of phospho-specific PKR and PKR antibodies (Cell Signaling Technology). Equal protein loading was assessed with human β-actin antibodies (Sigma). The membrane blots were rinsed three times with PBST buffer for 5 min each. Washed blots were incubated with 1:2000 dilutions of the horseradish peroxidase conjugated-secondary antibody and again washed three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (GE Healthcare).

PKR in Vitro Kinase Assay. Recombinant PKR (Upstate Biotech, Lake Placid, NY) and its substrate eIF2-α (Invitrogen) were used for in vitro kinase reactions in kinase reaction buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl₂, 1% Triton X-100, 20 mM ATP, and 5 μCi of [γ-^{32}P]ATP. Whenever indicated, poly(dI/dC) was added to a final concentration of 1 μg/ml; shikonin was used at 2 concentrations, 0.1 and 1 μM; and kinase reactions were incubated at 30°C for 30 min. The phosphorylation of test proteins was examined after fractionation in SDS-PAGE by autoradiography.

Results

Nontoxic Doses of Shikonin Efficiently Suppress TNF-α Protein Expression. Shikonin is effective in the treatment of dermatitis (Chen et al., 2002) but is known to decrease cellular metabolic rate (Nakaya and Miyasaka, 2003) and inhibit global transcription (Staniforth et al., 2004). We questioned whether this therapeutic action derives from a cell-death-induced detoxification or a more sophisticated regulation of cellular processes.

We evaluated the toxicity of shikonin on human monocyte cells, using trypan blue exclusion for 1–3 days. Doses at <1 μM in THP-1 cells and in monocytes (Fig. 1B) had no detectable toxic effects. Minor toxicity was first noted at ≥1 μM, and the toxic IC$_{50}$ was approximately 4 μM in THP-1 cells. We are therefore confident that shikonin lacks nonspecific cytotoxic activity below 1 μM.

We next examined the inhibition of shikonin on TNF-α protein in cultures stimulated with LPS (100 ng/ml). There was a dose-dependent decrease of TNF-α protein expression in monocytes and THP-1 cells between 0.1 and 1.0 μM shikonin (Fig. 1C) after 3 h. Thus, shikonin could suppress LPS-induced TNF-α expression at nontoxic concentrations.

Shikonin Specifically Inhibits TNF-α mRNA Expression. To examine whether shikonin suppresses TNF-α at the mRNA level, we performed quantitative real-time PCR assays in LPS-induced THP-1 cells. Shikonin caused a significant decrease of the steady-state mRNA level of TNF-α but not another LPS-inducible cytokine gene, IL-8, or the constitutively expressed housekeeping gene GAPDH (Fig. 2A) after 30 min. Because shikonin has been reported to inhibit general transcription by interfering with TATA-box binding protein binding to TATA box elements (Staniforth et al., 2004), we analyzed whether the overt down-regulation of TNF-α mRNA was the transient result of a relatively unstable transcript under global gene suppression. Although shikonin inhibited bromo-UTP incorporation into nascent mRNAs at concentrations ≥1 μM (Fig. 2B) after 16 h, the decrease in TNF-α expression was still observable at concentrations lower than those causing effective suppression of general transcription (Fig. 1C). Together, our data indicate that shikonin's action on the inhibition of TNF-α expression takes

place at the RNA level and is independent of its suppressive effect on general transcription.

Shikonin Affects TNF-α mRNA Expression by Impairing the Splicing Process. To pinpoint exactly how shikonin mediates suppression of TNF-α RNA levels, we used RT-PCR to study the effect of shikonin on the different splicing stages of TNF-α RNA species. The various primers and their predicted TNF-α products are depicted in Fig. 3A. To our surprise, the addition of ≥0.1 μM shikonin to monocytes caused a dose-dependent accumulation of TNF-α pre-mRNA that was not seen with IL-8 and GADPH pre-mRNAs (Fig. 3B) after treatment for 30 min.

TNF-α induction may occur in response to stimuli other than the inflammatory response. We investigated whether the shikonin-mediated effect on accumulation of TNF-α pre-mRNA was restricted only to the TLR4 pathway. CHX is known to cause superinduction of TNF-α mRNA but to shut down de novo protein synthesis (Jarrous et al., 1996). It enables one to directly assess signaling events, without interference from newly synthesized proteins. As seen with LPS-dependent stimulation, shikonin suppressed TNF-α mRNA induction by CHX (Fig. 3B), and the 810-base pair pre-mRNA intermediate rapidly accumulated at the expense of mature mRNA (Fig. 3C) after 1 h. Together, these data indicate that shikonin selectively inhibits TNF-α splicing independently of the signaling pathway leading to TNF-α expression.
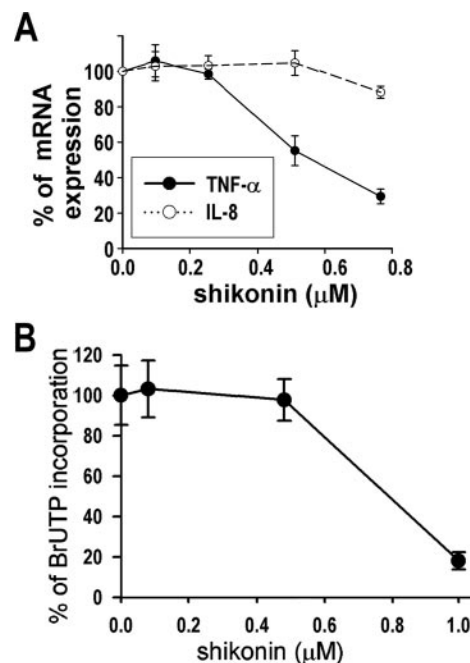

Fig. 2. Effect of shikonin on the expression of TNF-α mRNA. A, Shikonin selectively inhibits TNF-α mRNA expression. Total cellular RNA was harvested 30 min after treating THP-1 cells with 100 ng/ml LPS and different dosages of shikonin. The mRNAs of TNF-α and IL-8 were analyzed by quantitative real-time PCR and normalized to the GAPDH mRNA. Shikonin inhibited expression of TNF-α but not of the LPS-inducible cytokine IL-8 or the constitutively expressed housekeeping gene GAPDH. B, effect of shikonin on the rate of general transcription. THP-1 cells were treated with 100 ng/ml LPS and different dosages of shikonin for 16 h, and the general RNA transcription rate was measured by Br-UTP incorporation and analyzed by flow cytometry. Data were normalized against false-positive signals derived from DNA synthesis. The concentration of shikonin that effectively inhibited the general transcription of RNA was considerably higher than that required to inhibit TNF-α gene expression.

Comparison of the Effects of Specific Inhibitors for Different Signal Transduction Pathways. Regulation of TNF-α mRNA splicing can be mediated through a specific secondary RNA structure, called 2-AP responsive element (2APRE), located at the 3′ terminus of TNF-α pre-mRNA, via activation of the PKR pathway (Osman et al., 1999). 2-AP can block this process and lead to accumulation of unspliced TNF-α pre-mRNA species (Jarrous et al., 1996). Although 2-AP (10 mM) also led to accumulation of the 1724-base pair unspliced pre-mRNA species (Fig. 4A) in the presence of CHX (10 μg/ml), it also markedly affected the synthesis of TNF-α pre-mRNA. As seen in Fig. 4A, no mature mRNA molecules were detected in 2-AP-treated cells, which was not the case with 0.5 μM shikonin (Figs. 3C and 4A). In addition, 2-AP caused a substantial decrease in TNF-α pre-mRNA after 60 min. In contrast, shikonin caused a partial accumulation of mature TNF-α mRNA but did not affect the steady-state accumulation of pre-mRNA. We then compared the effect of shikonin and other TNF-α inhibitors on LPS-induced cytokine protein expression by ELISA after 6 h (Fig. 4B). We were surprised to find that both shikonin and 2-AP could

decrease the level of TNF-α protein and concomitantly increase the level of IL-8 protein in a dose-dependent manner (Fig. 4C). This effect was not observed with other known TNF-α inhibitors tested (Fig. 4B). We therefore suggest that 2-AP and shikonin may share a similar, yet distinguishable, mode of action and signaling pathways.

Shikonin Attenuates PKR Activation in THP-1 Cells. 2-AP is known to inhibit the PKR signaling pathway, so the effects of shikonin on this signaling system were examined in

Fig. 4. Comparison of the regulation of TNF-α pre-mRNA splicing between shikonin and 2-AP. A, shikonin and 2-AP cause accumulation of the 1724-base pair unspliced TNF-α pre-mRNA. RNA samples were collected from CHX (10 μg/ml)-stimulated THP-1 cells treated with 2-AP (10 mM) or shikonin (0.5 μM). B, effects of known TNF-α inhibitors on LPS (100 ng/ml)-stimulated cytokine expression after treatment for 6 h. Differential analysis of effects on TNF-α and IL-8 protein production suggests that shikonin shares a similar mode of action with 2-AP. C, dose-dependent effects of shikonin and 2-AP on expression of LPS-induced TNF-α and IL-8 was measured by ELISA at 6 h. Data are means ± S.E. of three independent duplicated experiments.
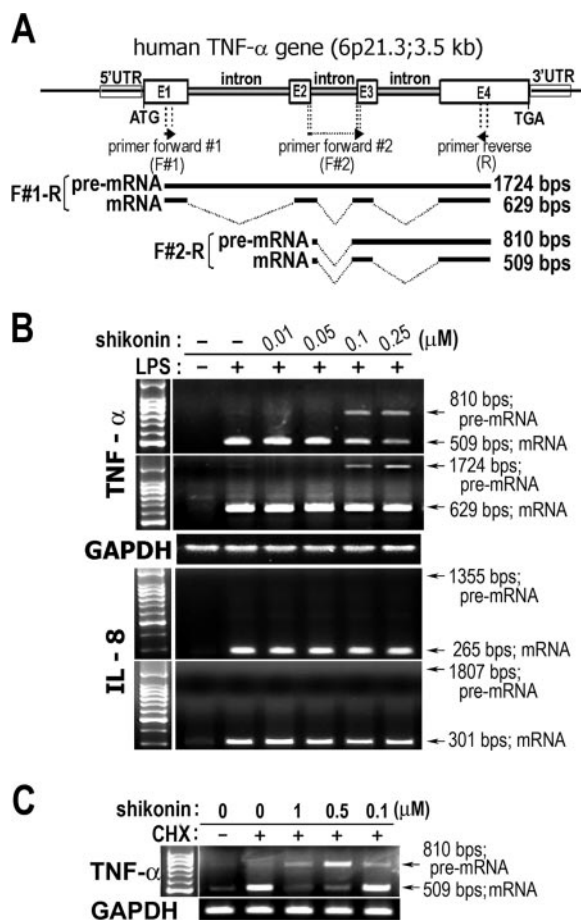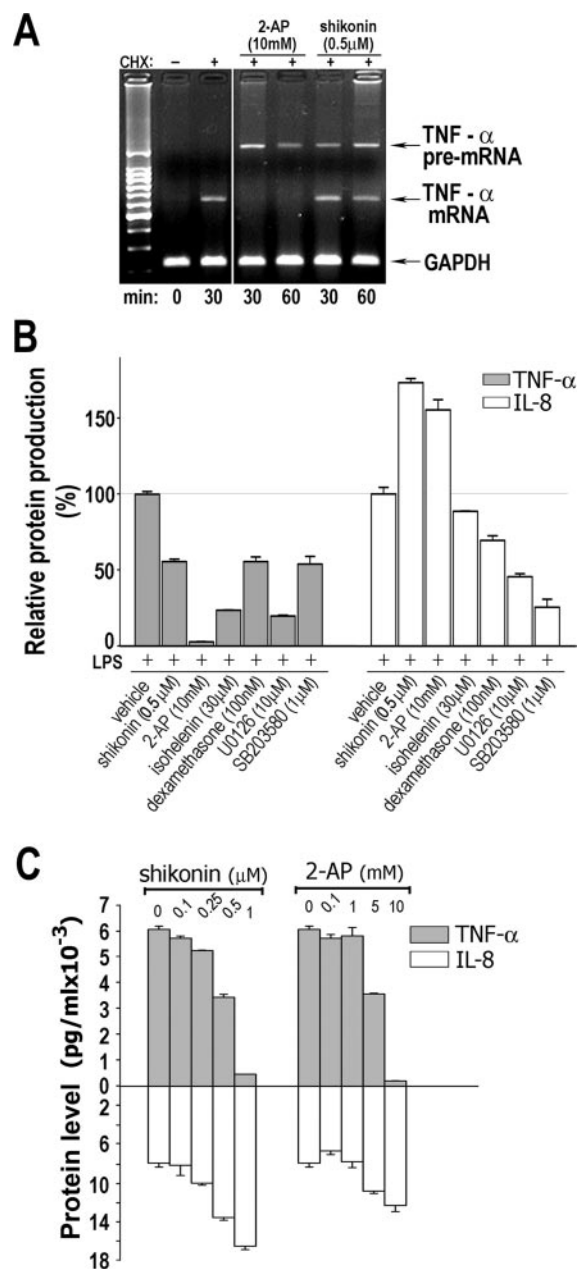
Fig. 3. Effect of shikonin on TNF-α pre-mRNA splicing. A, schematic representation of the DNA primers. The designed primer sets were used to discriminate different splicing stages of TNF-α mRNA. B, the selective inhibitory effect of shikonin on the splicing process of TNF-α pre-mRNA in a dose-dependent manner. Total RNA from LPS-stimulated THP-1 cells treated with different doses of shikonin for 30 min were analyzed by RT-PCR. PremRNA stalled in the splicing process was amplified by both sets of designed primers. GAPDH and IL-8 RNA expression were not affected. C, shikonin also suppressed CHX (10 ug/ml)-induced TNF-α RNA expression at the pre-mRNA splicing level after treatment for 60 min.

THP-1 cells using phosphospecific antibodies to detect activated PKR. The activation-dependent intracellular phosphorylation of PKR was effectively blocked by shikonin in THP-1 cells after treatment for 4 h (Fig. 5A). We next determined whether shikonin could directly interact with PKR and affect its activation. GST-fusion recombinant PKR and its substrate protein, eIF2α, were subjected to a cell-free, in vitro kinase assay. However, GST-fusion PKR phosphorylation of its substrate protein eIF-2α was not directly affected by shikonin (Fig. 5B). Taking the results of Fig. 5, A and B, we suggest that shikonin does not directly interact with PKR to block the signaling pathway of the PKR cascade.

Discussion

We have shown here that shikonin selectively inhibits the expression of human TNF-α mRNA in monocytic cells at noncytotoxic concentrations. This down-regulation occurs at the splicing level and is characterized by the prompt and extensive accumulation of newly transcribed splicing intermediate transcripts. The differential modulation of LPS-induced cytokines by shikonin was also shown to involve the PKR pathway.

Nontoxic Doses of Shikonin Inhibit the Splicing Process of TNF-α pre-mRNA. One explanation for the finding that shikonin causes short-lived unspliced TNF-α pre-mRNA to accumulate at the expense of mRNA may be that shikonin destabilizes TNF-α mRNA with a concomitant increase in transcription that leads to an increase in the precursor pool of RNA transcripts. However, another short-lived, inducible inflammatory cytokine gene, IL-8, did not respond in a similar fashion (Fig. 2A). There is no evidence that shikonin alone stimulates transcription of endogenous genes; in contrast, shikonin has been reported to inhibit the transcription of immune-related genes in vivo and in vitro (Subbaramaiah et al., 2001; Staniforth et al., 2004). Accordingly, we found that shikonin inhibited the expression of TNF-α in human primary monocytes (data not shown) and in the THP-1 cell line (Fig. 2A). In addition, we showed that at higher concentrations ($\geq$1 μM), shikonin inhibited global transcriptional activity (Fig. 2B), resulting in a gradual elimination of tran-

siently expressed RNA transcripts of TNF-α (Fig. 3C). We previously demonstrated that effects of this kind may be due to interference with TATA box binding protein by shikonin (Staniforth et al., 2004).

RT-PCR (Fig. 3B) revealed that treating THP-1 cells with shikonin resulted in deficient processing of TNF-α pre-mRNA. We also note that shikonin inhibits TNF-α gene expression in a manner distinct from other well known anti-inflammatory compounds or specific kinase inhibitors (Fig. 4C) (Swantek et al., 1997; Scherle et al., 1998; Mazor et al., 2000; Tsukagoshi et al., 2001). Shikonin specifically affected only the TNF-α pre-mRNA and not the inducible IL-8 gene or the constitutively expressed GAPDH gene. Because the RT-PCR analysis we designed was capable of simultaneously monitoring effects on transcription and on splicing (Fig. 3B and 3C), our data clearly show that expression of the TNF-α gene is selectively inhibited by shikonin at the splicing stage.

We conclude that the splicing inhibitory effect is not coupled to the inhibitory effect on transcription, because TNF-α pre-mRNA continued to accumulate, and the overall transcription rate was not affected by shikonin treatment (Fig. 2B). In other words, nontoxic concentrations of shikonin do not affect the initiation and synthesis of TNF-α pre-mRNA, and accumulation of TNF-α pre-mRNA results not only from stabilization of pre-existing pre-mRNA but also from the synthesis of short-lived nascent pre-mRNA transcript molecules.

Another important point is that we detected shikonin-affected TNF-α pre-mRNA at levels comparable with levels of mature mRNA seen without shikonin treatment. It would be interesting to investigate whether this shikonin-accumulated TNF-α pre-mRNA could enter the cytoplasm for later processing or whether it had any direct effect on expression.

Shikonin Down-Regulates Splicing Process of TNF-α pre-mRNA through Inactivation of the PKR Pathway. TNF-α pre-mRNA products of different lengths were evaluated with probes that monitored the excision of intron 3 as well as splicing within the primary transcripts at the intron 2-exon 3, exon 3-intron 3, and intron 3-exon 4 junctions. Shikonin blocked TNF-α splicing at multiple sites, whereas the control IL-8 transcript was spliced normally. These suggest that there is no rate limiting step for the processing of TNF-α introns and that the shikonin-mediated blockage affects every TNF-α splice site.

Shikonin inhibited the processing of TNF-α pre-mRNA, with an accumulation of precursor transcripts, after induction by both CHX and bacterial LPS (Fig. 3, B and C). The ability of shikonin to block expression of TNF-α mRNA in cells stimulated with CHX indicates that the shikonin-sensitive component is expressed in a functional form before induction. Previous reports indicated that the PKR-dependent pathway can regulate the fast processing of latent-pool TNF-α transcripts (Jarrous et al., 1996), which involves local activation of PKR through specific secondary structures found on RNA transcripts (Ben-Asouli et al., 2002). It is noteworthy that we found that the activation-dependent phosphorylation of PKR is suppressed upon treatment with shikonin (Fig. 5A), suggesting a key role of the PKR pathway in the shikonin-dependent splicing regulation (Fig. 6). Furthermore, the splicing of IL-8 mRNA proceeds unabated in shikonin-treated cells, indicating that the common steps of the splicing process were obviously not affected. Taken to-
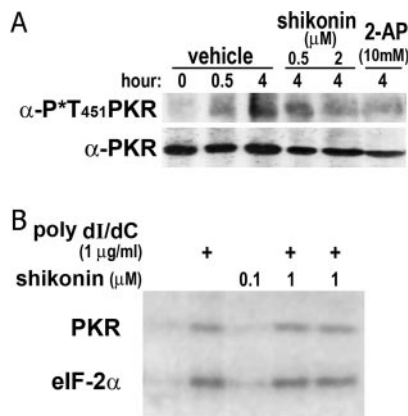

Fig. 5. Shikonin suppresses PKR activation. A, THP-1 lysates were harvested after the indicated treatments and subjected to SDS-PAGE analysis. Activation-dependent PKR phosphorylation at Thr451 (*top*) and whole PKR protein (*bottom*) were analyzed by Western blot. B, shikonin cannot reverse PKR activation after auto-phosphorylation. GST-fusion recombinant PKR activity was determined by in vitro kinase reaction, with purified eIF2α protein used as a substrate.

gether, these studies suggest shikonin may interfere with the autoregulatory loop of TNF-α splicing through local activation of PKR.

Our data (Fig. 4, A and B) indicate that shikonin and the PKR inhibitor 2-AP have distinct effects on TNF-α mRNA but similar effects on TNF-α protein. In conjunction with this finding, Fig. 5A shows a clear inhibitory effect of shikonin on PKR phosphorylation in the cellular context, which should relay upstream of PKR activation, because it has no effect once PKR is activated by dsRNAs in vitro. Thus, we suggest that the molecular action of shikonin might reside in the interference of PKR interaction with its upstream activating molecules, such as the 2APRE (Fig. 6) located within the 3′UTR of TNF-α transcript (Osman et al., 1999). Thus, it would be appealing to examine whether deleting the 2APRE sequence from the TNF-α gene would render splicing of the encoded precursor transcripts resistant to inhibition by shikonin or if introduction of 2APRE into the IL-8 gene would shift the inhibitory effect of shikonin to the IL-8 gene.

Altogether, our findings provide useful and additional mechanistic explanations of the anti-inflammatory effect of shikonin and highlight the nontoxic, highly specific, and effective pharmaceutical value of this extensively studied phytocompound. Several protein-based TNF-α inhibitors, including etanercept, infliximab, and adalimumab, have been approved for clinical use in inflammatory diseases by the U.S. FDA (Taylor, 2003). Although these injectable TNF-α inhibitors have demonstrated efficacy, they also show potentially serious side effects (Palladino et al., 2003). The natural compounds present in various Chinese traditional medicines have a long history of use in similar indications with fewer side effects. We speculate that there will be significant benefits in developing orally active, easily synthesized small molecules that target the specific signaling and synthesis pathways for TNF-α. Thus, the potency and specificity of these phytocompounds makes them an attractive prospect for the development of new anti-inflammatory therapies in both chronic and acute disease states.
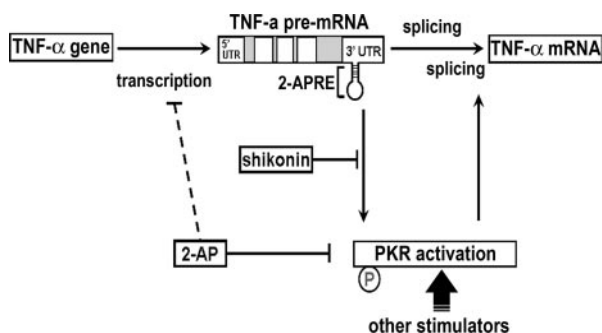

Fig. 6. A proposed scheme for the mechanism of action of shikonin on TNF-α. PKR can be inactivated by both 2-AP and shikonin in THP-1 cells, but the differential effects between shikonin and 2-AP observed implies that the transcriptional machinery for producing pre-mRNA is inhibited by 2-AP but not by shikonin.

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