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Growth compensatory role of sulindac sulfide-induced thrombospondin-1 linked with ERK1/2 and RhoA GTPase signaling pathways

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Abstract

Previously, we reported that non-steroidal anti-inflammatory drugs (NSAIDs) suppress cellular invasion which was mediated by thrombospondin-1 (TSP-1). As the extending study of the previous observation, we investigated the effect of NSAID-induced TSP-1 on the cellular growth and its related signaling transduction of the TSP-1 production. Among diverse NSAIDs, sulindac sulfide was most potent of inducing the human TSP-1 protein expression. Functionally, induced TSP-1 expression was associated with the growth-compensatory action of NSAID. TSP-1 expression was also elevated by mitogenic signals of ERK1/2 and RhoA GTPase pathway which had also growth-promotive capability after sulindac sulfide treatment. These findings suggest the possible mechanism through which tumor cells can survive the chemopreventive action of NSAIDs or the normal epithelium can reconstitute after NSAID-mediated ulceration in a compensatory way.

Keywords

Thrombospondin-1; ERK1/2; RhoA

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to relieve pain and inflammation but have also received considerable attention because of their protective effects against human cancer (Rao et al., 1995; Reddy et al., 1996). NSAIDs inhibit enzymatic activity of cyclooxygenases (COX) whose products prostaglandins are known to inhibit apoptosis, stimulate tumor growth, and enhance angiogenesis, tumor cell invasion and metastasis in many cancer models (Connolly et al., 2002; Yoshida et al., 2003). NSAIDs, by inhibiting COX activity, enhance apoptosis and exert anti-metastatic and anti-angiogenesis effects, thereby inhibiting tumor growth. However, some lines of evidences suggested that NSAIDs modulate tumor growth by cyclooxygenase-independent signaling pathways (Zhang et al., 1999; Tegeder et al., 2001).

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In terms of COX-independent pathway, evidence has been presented that NSAIDs suppress tumor cell invasion which was mediated by anti-metastatic factor thrombospondin-1 (TSP-1) via early growth response gene product-1 (EGR-1) (Moon et al., 2005). TSP-1 is a high-molecular-weight, multifunctional glycoprotein, which is synthesized and secreted by various cell types such as fibroblasts, smooth muscle cells, monocytes, macrophages, osteoblasts, and neoplastic cells (Adams, 2001; Adams and Lawler, 2004). However, the role of TSP-1 in tumor progression is very complex and controversial. Although the reduced expression of TSP-1 is known to correlate with a poor prognosis in cancer patients as well as animal tumor models (Yamaguchi et al., 2002), released TSP-1 is also capable of fostering the metastatic spread, angiogenesis as well as tumor cell survival in some progressive tumors such as invasive cervical cancer, pancreatic and ductal carcinoma, and breast carcinomas (Cleardin et al., 1993; Roberts, 1996). In addition to the effects on the tumor cell growth and metastasis, TSP-1 can promote the wound healing process in response to the external stresses as well (DiPietro et al., 1996; Anilkumar et al., 2002).

Generally speaking on the mitogenic signals, ERK1/2 MAP kinase signals function as the cytoprotective roles and maintain the cellular homeostasis by enhancing the cellular growth and proliferation after diverse injuries (Kohno and Pouyssegur, 2003; Sun and Sinicrope, 2005). However, whereas ERK1/2 signals can promote the recovery of the injured tissue, sometimes chronic stimulation of mitogenic signals can provide the tumor cells with the growth advantage over the normal tissues. Recently, we reported that the human intestinal epithelial cells can develop compensatory protection mechanisms after NSAID exposure (Moon et al., 2007). Mitogenic signals associated with ERK1/2 MAP kinase and early growth response gene 1 (EGR-1) during gastrointestinal injury mediated the epithelium recovery processes after NSAID exposure. Epithelial toxic stresses have been known to stimulate the compensatory signals of wound healing and tissue reconstitution including growth factor-activated ras-associated MAP kinase pathway and other small GTP-binding protein family signals as well (Guo et al., 2003; Tarnawski, 2005). Rho small GTPases such as RhoA, CDC42, and Rac1 GTPases are a multimember family of RAS small GTPase, which are also mitogenic and tumorigenic when overexpressed in the intestinal epithelium.

Based on the hypothesis that TSP-1 can mediate the cellular survival after NSAID treatment, we now evaluated the cellular growth in terms of TSP-1 production and its induction signaling pathways. Induction of TSP-1 by sulindac sulfide was mediated by mitogenic signals and the TSP-1 had growth modulating function in sulindac sulfide-treated HCT-116 cells. These findings provide the insight into the possible compensatory mechanism under the cytotoxic condition by chemopreventive NSAID intake. Although there are chances that NSAID-treated tumor cells can survive the chemopreventive action, the growth-compensatory actions of TSP-1 may contribute to the reconstitution after NSAID-mediated cytotoxic insults.

Materials and methods

Cell culture conditions and reagents

HCT-116 colonic adenocarcinoma cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Welgene bioscience, Daeu, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma Chemical Company, St. Louis, MO), 50 unit/ml penicillin (Sigma Chemical Company), and 50 µg/ml streptomycin (Sigma). During incubation with chemicals, cells were cultured in serum-free RPMI 1640 media. All the chemicals were purchased from Sigma.

Cellular viability assay

Colorimetric analysis of cell growth was performed with Thiazolyl Blue Tetrazolium Bromide (MTT, Fluka, USA) assay. Cells (5×10^4 /well) were cultured in 96-well plate for each time and the MTT (20 μ l from 5 mg/ml stock solution) was treated onto cells for 2 h. Supernatant was removed and dissolved with 200 μ l dimethyl sulfoxide (DMSO). Optical density at 560 nm was measured and was the background OD at 670 nm was subtracted from OD at 560 nm. Corrected optical density was directly correlated with cell quantity.

Western immunoblot analysis

Levels of protein expression were compared using Western immunoblot analysis using rabbit polyclonal anti-human Actin antibody (Santacruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-human TSP-1 antibody (Ab-11, NeoMarkers, Fremont, CA), and rabbit polyclonal anti-p-p44/42 MAP kinase antibody (anti-ERK1/2 antibody, Cell signaling technology, Beverly, MA). Cells were washed with ice-cold phosphate buffer, lysed in boiling lysis buffer [1% (w/v) SDS, 1.0 mM sodium *ortho*-vanadate, and 10 mM Tris, pH 7.4], and sonicated for 5 s. Lysates containing proteins were quantified using BCA protein assay kit (Pierce, Rockford, IL). Fifty micrograms of protein was separated by Bio-Rad gel mini-electrophoresis. Proteins were transferred onto PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and the blots were blocked for 1 h with 5% skim milk in Tris-buffered saline plus Tween 0.05% (TBST) and probed with each antibody for 2 h at room temperature or overnight at 4 °C. After three times washing with TBST, blots were incubated with horseradish-conjugated secondary antibody for 1 h and washed with TBST three times. Protein was detected by Enhanced Chemiluminescence (ECL) substrate (Amersham Pharmacia Biotech, Piscataway, NJ).

Traditional reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA (100 ng) from each sample was transcribed to cDNA by Prime RT-Premix RNase H-Reverse-transcriptase (Genet Bio, Nonsan, Korea). The amplification was performed with HS Taq DNA Polymerase (Genet Bio, Nonsan, Korea) in Mycycler Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) using the following parameters: denaturation at 94 °C for 2 min and 25 cycles of reactions of denaturation at 98 °C for 10 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 45 s. An aliquot of each PCR product was subjected to 1.2% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide. The 5' forward and 3' reverse-complement PCR primers for amplification of each gene were as follow: human thrombospondin-1 (5'-AGAATGCTGTCCTCGCTGTT-3' and 5'-TTTCTTGCAGGCTTTGGTCT), and human GAPDH (5'-TCAACGGATTTGGTCGTATT-3' and 5'-CTGTGGTCATGAGTCCTTCC-3').

Construction of plasmids

Human TSP-1 promoter (from -954 to 147) was cloned into pGL3 basic vector that were generated. After PCR of promoter region with *Pfu*Turbo DNA polymerase (Stratagene, La Jolla, CA) from human genomic DNA, the fragment was cloned into the TA vector (Invitrogen, Carlsbad, CA), sequenced, and further cloned into the pGLBasic3 vector. To inhibit ERK1/2 activity, MEK1 dominant negative plasmid was used. pMEV (control vector) and pMEV-MEK1-DN (dominant negative) were provided from Biomyx Technology (San Diego, CA). The activated form of RhoA expression plasmid (RhoAV; Q63V substitution) and dominant negative RhoA plasmid (RhoAN; T19N substitution) was provided from Upstate Biotech (Lake Placid, NY).

Transfection

Cells were transfected with mixture of plasmids using Trans-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. For transfection of the luciferase reporter gene, a mixture of 1.5 µg firefly luciferase reporter and 0.15 µg *renilla* luciferase, pRL-null vector (Promega, Madison, WI) per 4.5 µl of Trans-LT1 reagent was applied for a 6 well culture plate. For the luciferase assay, at 18 h after transfection, cells were exposed to chemicals for the next 24 h and lysed for dual-luciferase reporter assay system (Promega, Madison, WI). All transfection efficiency was maintained at around 50 to 60%, which was confirmed with pMX-enhanced GFP vector. To create the stable cell lines, cells were transfected with single plasmid (1.5 µg DNA per 35 mm dish cells) using Trans-LT1 reagent. After 48 h, the cells were subjected to selection for stable integrants by exposure to 400 µg/ml G418 (Invitrogen, Carlsbad, CA) in complete medium containing 10% fetal bovine serum. Selection was continued until monolayer colonies formed. The transfectants were then maintained in medium supplemented with 10% fetal bovine serum and 200 µg/ml G418.

Luciferase assay

Cells were washed with cold PBS, lysed with passive lysis buffer (Promega, Madison, WI) and then centrifuged at 12,000 g for 4 min. The supernatant was collected isolated and stored at -80 °C until assessment of luciferase activity. Luciferase activity was measured with a dual-mode luminometer (Model TD-20/20, Turner Designs Co., Sunnyvale, CA) after briefly mixing the supernatant (10 µl) with 50 µl *firefly* luciferase assay substrate solution, followed with 50 µl stopping *renilla* luciferase assay solution (Promega, Madison, WI). The *firefly* luciferase activity was normalized against *renilla* luciferase activity using the following formula: *firefly* luciferase activity/*renilla* luciferase activity.

Statistical analyses

Data were analyzed using SigmaStat for Windows (Jandel Scientific, San Rafael, CA). For comparison of two groups of data, Student's *t* test was performed. For comparison of multiple groups, data were subjected to ANOVA and pairwise comparisons made by the Student–Newman–Keuls (SNK) method. Data not meeting normality assumption were subjected to Kruskal–Wallace ANOVA on ranks and then pairwise comparisons made by the SNK method.

Results

Induction of human thrombospondin-1 gene expression and its effects on the cellular proliferation in presence of NSAIDs

HCT-116 cells were selected to investigate the effect of NSAIDs because they serve as a well established cell line model for the study of chemopreventive effects on colon cancer in cyclooxygenase (COX)-2-independent way (Baek et al., 2002; Maier et al., 2005). We first needed to determine whether COX inhibitors would increase the expression of thrombospondin-1 in HCT-116 cells as observed in other cancer cell line according to our previous report (Moon et al., 2005). HCT-116 cells were treated with concentrations of NSAIDs and TSP-1 protein expression was measured (Fig. 1A). Among the tested NSAIDs, sulindac sulfide was a most potent inducing agent of TSP-1 gene expression, followed by indomethacin and ibuprofen whereas structurally similar sulindac sulfone did not induce TSP-1 gene expression. Strong TSP-1 induction by sulindac sulfide can be expected from the similar inductive patterns in TSP-1-inducing EGR-1 (Moon et al., 2005). Our treatment concentrations of sulindac sulfide well agree with the dose levels reported in the other study, which is measured up to 50 µM in the human plasma depending on the treatment regime (Davies and Watson, 1997). Moreover, sulindac sulfide is locally concentrated in the gastrointestinal epithelium to levels that are at least 20-fold higher than those achieved in serum (Duggan et al., 1980). In

following studies, sulindac sulfide was used as the standard inducer of TSP-1 gene expression while indomethacin was also used for comparison and to further validate our findings. TSP-1 mRNA level was elevated by sulindac sulfide or indomethacin, and the TSP-1 mRNA induction by sulindac sulfide was maximal around 2–4 h (Fig. 1B and C). Moreover, sulindac sulfide was positive transcriptional regulator by enhancing reporter activity of TSP-1 promoter luciferase construct (Fig. 1D). To assess the contribution of TSP-1 to the cellular proliferation after NSAID treatment, the action of released TSP-1 on the neighboring cells was inhibited using its specific antibody. Blocking of TSP-1 action significantly exacerbated the growth suppression by NSAIDs (Fig. 2A and B). The blocking antibody Ab-1 from clone A4.1 (NeoMarkers, Fremont, CA) is known to inhibit TSP-1 binding to TSP-1 receptor/CD36, thus antagonizing TSP-1 action. Next, we analyzed the TSP-1 inductive signals and measured the growth regulatory action by TSP-1-inducing signaling pathway.

Role of ERK1/2 MAP kinases in inducing thrombospondin-1 and cellular viability

Since MAP kinase and PI3 kinase signaling pathways are known to play critical roles in TSP-1 induction (Nakagawa et al., 2005; Kim et al., 2006; McGillicuddy et al., 2006), we tested the effects of each kinase inhibitors on sulindac sulfide-induced TSP-1 expression. Pretreatment with each specific MAP kinase inhibitor (U0126 [a specific MEK1/2 inhibitor], SP600125 [JNK inhibitor], or SB203580 [p38 MAP kinase inhibitor]) or PI3 kinase inhibitor (LY294002) was performed and then sulindac sulfide was administered to detect TSP-1 transcription. Among these inhibitors, only ERK1/2 signaling inhibitor U0126 efficiently suppressed sulindac sulfide-induced TSP-1 promoter activation (Fig. 3A). When the TSP-1-producing ERK1/2 signals were blocked, the cellular proliferation was significantly more suppressed than the levels in only sulindac sulfide treatment (Fig. 3B). These results indicated that the survival signals from NSAID-mediated cytotoxicity may be, at least in part, mediated by ERK1/2 signals that were involved in TSP-1 expression. Sulindac sulfide also activated ERK1/2 phosphorylation, all of which imply the positive signaling association between ERK1/2 and TSP-1 gene expression (Fig. 3D). TSP-1 protein expression and the transcriptional activity were also inhibited by dominant negative MEK1 expression (Fig. 3C and E).

Contribution of RhoA small GTPase to thrombospondin-1 induction by sulindac sulfide and cellular growth

Small GTP-binding proteins of the Ras families are essential components of the cellular proliferation and mobility. As the second signal in regulating TSP-1 production, we investigated effects of Rho GTPase family including representative RhoA, cdc42, and Rac1. Among these small GTPase, only RhoA was potent regulator in TSP-1 expression (data not shown). Activated RhoA protein enhanced sulindac sulfide-induced TSP-1 production whereas dominant negative RhoA expression suppressed the TSP-1 levels (Fig. 4A). Moreover, external RhoA activation also increased TSP-1 gene expression regardless of NSAID action. The TSP-1 protein expression was confirmed at the transcription levels using TSP-1 promoter luciferase plasmid. RhoA was positively associated with sulindac sulfide-induced TSP-1 expression in HCT-116 cells (Fig. 4B). However, in our preliminary study, we could not observe any increase in RhoA GTPase activity after sulindac sulfide treatment in HCT-116 cells. Therefore, it can be speculated that intracellular status of RhoA activation can enhance TSP-1 gene expression without regard to sulindac sulfide exposure. To test contribution of RhoA signals in the sulindac sulfide-treated cytotoxicity in HCT-116 cells, stable cell lines were created by stably transfecting with empty vector, activated RhoA (RhoAV), or dominant negative RhoA (RhoAN) expression plasmid. Cellular growth was compared within each group of cells, and it was demonstrated that RhoA signals had the enhancing effect on the cellular viability after sulindac sulfide exposure in the HCT-116 cells (Fig. 4C). Taken together, cellular state of RhoA signals affected TSP-1 levels and promoted cellular growth in chemical-injured cells.

Discussion

This study investigated the influence of sulindac sulfide on the induction of TSP-1 in the colonic HCT-116 cells and demonstrated that the enhanced TSP-1 production was associated with survival in sulindac sulfide-treated cells. The TSP-1 gene induction mechanism was linked with ERK1/2 and RhoA GTPase signaling pathways (Fig. 5). TSP-1 has been generally associated with both the tumor progression and the tumor suppression. Many different and sometimes opposite functions of TSP-1 have been described as an oncogene (Clezardin et al., 1993; Roberts, 1996) or as a tumor suppressor gene (Yamaguchi et al., 2002; Gutierrez et al., 2003). While we demonstrated that TSP-1 in lung adenocarcinoma cells functions as mediator of tumor invasion (Moon et al., 2005), the present study suggests some growth-advantage roles of TSP-1 in sulindac sulfide-treated cells. Several studies also describe the growth-promoting effects of TSP-1 as well as the paracrine factor (Ichii et al., 2002; Maile and Clemmons, 2003). In view of tumorigenesis process, TSP-1 thus can provide cancer cells with the resistance to the NSAID chemoprevention or inefficacy of the drugs in the cancer cells. However, in case of non-transformed cells, TSP-1 could have beneficial effects on the injured tissue by enhancing the cellular proliferation after NSAID intake. This pattern was similarly implicated in our previous study (Moon et al., 2007). Human non-transformed epithelial cells enhanced EGR-1 protein after sulindac sulfide, which increased cellular viability as a compensatory mediator. EGR-1 is also one of the important mediators of TSP-1 production (Moon et al., 2005) and it is thus possible that EGR-1-enhanced TSP-1 may play the protective roles in the normal tissues for NSAID toxicity as well. Stress conditions in many other systems can turn on the immediately expressed genes like EGR-1 which contributes to the TSP-1 production associated with tissue repair in response to the acute insults (Majack et al., 1987; DiPietro et al., 1996).

In our result, RhoA was the positive modulator of TSP-1 production as well as cellular growth. However, sulindac sulfide does not activate RhoA GTPase and even some report demonstrated the decrease in Rho activity by NSAIDs (Zhou et al., 2003). In our study, status of RhoA activity in the cells could determine the TSP-1 gene expression without regard to action of sulindac sulfide. Generally speaking, inflamed and transformed tissues tend to elevate RhoA signaling status (Horiuchi et al., 2003; Rolfe et al., 2005) and thus NSAID-induced TSP-1 levels could be affected by this intracellular level of RhoA activity depending on the cellular state and its environment. With the importance in RhoA-mediated carcinogenesis, RhoA signal plays critical roles in the cellular proliferation and tissue protection for the epithelial stresses (Guo et al., 2003; Desai et al., 2004). In addition to RhoA signals, the intestinal epithelial injury turns on some other protective mediators like ERK1/2 signals. Target genes in ERK1/2-mediated downstream can be involved in the mitogenic and wound healing process (Liu et al., 1998; Braddock, 2001). Therefore, the mitogenic ERK1/2 and RhoA signals are supposed to elicit lines of cellular protective signals along with TSP-1 protein. However, tissue recovery via the mitogenic signals after tissue injury is not always beneficial to the body. (Sun and Sinicrope, 2005). Chronic accumulation of the mitogenic hits can be a critical cause of the epithelial tumorigenesis. Chronic insult with the mitogenic signals of ERK1/2 and RhoA pathway can be linked to the oncogenic stimulation and promote epithelial tumorigenesis. Considering the mitogenic activity of ERK1/2 and RhoA signals and its mediated TSP-1 induction, much careful examination should be made in terms of the assessment of the cancer-preventive action of NSAID. Taken all together, induction of TSP-1 via mitogenic signaling pathway could be beneficial for the acutely injured tissues by NSAID because of growth-enhancing function of TSP-1. However, the actions of TSP-1 in the epithelial tumor progression need to be further investigated to estimate the exact benefit or harmfulness of NSAID as the chemopreventive agent in the cancer.

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Fig. 1.

Effects of NSAIDs on TSP-1 expression. Each NSAID was tested for its effect on TSP-1 expression in HCT-116 cells (A). The HCT-116 cells were treated with each chemical for 24 h (A) or 4 h (B) to measure protein or mRNA, respectively. Cells treated with 30 μ M sulindac sulfide were analyzed for the TSP-1 mRNA at each exposure time point (C). TSP-1 luciferase plasmid was introduced into HCT-116 cells which were then treated with sulindac sulfide for 24 h to detect promoter activity (D). Data are mean $\pm$ SD ($n=4$). Groups with asterisk are significantly different ($p<0.05$) from the vehicle group by Student's t test. Results are representative of three independent experiments.

Fig. 2.

Effects of blocked thrombospondin-1 on viability in NSAID-treated cells. HCT-116 cells were subjected to vehicle or each NSAID (sulindac sulfide (A) or indomethacin (B)) in the indicated doses for 24 h. Each group was also co-treated either with anti-TSP-1 antibody (α-TSP-1 IgM isotype [Ab-1, clone A4.1], 10 μg/ml) or immunoglobulin M (IgM, 10 μg/ml) as indicated. Data are mean±SD ($n=12$). Groups with asterisk are significantly different ($p<0.05$) from the IgM group at each dose point by Student's t test. Results are representative of three independent experiments.

Fig. 3.

Involvement of ERK1/2 MAP kinases in TSP-1 induction by sulindac sulfide. A. TSP-1 luciferase plasmid was introduced into HCT-116 cells which were then treated with 30 μM sulindac sulfide in presence of DMSO, 5 μM LY294002 (LY, PI3 kinase inhibitor), each MAP kinase inhibitor (1 μM U0126 [a specific MEK1/2 inhibitor], 5 μM SP600125 [SP, JNK inhibitor], or 2 μM SB203580 [SB, p38 MAP kinase inhibitor]) for 24 h to detect promoter activity. Data are mean±SD (*n*=4). Bars with different letters are significantly different (*p*<0.05). B. HCT-116 cells were subjected to vehicle or each dose of sulindac sulfide with or without U0126 for 24 h. Data are mean±SD (*n*=12). Groups with asterisk are significantly different (*p*<0.05) from the DMSO group at each dose of sulindac sulfide by Student's *t* test.

C. HCT-116 cells were transfected with empty vector or DN-MEK1 expression plasmid. After 24 h transfection, chemical was treated for another 24 h. D. Sulindac sulfide (30 μ M) was administered to HCT-116 cells for the indicated time. Total cellular lysate was analyzed using Western blot. E. TSP-1 luciferase plasmid plus empty vector or DN-MEK1 expression plasmid was introduced into HCT-116 cells which was then treated with 30 μ M sulindac sulfide for 24 h. Data are mean $\pm$ SD ($n=4$). Bars with different letters are significantly different ($p<0.05$). All results are representative of three independent experiments.

Fig. 4.

Effects of RhoA on TSP-1 expression and cellular viability. A. Stably transfected HCT-116 cells with each of plasmids [vector, the activated form of RhoA expression plasmid (RhoAV) and dominant negative RhoA plasmid (RhoAN)] were treated with each chemical combination (sulindac sulfide, U0126, or DMSO) for 24 h. Protein lysate was analyzed using Western blot. B. Stably transfected HCT-116 cells with each plasmids (vector, RhoAV, RhoAN) were transiently transfected with TSP-1 luciferase reporter plasmid and then treated with sulindac sulfide or DMSO for 24 h. C. Stably transfected HCT-116 cells with each plasmids (vector, RhoAV, RhoAN) were treated with sulindac sulfide or DMSO for 24 h to perform MTT viability assay. Sign # stands for the significant difference from the control group without

sulindac sulfide and sign * for the significant difference from the empty vector-transfected cells at each dose level of sulindac sulfide ($p<0.05$). Results are representative of two independent experiments.

Fig. 5.

Proposed contribution of TSP-1 linked with ERK1/2 and RhoA signaling pathway in NSAID-treated cells. Sulindac sulfide usually causes cellular growth suppression or cytotoxicity. As the compensatory mechanism, it also induces TSP-1 expression via ERK1/2 signaling pathway. Moreover, activated RhoA signals can enhance TSP-1 induction levels. All of the compensatory signals elevated cellular viability under the cytotoxic NSAID action.