

Suppressive effect of isofraxidin on the overexpression of IL-6 and its molecular mechanism

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Research Article

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Abstract

Interleukin-6 (IL-6) is a pleiotropic cytokine that has many biological activities, including inflammation, hematopoiesis, bone metabolism, embryonic development, and other fundamental processes. Recently, IL-6 has been widely recognized as an important pro-inflammatory cytokine involved in cytokine storm pathogenesis during severe inflammatory diseases, such as coronavirus disease 2019 (COVID-19). Therefore, IL-6 is considered to be a therapeutic target for inhibiting cytokine storm. In the present study, we investigated the suppressive effect of isofraxidin, a major coumarin compound of *Acanthopanax senticosus*, on the overexpression of IL-6 and its molecular mechanism. When human hepatocellular carcinoma cell lines, HuH-7 and HepG2, were treated with 12-O-tetradecanoylphorbol 13-acetate (TPA), a marked induction of IL-6 mRNA expression was observed in HuH-7 cells compared with HepG2 cells. Isofraxidin significantly suppressed TPA-induced IL-6 mRNA expression in HuH-7 cells in a dose-dependent manner. Furthermore, isofraxidin inhibited TPA-induced phosphorylation of ERK1/2 in a dose dependent manner. Similarly, the MAPK/ERK inhibitor U0126 suppressed TPA-induced IL-6 mRNA expression. However, isofraxidin had no effects on TPA-induced phosphorylation of SAPK/JNK, Akt (Ser473), and STAT3 (Tyr705), nuclear translocation of NF- κ B p65, and degradation of I κ B. Taken together, isofraxidin suppresses TPA-induced overexpression of IL-6 mRNA by selectively inhibiting the activation of the MAPK/ERK pathway in HuH-7 cells, indicating that isofraxidin may be an effective anti-inflammatory agent for treating cytokine storm.

Introduction

Cytokine storm is a systemic inflammatory response that is commonly triggered by bacteria or viral infections and chimeric antigen receptor T cell therapy, and is one of the leading causes of morbidity and mortality in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-induced coronavirus disease 2019 (COVID-19) [1–4]. Cytokine storm is characterized by the overexpression and excessive production of pro-inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α), which may cause acute respiratory distress syndrome (ARDS) and subsequent severe tissue damage [1–4]. Among the pro-inflammatory cytokines, IL-6 is one of the most important cytokines involved in the initiation of cytokine storm, and induces other pro-inflammatory cytokines and the marker of endothelial injury plasminogen activator inhibitor-1 (PAI-1). Therefore, IL-6 is considered to be a therapeutic target for inhibiting cytokine storm. In fact, the blockade of IL-6 trans-signaling using a humanized monoclonal antibody against human IL-6 receptor (anti-IL-6R antibody) has been shown to be effective in patients with severe COVID-19 [1].

Moreover, it has been revealed that intracellular signaling pathways, such as MAPK/ERK, SAPK/JNK, PI3K/Akt, NF- κ B/I κ B, and JAK/STAT3, are activated in host cells infected with SARS-CoV-2, resulting in the overexpression and excessive production of pro-inflammatory cytokines, such IL-6, in host cells [4–8]. Therefore, the inhibition of these signaling pathways using pharmacological agents may be an effective therapeutic strategy for counteracting cytokine storm in addition to the blockade of IL-6 trans-signaling

using an anti-IL-6R antibody. However, only a limited number of pharmacological agents are available for the treatment of cytokine storm.

Glucocorticoids have been used in the treatment of inflammation-related diseases, such as rheumatoid arthritis, sepsis, and COVID-19 [9–11]. Glucocorticoids bind intracellular glucocorticoid receptors (GRs) and subsequently downregulate the expression of pro-inflammatory cytokines by inhibiting the activation of intracellular signaling pathways and transcriptional factors [12]. However, the anti-inflammatory effects of glucocorticoids are often accompanied by several intolerable adverse effects, including indigestion, vomiting, weight gain, osteoporosis, hyperglycemia, cardiovascular diseases, and infections [13, 14]. Thus, it is urgent to develop new anti-inflammatory agents that are effective for cytokine storm and exhibit tolerable adverse effects. Phytochemical compounds have become the primary sources for the discovery of new anti-inflammatory agents, due to their ability to alleviate inflammation with minimal side effects [15]. To date, the anti-inflammatory effects of various phytochemical compounds, including epigallocatechin-3-gallate (EGCG) and curcumin, have been reported [15–17].

Isofraxidin is a well-known coumarin compound that is naturally present plants, including *Acanthopanax senticosus*, and has been reported to exhibit several pharmacological activities, such as anti-inflammatory, anti-cancer, anti-depressive, anti-oxidant, and cardio-protective effects [18–27]. In 2007, we first reported the anti-inflammatory effects of isofraxidin using the SW982 human synovial sarcoma cell line [18]. In this study, we performed an examination of the effect of isofraxidin on IL-6 using ELISA and RT-PCR methods. Recently, reports have described the anti-inflammatory effects of isofraxidin using mouse peritoneal macrophages, human osteoarthritis chondrocytes, or LPS-injected mice [19–21]. However, these studies were not designed to elucidate the detailed molecular mechanisms underlying the effect of isofraxidin on the overexpression of IL-6. Therefore, the present study examined the effect of isofraxidin on TPA-induced IL-6 mRNA expression and activation of intracellular signaling pathways involved in the transcriptional regulation of the IL-6 gene using HuH-7 cells. The results of the present study may contribute to the development of effective treatments for cytokine storm.

Materials and Methods

Cell culture

The human hepatocellular carcinoma cell lines, HuH-7 (RRID: CVCL_0336) and HepG2 (RRID: CVCL_0027) were obtained from JCRB (Osaka, Japan) and RIKEN BRC Cell Bank (Tsukuba, Japan), respectively. The cells were authenticated by STR profiling and mycoplasma DNA was not detected by PCR. These cells were routinely maintained in DMEM medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

Treatment of cells with isofraxidin, dexamethasone, and signal transduction inhibitors

Cells were plated onto 60-mm plastic dishes at a density of 3×10^4 cells/cm² in DMEM medium supplemented with 10% FBS. The next day, the medium was replaced with serum-free DMEM, and the day after that, cells were treated with 100 nM TPA (Sigma, St. Louis, MO, USA) and isofraxidin or dexamethasone (FUJIFILM Wako, Osaka, Japan) at the indicated concentrations in serum-free DMEM for 2, 8, and 24 h. Untreated cells were used as controls. For the inhibition of intracellular signaling pathways, individual signal transduction inhibitors (MAPK/ERK inhibitor U0126, PI3K inhibitor LY294002, JNK inhibitor SP600125, JAK2 inhibitor AZD1480, and NF- κ B inhibitor PDTC (Cayman Chemicals, Ann Arbor, MI, USA)) were added in place of isofraxidin.

MTT assay

Cells were plated onto 96-well plates at a density of 3×10^4 cells/cm² in DMEM supplemented with 10% FBS. The next day, the medium was replaced with serum-free DMEM, and the day after that, cells were treated with or without isofraxidin at the indicated concentrations in serum-free DMEM for 2 and 24 h. After treatment with isofraxidin, cells were incubated with MTT (FUJIFILM Wako, 10 μ L, 5 mg/mL) for 4 h. The formazan precipitates were dissolved in 100 μ L DMSO, and the optical density (OD) values were read at 540 nm using a microplate reader.

Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed with the MyGo Mini Real Time PCR system (IT-IS Life Science, Dublin, Ireland) using Precision PLUS OneStep RT-qPCR Master Mix (Primerdesign Ltd, Chandler's Ford, UK), consisting of 10 μ L of 2 $\times$ OneStep RT-qPCR Master Mix, 0.5 μ L of each 10 μ M (0.25 μ M final) forward and reverse primers, 2.5 μ L (25 ng) of template RNA and 6.5 μ L of RNase/DNase-free water was made to a total volume of 20 μ L. The real-time PCR cycling conditions were: 55°C for 10 min, 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. The PCR primers used for amplification are as follows: IL-1 β : sense 5'-ttcgacacatgggataacgagg-3', antisense 5'-aggacatggagaacaccacttg-3'; IL-6: sense 5'-gtagccgccccacacagacagcc-3', antisense 5'-gccatctttggaagggtc-3'; TNF- α : sense 5'-tcttctgaacccccgagtga-3', antisense 5'-cctctgatggcaccaccag-3'; IL-8: sense 5'-ttggcagccttctctgattc-3', antisense 5'-atttctgtgttggcgcagtg-3'; MCP-1: sense 5'-tgctcatagcagccaccttc-3', antisense 5'-tctccttgccacaatggtc-3'; GAPDH: sense 5'-ggtggtctcctctgacttcaaca-3', antisense 5'-gttgctgtagccaaattcgttg-3'. GAPDH was used as an internal control.

Western blot analysis

Cell lysates and nuclear extracts were prepared using cell lysis buffer (Mammalian Cell PE LB, Biosciences, St. Louis, MO, USA) and nuclear extraction buffer (LysoPure Nuclear and Cytoplasmic Extraction Kit, FUJIFILM Wako), respectively. Protein concentrations were measured using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Cell lysates (10–30 μ g) and nuclear extracts (10 μ g) were treated with 5 $\times$ SDS sample buffer, separated by 10% SDS-polyacrylamide gels (SDS-PAGE), and then transferred to PVDF membranes. After the membranes were blocked with 2% blocking reagent in TBS-T buffer, they were incubated with 1:1000 dilution of anti-phospho (P)-ERK1/2, anti-ERK1/2, anti-P-SAPK/JNK, anti-

SAPK/JNK, anti-P-Akt (Ser473, Thr308), anti-Akt, anti-P-STAT3 (Tyr705), anti-STAT3, anti-NF- κ B p65, anti-I κ B- α (Cell Signaling Technology, Danvers, MA, USA), anti-IL-6 (GeneTex, Taipei, Taiwan, ROC), and anti-actin (Sigma) in TBS buffer for 24 h at 4°C, and then incubated with 1:2000-1:10000 dilution of anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology) in TBS-T buffer for 1 h at room temperature. Actin was used as an internal control. Detection of proteins was carried out using ECL Prime Western Blotting Detection System (GE Healthcare, Little Chalfont, UK). Protein band intensities were quantitated using Image J (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical significance was determined by analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparison.

Results

Effect of isofraxidin on TPA-induced IL-6 mRNA expression in HuH-7 and HepG2 cells

In order to elucidate whether isofraxidin suppresses the overexpression of IL-6, we first examined the effect of isofraxidin on TPA-induced IL-6 mRNA expression in HuH-7 and HepG2 cells using quantitative real-time PCR. When HuH-7 and HepG2 cells were treated with TPA for 2 h, a marked induction of IL-6 mRNA expression was observed in HuH-7 cells, whereas a modest induction was observed in HepG2 cells. In addition, isofraxidin significantly suppressed TPA-induced IL-6 mRNA expression in HuH-7 cells in a dose-dependent manner (Fig. 1). However, isofraxidin only slightly suppressed TPA-induced IL-6 mRNA expression in HepG2 cells.

Time-course experiments of IL-6 mRNA expression in TPA-treated HuH-7 cells

In order to determine the optimal treatment time in the suppression of IL-6 mRNA expression by isofraxidin, we performed time-course experiments. The effect of isofraxidin on TPA-induced IL-6 mRNA expression was examined in HuH-7 cells at 2, 8, and 24 h of treatment. The most marked induction by TPA and the most marked suppression by isofraxidin were observed at 2 h (Fig. 2A). In addition, the suppression of IL-6 protein expression was also observed at 24 h of treatment (Fig. 2B). Moreover, the effect of isofraxidin on cell viability of HuH-7 cells was evaluated using the MTT assay. The viability of HuH-7 cells was not significantly affected by treatment with isofraxidin at any concentration at 2 and 24 h (Fig. 2C), indicating that the suppressive effect of isofraxidin on TPA-induced IL-6 mRNA expression is not due to cell toxicity. Based on these results, the following quantitative real-time PCR experiments were performed at 2 h of treatment.

Effects of isofraxidin on TPA-induced mRNA expression of other pro-inflammatory cytokines and chemokines in HuH-7 cells

In addition to IL-6, other pro-inflammatory cytokines (such as IL-1 β and TNF- α) and chemokines (such as IL-8 and MCP-1) are also involved in cytokine storm pathogenesis. Therefore, we examined whether isofraxidin suppresses TPA-induced mRNA expression of other cytokines and chemokines. TPA treatment significantly induced the expression of IL-1 β , TNF- α , and IL-8 mRNA. Isofraxidin tended to suppress IL-1 β and TNF- α mRNA expression (Fig. 3A). However, no suppression of IL-8 and MCP-1 mRNA expression was observed by treatment with isofraxidin at all concentrations tested (Fig. 3B).

Comparison of the suppressive effect of isofraxidin on TPA-induced IL-6 mRNA expression with dexamethasone in HuH-7 cells

Dexamethasone is a well-known synthetic glucocorticoid that is commonly used in the treatment of inflammation-related diseases. Therefore, we compared the suppressive effect of isofraxidin on TPA-induced IL-6 mRNA expression with that of dexamethasone. At 50–200 μ M, isofraxidin showed a dose-dependent suppression of TPA-induced IL-6 mRNA expression that was similar to dexamethasone (Fig. 4)

Involvement of intracellular signaling pathways in TPA-induced IL-6 and IL-8 mRNA expression in HuH-7 cells

Intracellular signaling pathways, such as MAPK/ERK, SAPK/JNK, PI3K/Akt, NF- κ B/I κ B, and JAK/STAT3, are known to be involved in the transcriptional regulation of IL-6 gene expression. Therefore, we confirmed the involvement of these signaling pathways in TPA-induced IL-6 mRNA expression in HuH-7 cells using signal transduction inhibitors. The addition of the MAPK/ERK inhibitor U0126, JNK inhibitor SP600125, Akt inhibitor LY294002, or JAK2 inhibitor AZD1480 resulted in a concentration-dependent suppression of IL-6 mRNA expression at 1–25 μ M (Fig. 5A, B, C, D). The NF- κ B inhibitor PDTC did not suppress IL-6 mRNA expression at 1–25 μ M, but suppressed at 50–200 μ M (Fig. 5E). As a reference, we also examined the effect of each inhibitor on TPA-induced IL-8 mRNA expression. U0126, SP600125, and PDTC suppressed IL-8 mRNA expression, but only weakly (Fig. 5A, B, E), while LY294002 and AZD1480 did not suppress IL-8 mRNA expression (Fig. 5C, D). These results indicate that the activation of the MAPK/ERK, SAPK/JNK, PI3K/Akt, NF- κ B, and JAK/STAT3 pathways may be involved in TPA-induced IL-6 mRNA expression in HuH-7 cells. In addition, the present results indicate that the regulatory mechanism of IL-6 is different from that of IL-8 in TPA-treated HuH-7 cells.

Time-course experiments for the detection of intracellular signaling molecules in TPA-treated HuH-7 cells

In Fig. 5, we showed that the activation of the MAPK/ERK, SAPK/JNK, PI3K/Akt, NF- κ B/I κ B, and JAK/STAT3 pathways may be involved in TPA-induced IL-6 mRNA expression in HuH-7 cells. For the detection of the phosphorylation of intracellular signaling molecules, it is important to examine their time-

courses and to determine their inhibition kinetics. The phosphorylation of ERK1/2 was induced by TPA and inhibited by U0126 at all time points examined (15 min, 1 h, and 2 h), although U0126 inhibition was most prominent at 15 min (Fig. 6A). The phosphorylation of SAPK/JNK was induced by TPA and inhibited by SP600125 only at 2 h (Fig. 6B). The phosphorylation of Akt (Ser473) was induced by TPA and inhibited by LY294002 only at 15 min (Fig. 6C). The phosphorylation of STAT3 (Tyr705) was not induced by TPA, but was inhibited by AZD1480 at all time points (15 min, 1 h, 2 h, Fig. 6D). In contrast, I κ B was degraded with TPA treatment, and PDTC clearly inhibited the degradation of I κ B at 15 min (Fig. 6E). The total protein levels of these signaling molecules and actin remained unchanged. Based on the results obtained in the present experiments, we next examined the effects of isofraxidin on intracellular signaling molecules.

Effects of isofraxidin on intracellular signaling molecules in TPA-treated HuH-7 cells

In order to elucidate the mechanisms responsible for the suppressive effect of isofraxidin on TPA-induced IL-6 mRNA expression, we examined the effects of isofraxidin on the phosphorylation of intracellular signaling molecules, nuclear translocation of NF- κ B p65, and degradation of I κ B using western blotting. As shown in Fig. 7A and B, TPA significantly induced the nuclear translocation of NF- κ B p65 and degradation of I κ B. However, isofraxidin did not inhibit these effects at the concentration range studied (25–200 μ M). Furthermore, isofraxidin did not inhibit TPA-induced phosphorylation of SAPK/JNK, Akt (Ser473), and STAT3 (Tyr705). On the other hand, isofraxidin inhibited TPA-induced ERK1/2 phosphorylation in a dose-dependent manner. The phosphorylation of Akt (Thr308) was not induced by TPA and was not inhibited by isofraxidin. The total protein levels of these signaling molecules and actin remained unchanged. These results indicate that isofraxidin selectively inhibits the MAPK/ERK pathway among several signaling pathways examined in TPA-treated HuH-7 cells.

Discussion

IL-6 is a pleiotropic cytokine with many biological activities, including inflammation, hematopoiesis, bone metabolism, embryonic development, and other fundamental processes [28]. Recently, IL-6 was considered to be a therapeutic target for inhibiting cytokine storm [1]. To date, although an anti-IL-6R antibody, dexamethasone, mRNA vaccines, and antiviral agents have been used for the treatment of COVID-19 [1, 11, 29, 30], only a few of these agents (anti-IL-6R antibody [1] and dexamethasone [11]) have been shown to be effective for cytokine storm. In the present study, we investigated the suppressive effect of isofraxidin on the overexpression of IL-6 and its regulatory mechanism. The results of the present study suggest that isofraxidin may be an effective agent for the treatment of cytokine storm.

Although TPA is one of the most utilized tumor promoters used to study the mechanisms of carcinogenesis [31], it is also frequently used as a stimulus for anti-inflammatory research because of its ability to induce inflammatory reactions [32, 33]. Compared to viral infection experiments with SARS-CoV-2, *in vitro* experiments using TPA have the advantages of safety and simplicity. When HuH-7 and HepG2

cells were treated with TPA, a marked induction of IL-6 mRNA expression was observed in HuH-7 cells compared with HepG2 cells, although IL-6 induction was significantly elevated in both cell lines. Similarly, TPA was found to markedly activate several intracellular signaling pathways and induce the expression of IL-1 β , TNF- α , and IL-8 mRNAs in HuH-7 cell, confirming that TPA is a useful stimulator for the present study.

In the present study, the effect of isofraxidin on TPA-induced mRNA expression of several pro-inflammatory cytokines was evaluated using quantitative real-time PCR, and IL-6 mRNA expression was found to be significantly suppressed in a dose-dependent manner. In contrast, IL-1 β and TNF- α mRNA expression were suppressed only at 200 μ M. Moreover, the results also indicated that the suppressive effect of isofraxidin on TPA-induced IL-6 mRNA expression is more evident in HuH-7 cells than in HepG2 cells, suggesting that the effect of isofraxidin may exhibit the cell type differences. Thus, we have used TPA-treated HuH-7 cells as an *in vitro* model for further studies. Similarly, HuH-7 cells have been used in studies targeting the inhibition of cytokine storm in COVID-19 [4].

As described above, dexamethasone has been used as a therapeutic for treating cytokine storm [11]. Comparisons of isofraxidin with dexamethasone in the present study showed that both agents suppress TPA-induced IL-6 mRNA expression in HuH-7 cells at 50–200 μ M. However, a study using the A549 human lung cancer cell line, showed that dexamethasone suppressed IL-6 mRNA expression at 10–10,000 nM following IL-1 β stimulation [34]. Determining whether isofraxidin suppresses IL-6 mRNA expression at lower concentrations (less than 50 μ M) in HuH-7 cells under improved culture conditions is under investigation.

It is known that intracellular signaling pathways, such as MAPK/ERK, SAPK/JNK, PI3K/Akt, NF- κ B/I κ B, and JAK/STAT3, are involved in the transcriptional regulation of the IL-6 gene [4, 8, 17, 33, 35–37]. Among these intercellular signaling molecules, isofraxidin only inhibited the TPA-induced phosphorylation of ERK1/2. We previously reported that isofraxidin suppresses TPA-induced MMP-7 expression in HuH-7 cells by inhibiting the phosphorylation of ERK1/2, but not of SAPK/JNK or p38 and activation of transcription factors AP-1 and NF- κ B [22]. However, other investigators have reported that isofraxidin inhibits the PI3K/Akt, SAPK/JNK, and NF- κ B/I κ B pathways in studies using human lung cancer cells and others [20, 21, 23, 24, 25, 26]. However, the present results shown in Fig. 7 indicated no inhibition of these signaling molecules by isofraxidin. These findings suggest that isofraxidin suppresses TPA-induced overexpression of IL-6 mRNA through selective inhibition of the MAPK/ERK pathway in HuH-7 cells. Recently, it has been reported that the MEK1/2 inhibitor ATR-002 alleviates SARS-CoV-2-induced expression of pro-inflammatory cytokines and chemokines [8], indicating that the inhibition of MAPK/ERK pathway may be useful in the treatment of cytokine storm. In addition, the PI3K/Akt, NF- κ B/I κ B, and JAK-STAT3 pathways were also reported to be important in the development of cytokine storm [4, 5, 7]. Taken together, use of isofraxidin alone or in combination with other agents that inhibit signaling pathways other than MAPK/ERK, may represent a more effective treatment of cytokine storm.

Recently, the anti-inflammatory potential of phytochemical compounds, as well as their underlying mechanisms, have been actively studied. For example, luteolin, a flavonoid compound of celery or green pepper, suppresses IL-6 mRNA expression and protein production through the inhibition of phosphorylation of JNK and activity of AP-1 [37]. EGCG inhibits IL-6 synthesis and suppresses trans-signaling by inducing the production of soluble gp130 [16]. Curcumin, an active compound of turmeric, suppresses IL-6 expression by inhibiting the nuclear translocation of NF- κ B p65 [17]. The present results suggest that the molecular mechanism by which isofraxidin suppresses IL-6 expression may be different from that of the aforementioned phytochemicals, although further studies are needed to verify this possibility.

MicroRNAs (miRNAs) have been revealed to be involved in the regulation of activation and specificity of the MAPK/ERK pathway, with several miRNAs reported to decrease the activity of the MAPK/ERK pathway [38]. In gall bladder cancer cells, miR-29c-5p induces apoptosis through reducing the phosphorylation of MEK1/2, ERK1/2, and Akt (Ser473) [39]. MiR-101 is a down-regulated miRNA in hepatocellular carcinoma cells. The overexpression of miR-101 suppresses the proliferation of HepG2 cells through decreasing the activity of ERK1/2 [40]. MiR-148a inhibits the proliferation and invasion of esophagus squamous cell carcinoma cells through targeting MAP3K9 by the MAPK/ERK pathway [41]. Whether and how miRNAs are involved in the suppressing expression of IL-6 induced by isofraxidin is under investigation.

In conclusion, isofraxidin suppresses the TPA-induced overexpression of IL-6 mRNA by selectively inhibiting the activation of the MAPK/ERK pathway in HuH-7 cells, indicating that isofraxidin may be an effective anti-inflammatory agent for treating cytokine storm.

Declarations

Author contributions TY and TT designed the study. TY performed the experiments and analyzed the data. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Figures

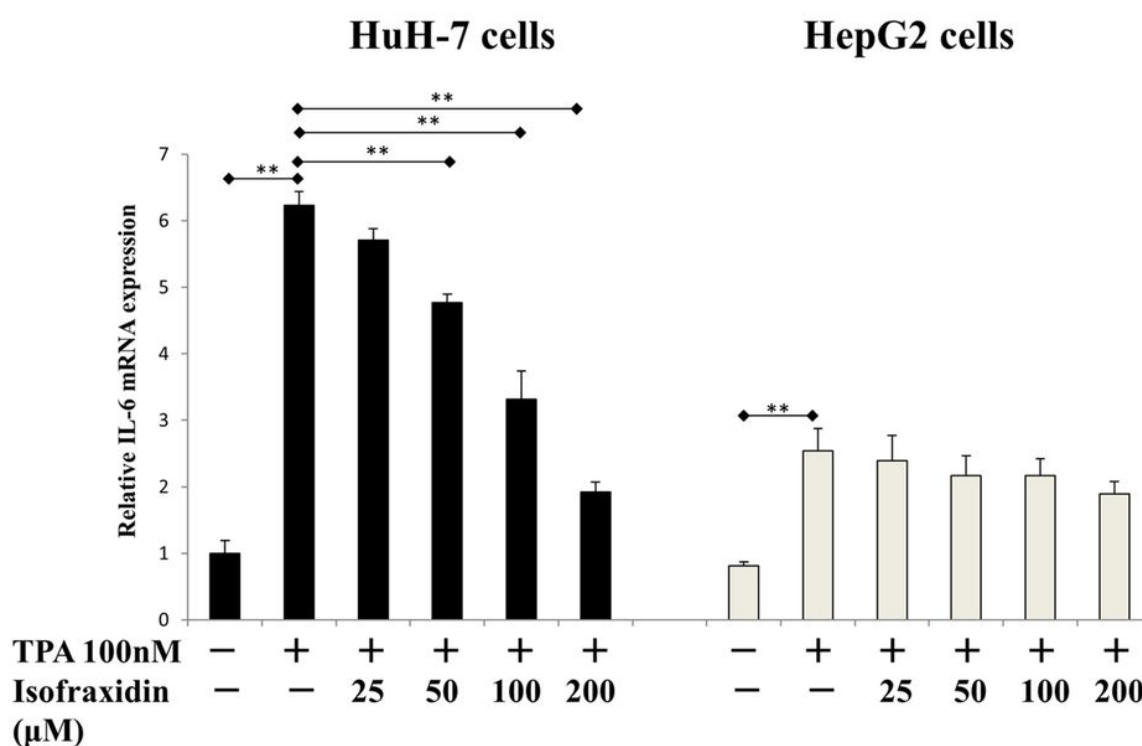


Fig. 1

Figure 1

Effect of isofraxidin on TPA-induced IL-6 mRNA expression. HuH-7 and HepG2 cells were treated with 100 nM TPA and various concentrations of isofraxidin for 2 h. IL-6 mRNA expression was determined by quantitative real-time PCR. Levels are expressed relative to those of untreated cells, which were arbitrarily set at 1. Values represent the mean±standard deviation of three independent experiments. Statistical significance was determined by analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparison (* $p<0.05$, ** $p<0.01$ compared with the TPA treatment only).

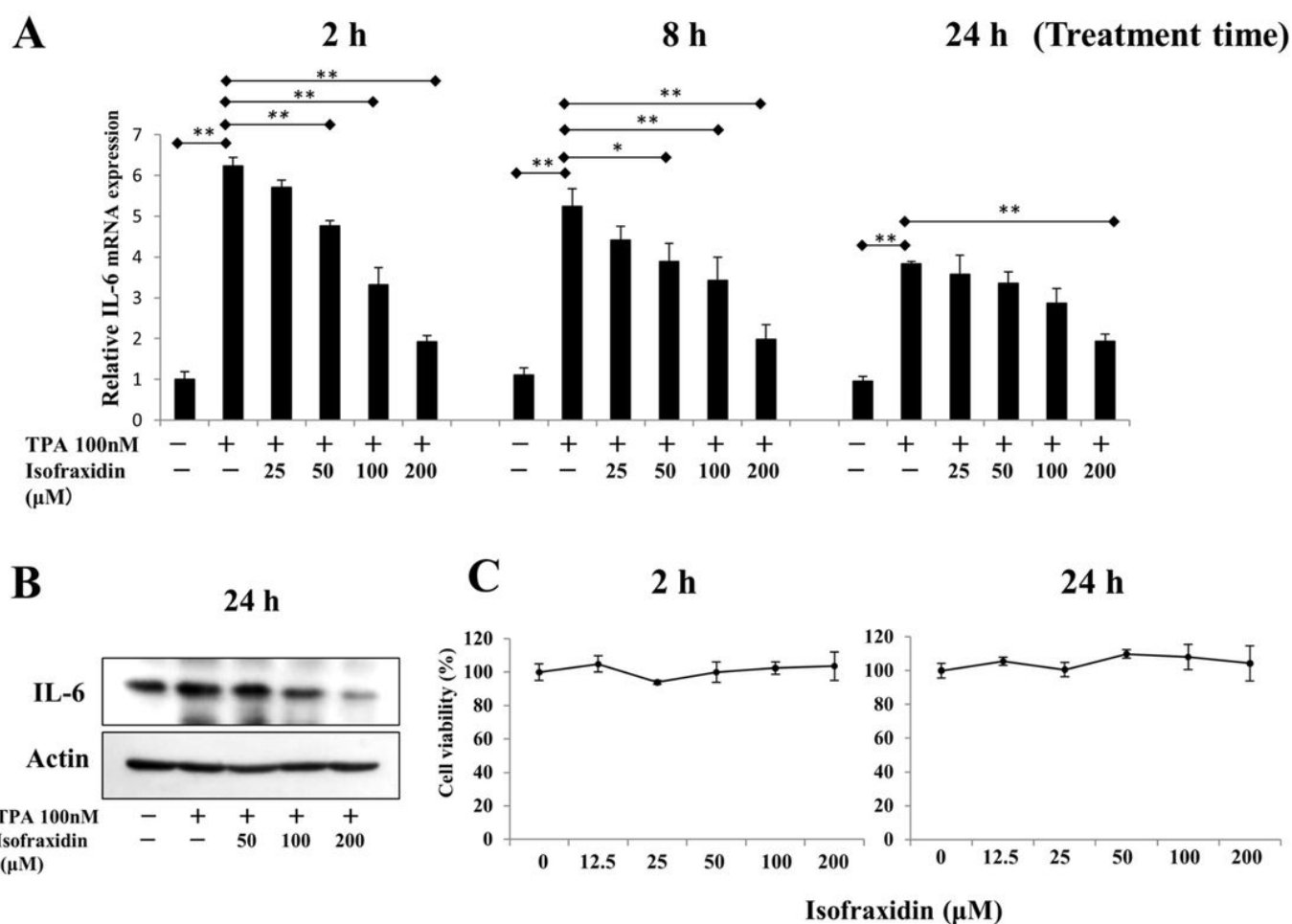


Fig. 2

Figure 2

Time-course experiments of IL-6 mRNA expression. **A** Quantitative real time-PCR analysis. HuH-7 cells were treated with 100 nM TPA and various concentrations of isofraxidin for 2, 8, and 24 h. IL-6 mRNA expression was determined by quantitative real-time PCR. Relative expression levels were calculated as describe in the legend to Fig. 1. **B** Western blot analysis of IL-6. HuH-7 cells were treated with 100 nM TPA and various concentrations of isofraxidin for 24 h. IL-6 protein expression was determined by western blotting. Results are representative of two independent experiments. **C** MTT assay. The cell viability of HuH-7 cells was evaluated using MTT assay.

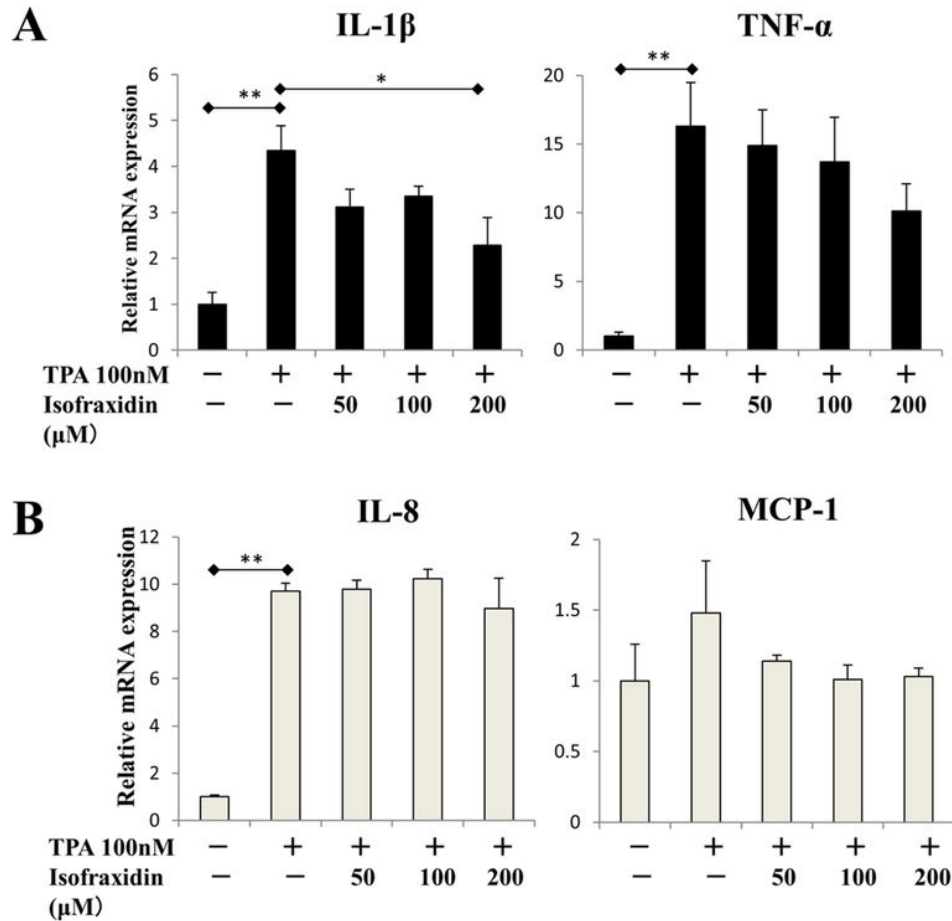


Fig. 3

Figure 3

Effects of isofraxidin on the TPA-induced mRNA expression of other pro-inflammatory cytokines and chemokines. HuH-7 cells were treated with 100 nM TPA and various concentrations of isofraxidin for 2 h. The mRNA expression of pro-inflammatory cytokines and chemokines was determined by quantitative real-time PCR. Relative expression levels were calculated as describe in the legend to Fig. 1.

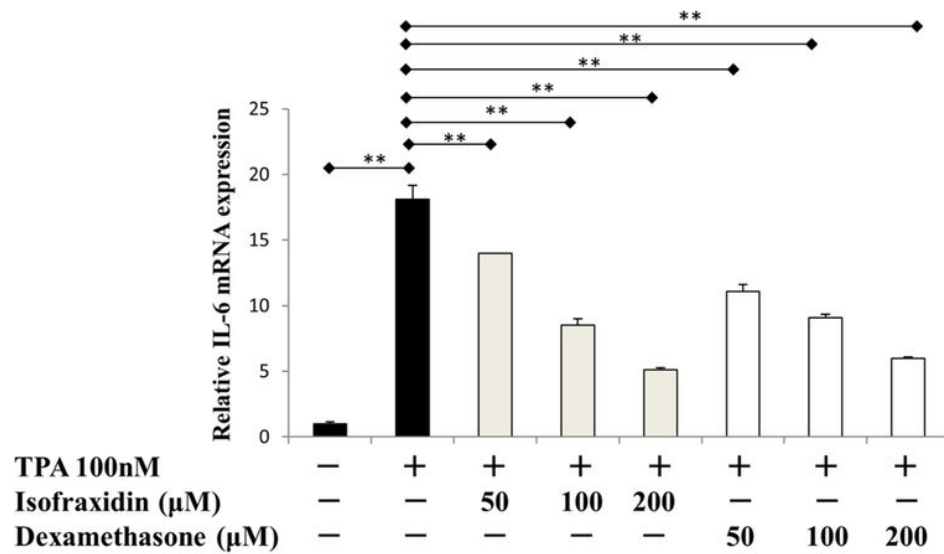


Fig. 4

Figure 4

Comparison of the suppressive effect of isofraxidin on TPA-induced IL-6 mRNA expression with dexamethasone. HuH-7 cells were treated with 100 nM TPA and various concentrations of isofraxidin or dexamethasone for 2 h. IL-6 mRNA expression was determined by quantitative real-time PCR. Relative expression levels were calculated as describe in the legend to Fig. 1.

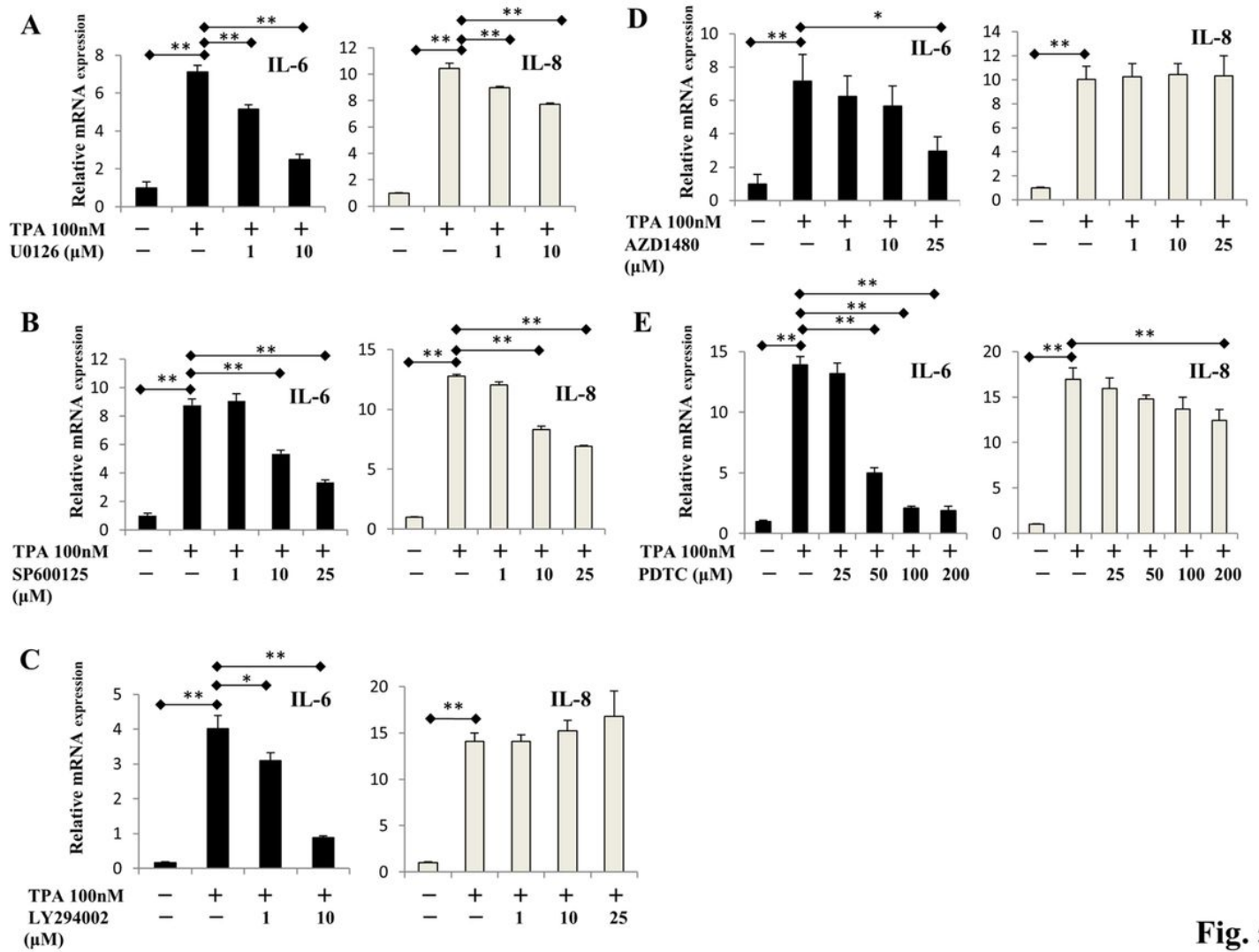


Fig. 5

Figure 5

Involvement of intracellular signaling pathways in TPA-induced IL-6 and IL-8 mRNA expression. HuH-7 cells were treated with 100 nM TPA and various concentrations of each signal transduction inhibitors for 2 h. IL-6 and IL-8 mRNA expression were determined by quantitative real-time PCR. Relative expression levels were calculated as describe in the legend to Fig. 1.

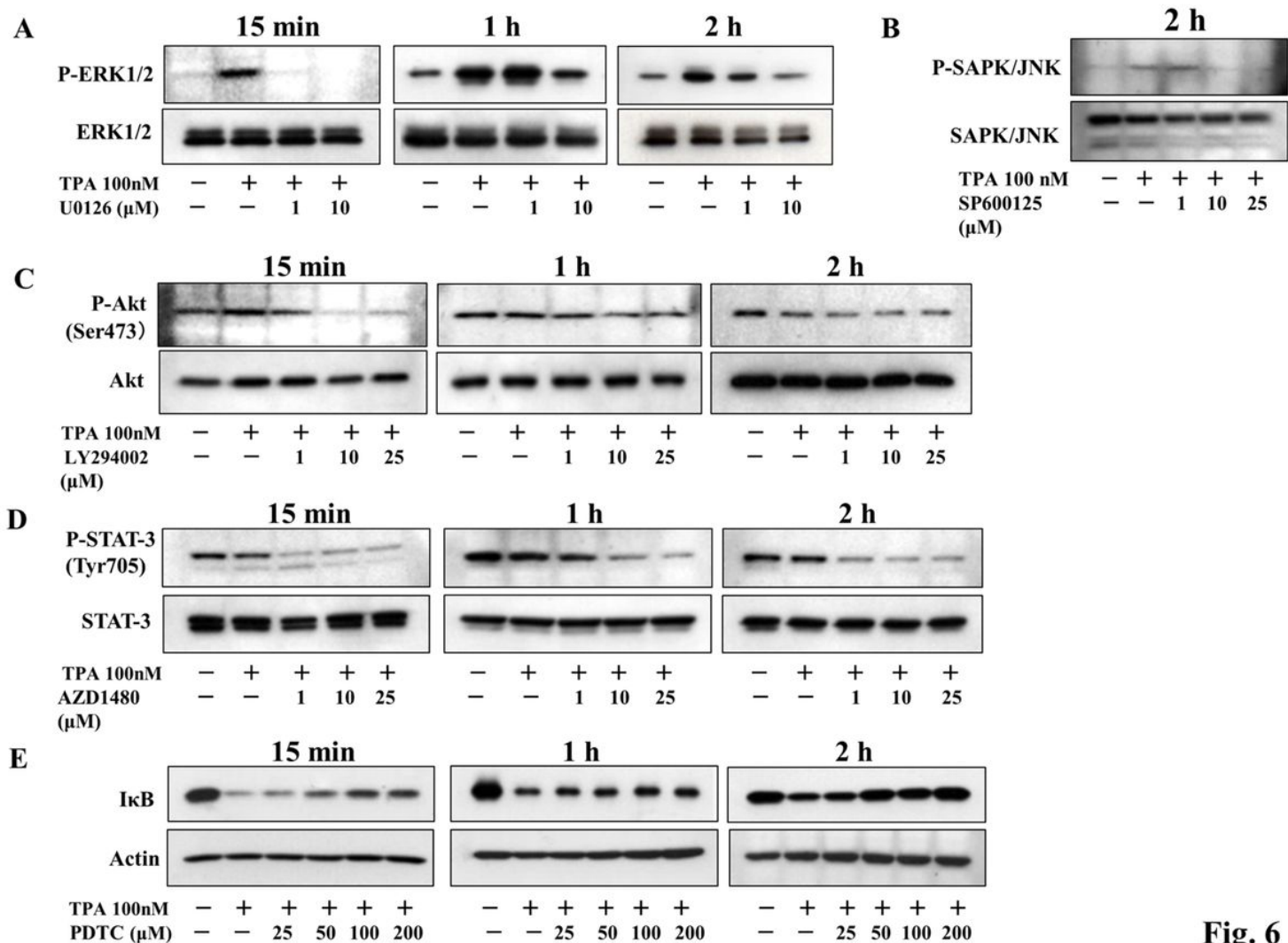


Fig. 6

Figure 6

Time-course experiments for the detection of intracellular signaling molecules. HuH-7 cells were treated with 100 nM TPA and various concentrations of each signal transduction inhibitors for 15 min, 1h, and 2 h. The phosphorylation of intracellular signaling molecules and degradation of IκB were determined by western blotting. Results are representative of two independent experiments.

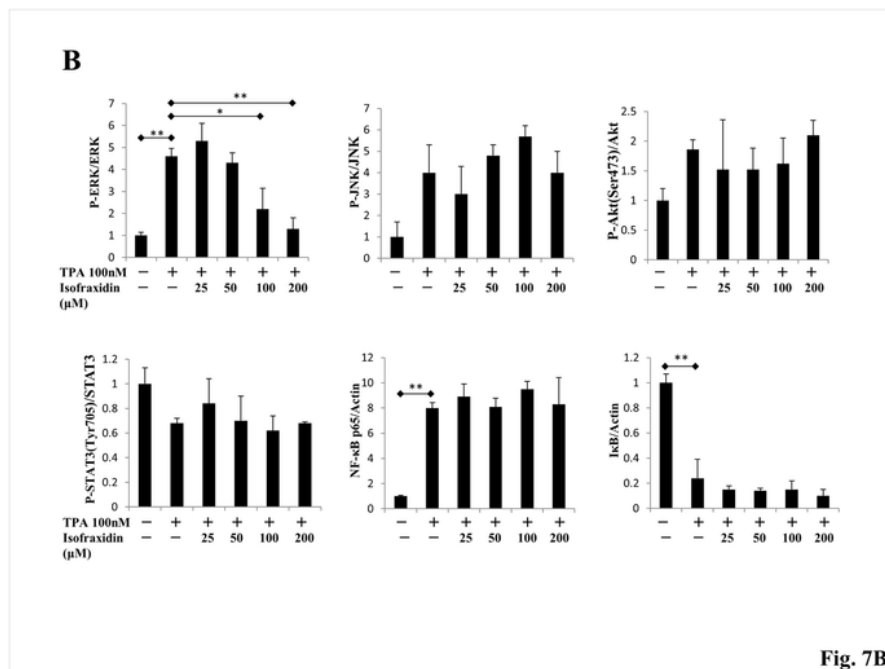
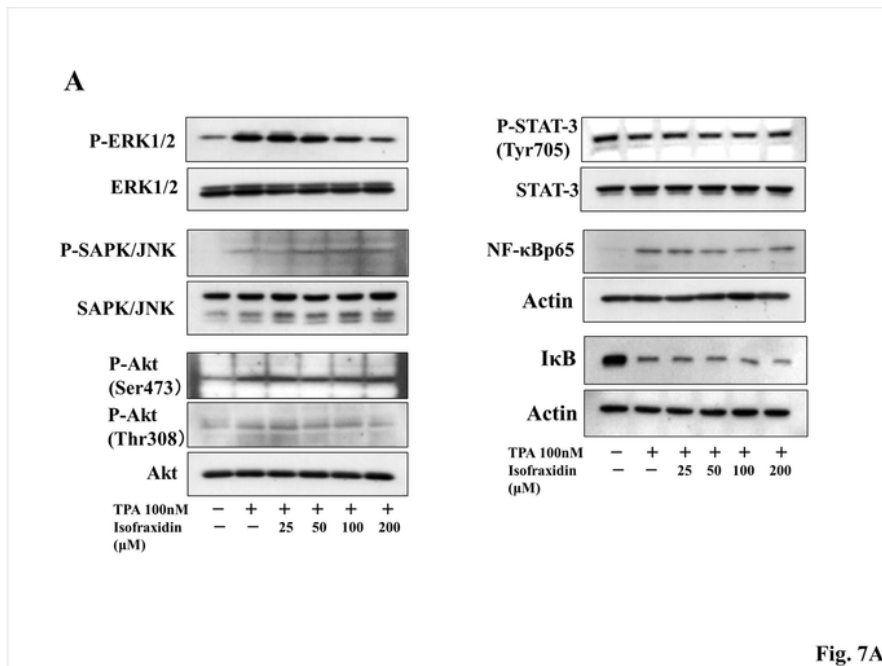


Figure 7

Effects of isofraxidin on intracellular signaling molecules. **A** Western blot analysis of intracellular signaling molecules. **B** Densitometric analysis of western blotting results. HuH-7 cells were treated with 100 nM TPA and various concentrations of isofraxidin for 15 min (ERK1/2, Akt, STAT3, NF-κB p65, and IκB) and 2 h (SAPK/JNK). The phosphorylation of intracellular signaling molecules, nuclear translocation of NF-κB p65, and degradation of IκB were determined by western blotting. Protein band intensities were

quantitated by Image J. Levels are expressed relative to those of untreated cells, which were arbitrarily set at 1. Values represent the mean $\pm$ standard deviation of three independent experiments. Statistical significance was determined by analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparison (* p <0.05, ** p <0.01 compared with the TPA treatment only).