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Prostaglandin E_1 reduces the keratinocyte toxicity of sorafenib by maintaining signal transducer and activator of transcription 3 (STAT3) activity and enhancing the cAMP response element binding protein (CREB) activity

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Abstract

Hand-foot skin reaction (HFSR) is a common side effect of multiple tyrosine kinase inhibitors (mTKIs). HFSR can necessitate dose reductions or interruption of therapy owing to its negative effect on the quality of life. Therefore, effective use of mTKIs requires measures to prevent HFSR. We evaluated the effect of prostaglandin E_1 (PGE_1) on HFSR, because PGE_1 is already used to treat bed sores and skin ulcers and has established angiogenic and antiproliferative effects in keratinocytes. We found that the pathogenesis of sorafenib-induced HFSR is characterized by a decrease in levels of a phosphorylated signal transducer and activator of transcription 3 (STAT3). We investigated the effect of PGE_1 on the sorafenib-mediated reduction in phosphorylated STAT3 levels in HaCaT human epidermal keratinocytes. In cells treated with sorafenib, phosphorylated STAT3 levels decreased in a concentration-dependent manner, and this effect was blocked in cells treated with sorafenib and PGE_1. Furthermore, the expression of phosphorylated STAT3, the antiapoptotic proteins myeloid cell leukemia-1 (Mcl-1) and survivin decreased in cells pretreated with an inhibitor of cAMP response element binding protein (CREB). Cell viability increased in cells treated with sorafenib and PGE_1 compared with that in cells treated with sorafenib alone, and these effects were not observed in STAT3 knockdown HaCaT cells. Collectively, these findings indicate that PGE_1 blocks the inhibitory effects of sorafenib on cell growth by maintaining the activity of STAT3 and

enhancing the CREB activity. Therefore, PGE_1 might represent an effective treatment for the prevention of sorafenib-induced HFSR.

Keywords

Sorafenib, Prostaglandin E_1, STAT3, Keratinocyte, Hand-foot skin reaction

1. Introduction

In recent years, the outcomes of metastatic kidney cancer and unresectable liver cancer have markedly improved owing to the clinical development of multiple tyrosine kinase inhibitors (mTKIs) [1,2]. These mTKIs inhibit kinases that are commonly upregulated in cancer cells, including Raf-1, vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), c-Kit, and rearranged during transfection (RET) [3]. In addition, mTKIs are associated with fewer side effects than traditional cytotoxic anticancer agents; however, hand–foot skin reaction (HFSR) is a common side effect of mTKIs, and this condition can substantially decrease the quality of life (QOL). HFSR occurs in approximately 60% of patients treated with sorafenib [4] and presents with inflammation, ulcers, parakeratosis, and functional defects in the hand or foot affected by the lesion. It is a serious side effect that can necessitate dose reductions or interruption of therapy owing to its negative effects on QOL. The incidence of HFSR is proposed to be directly associated with the therapeutic response to the mTKI [5]. Therefore, measures to prevent HFSR remain a critical unmet need in cancer treatment [6].

We recently demonstrated that HFSR is associated with a decrease in the activity of signal transducer and activator of transcription 3 (STAT3), a key mediator of proliferation in epidermal keratinocytes [7]. STAT3 is phosphorylated by Janus kinases, and phosphorylated STAT3

translocates to the nucleus [8,9]. STAT3 promotes the transcription of target genes associated with apoptosis, including the genes encoding the antiapoptotic factors such as myeloid cell leukemia-1 (Mcl-1) and survivin. Mcl-1 inhibits the proapoptotic factor Bim [10] and survivin inhibits apoptosis by inhibiting Caspase-3, -7, and -9 [11]. Recent reports reveal that Mcl-1 is associated with keratinocyte cornification [12] and that survivin induces keratinocyte proliferation [13]. Therefore, STAT3-mediated regulation of Mcl-1 and survivin might play an important role in the homeostasis of the epidermis.

These findings suggest that drugs capable of maintaining STAT3 activity in the presence of sorafenib might prevent HFSR. Therefore, we investigated the prostaglandin E (PGE)-mediated activation of STAT3 by stimulating the E-prostanoid (EP) 2-β-Arrestin 1 pathway [14]. PGE_1 and PGE_2 are well-characterized PGE subtypes. EP receptors are classified into 1 of 4 subgroups according to their function. In the epidermis, the G protein-coupled receptor EP2 enhances cyclic adenosine monophosphate (cAMP) levels and stimulates PGE_2, there by inducing cell growth and VEGFR-induced effects [15]. As PGE_1 exerts similar effects, it has been used to treat bed sores and skin ulcers in the clinical setting [15-18]. Based on its ability to potently activate skin cell proliferation, we hypothesized that PGE_1 might have a protective effect against HFSR. Therefore, we examined the effects of PGE_1 on sorafenib-induced keratinocyte toxicity and skin structure

alteration.

2. Material and methods

2.1. Chemicals

Sorafenib and PGE_1 were purchased from LKT Laboratories (St. Paul, MN, USA). CREB binding protein-CREB interaction inhibitor (CREB-I) was purchased from Merck Millipore (Darmstadt, Germany).

2.2. Antibodies

Rabbit anti-phosphorylated STAT3 (Tyr705), anti-STAT3, anti-survivin, anti-Mcl-1, anti-phosphorylated CREB (Thr202/Tyr204), anti-CREB anti-β-actin, and anti-rabbit horseradish peroxidase (HRP)-conjugated IgG were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA).

2.3. Cells and cell culture

The human epidermal keratinocytes cell line HaCaT was provided by Professor Norbert Fusenig (German Cancer Research Centre, Heidelberg, Germany) [19]. The cells were maintained in

Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals, Solon, OH, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies Corporation, Carlsbad, CA, USA). Cells seeded in culture dishes were grown in a humidified atmosphere at 37°C with 95% air and 5% CO_2. The cells were subcultured with 0.25% trypsin and 0.02% EDTA (Life Technologies Corporation).

2.4. STAT3 knockdown

STAT3 expression was inhibited using a Silencer® Select Pre-Designed small interfering RNA (siRNA) against STAT3 (Applied Bio, siRNA ID: s743). The STAT3-siRNA (10 nM) and a nonspecific control siRNA (NC siRNA) was transfected into cells cultured in a serum-free medium, Opti-MEM (Life Technologies), using HiperFect Transfection Reagent (QIAGEN, Hilden, German) according to the manufacture's instructions. The transfection was conducted at room temperature for 10 min to promote the formation of transfection complexes. HaCaT cells grown to 90% confluence were detached from the plates using a 0.25% trypsin solution and cell suspensions were prepared at the appropriate concentrations. After the transfection complexes were added to the cell suspension, the cells were seeded on plates or culture dishes.

2.5. WST-8 colorimetric assay

The inhibitory effect of sorafenib on cell growth in HaCaT cells was evaluated using the water-soluble tetrazolium salts 8 (WST-8; Dojindo Laboratories, Kumamoto, Japan) assay, as previously described [20]. The cells (2×10^3 cells/well) were seeded on 96-well plates and precultured for 24 h. The medium was exchanged for fresh media supplemented with sorafenib at the indicated concentrations, and the cells were incubated for an additional 48 h at 37°C. Furthermore, the culture medium was replaced with fresh medium supplemented with the WST-8 reagent and incubated for an additional 2.5 h. The absorbance in each well at 450 nm was determined using a microplate reader with a reference wavelength of 630 nm (Infinite 200 Pro, TECAN Co. Ltd., Switzerland).

2.6. Western blot analysis

The proteins in the total cell lysates were extracted using 0.7% CHAPS Buffer (20 mM Tris [pH 7.5], 120 mM NaCl, 1 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, and 50 mM NaF) supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin. The proteins were separated using 8% or 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were electrotransferred to a Hybond-P nitrocellulose

membrane (GE Healthcare Ltd, Little Chalfont UK). Moreover, the membrane was blocked in wash buffer (10 mM Tris [pH 7.5], 150 mM NaCl, and 0.05% Tween-20) and supplemented with 2% skim milk for 30 minutes. The membrane was washed with specific primary antibodies diluted buffer and subsequently incubated with HRP-conjugated secondary antibodies for 1 h. Antibody-bound proteins were visualized using an enhanced luminol-based chemiluminescent method, and the protein bands were imaged using ChemiStage 16-CC (KURABO Industries Ltd., Osaka, Japan). The intensity of protein bands was measured using ImageJ (NIH) [21]. Where indicated, some membranes were stripped and probed with a different antibody.

2.7. 3D skin assay

The 3D human epidermis tissue model consisted of multilayered cell normal human epidermal keratinocytes (NHEKs) composed of a basal layer, spinous layer, granular layer, and cornified layer was purchased from MatTek corporation (Homer Ave, Ashland, MA, USA). The experimental protocol was based on the skin irritation test (SIT) method [22]. Sorafenib was administered under the basal layer and PGE_1 was administered on the cornified layer. The control group was treated with PBS. The treatments were uniformly applied using nylon mesh for 1 week after preculturing the cells for 3 days. Hematoxylin and eosin (H & E) staining was performed using paraffin-embedded 3D skin

model. The thickness between the basal and granular layers was measured using ImageJ (NIH). Nine randomly-selected regions from each image were measured and the average was calculated. The values are presented as percentages of control thickness. Protein levels were analyzed by western blot assays using total tissue lysates.

2.8. Statistical analysis

The data are expressed as the mean ± S.D. or S.E. as indicated. Comparisons between two and among more than three groups were performed with Student's unpaired *t*-test and the repeated or nonrepeated one-way analysis of variance (ANOVA), followed by Tukey's test. P-values of less than 0.05 (two-tailed) were considered to be statistically significant.

3. Results

3.1. PGE_1 inhibited the sorafenib-mediated decrease in phosphorylated STAT3 levels

The HaCaT cells treated with sorafenib showed decreased levels of phosphorylated STAT3 (p-STAT3) in a concentration-dependent manner, and this effect was attenuated by PGE_1 (Fig. 1A and 1B). As substantial reductions in p-STAT3 levels were observed in cells treated with 5 μM

sorafenib, this concentration was used in subsequent experiments. In the PGE_1 + sorafenib group, p-CREB levels rapidly increased 1-2 h after treatment and decreased thereafter (Fig. 1C and 1D). p-STAT3 levels remained elevated in the PGE_1 + sorafenib compared with sorafenib group 6 h after treatment (Fig. 1C and 1D).

3.2. PGE_1 rescued sorafenib-induced cell growth inhibition

Sorafenib inhibited proliferation in HaCaT cells in a concentration-dependent manner, but this effect was attenuated in cells treated with both sorafenib and PGE_1 (Fig. 2A). Thirty-five percents decrease in cell proliferation was observed in cells treated with 5 μM sorafenib, whereas a significant 1.4-fold increase in proliferation was observed in cells treated with 5 μM sorafenib in combination with PGE_1. This effect was inhibited in cells treated with sorafenib + PGE_1 pretreated with CREB-I. In contrast, PGE_1 did not rescue the sorafenib-induced reduction in proliferation in STAT3 knockdown HaCaT cells (Fig. 2B). Moreover, there was no significant difference in the rate of the cell proliferation between the sorafenib group and the sorafenib + PGE_1 group pretreated with the CREB inhibitor.

3.3. CREB inhibition disrupts the effect of PGE_1 on STAT3 signaling

Despite pretreatment with a CREB inhibitor, p-CREB levels increased in the forskolin (Fsk) group and the sorafenib + PGE_1 group compared with the untreated control cells in the treatment for 1 h (Fig. 3A). Elevated p-CREB levels were not observed in any of the treatment groups 24 h after treatment (Fig. 3B). Compared with the sorafenib group, the expression levels of p-STAT3, Mc1-1, and the anti-apoptotic factor survivin increased in the sorafenib + PGE1 group, and this effect was inhibited by the addition of the CREB inhibitor (Fig. 3B and 3D); however, in STAT3 knockdown, the HaCaT cells, p-STAT3, Mc1-1, and survivin were similar in the sorafenib group and the sorafenib + PGE_1 group (Fig. 3C and 3E).

3.4. Effects of sorafenib and PGE_1 in a 3D skin model

In a 3D human skin culture model, the thickness between the basal and granular layers decreased in the sorafenib group, and this effect was inhibited by PGE_1 (Fig. 4A and 4B). Similarly, p-STAT3 and Mc1-1 levels decreased in the treatment of sorafenib and this effect was also inhibited by PGE_1 (Fig. 4C).

5. Discussion

In this study, we demonstrated that PGE_1 maintains STAT3 signaling by activating CREB and that PGE_1 ameliorates sorafenib-induced cytotoxicity and pathological changes of skin model.

As previously reported, p-STAT3 levels decreased in sorafenib-treated HaCaT cells in a concentration-dependent manner [7]. In contrast, p-STAT3 levels increased in cells treated with a combination of sorafenib and PGE_1, whereas p-STAT3 levels decreased in sorafenib-treated cells in a time-dependent manner (Fig. 1A and 1B). Although p-CREB levels recovered in cells treated with a combination of sorafenib and PGE_1, this effect was not observed until 1 h after treatment. The CREB pathway, which is activated at the downstream of EP2-β-Arrestin 1, was transiently activated in sorafenib-treated cells, suggesting that the EP2-β-Arrestin1-CREB pathway is not greatly affected by sorafenib (Fig. 2B). The binding affinity of PGE_1 for different EP receptors has been described as EP3 ≥ EP4 > EP2 > EP1 [23]. EP3 is a Gi protein-coupled receptor, and it is reported to promote a decrease in cAMP reduction in conjunction with PGE_1. Similar to EP2 function, EP4 is also known to promote an increase in the intracellular concentration of cAMP [24,25]. As the findings suggest that CREB was activated in HaCaT cells in present study, we assumed that HaCaT cells express very low levels of EP3. Hence, we propose that PGE_1 primarily activates the EP2 or EP4 receptor and thus activates CREB by inducing an increase in cAMP levels.

The results show that proliferation decreased in sorafenib-treated HaCaT cells in a

concentration-dependent manner; however, sorafenib-induced cell growth inhibition was rescued in cells treated with a combination of sorafenib and PGE_1. Interestingly, treatment with a CREB-I blocked the proliferative effect of PGE_1. These results conclude that PGE_1-induced CREB activation promotes cell proliferation (Fig. 2A).

PGE_1 rescued the sorafenib-induced decrease in p-STAT3 levels and enhanced levels of Mcl-1, a downstream factor of STAT3 activation. Pretreatment with the CREB-I also inhibited the effects of PGE_1 on Mcl-1 (Fig. 3A); however, PGE_1 had no effect on STAT3 knockdown cells (Fig. 2B and 3C). These findings indicate that PGE_1 activates STAT3 signaling via the activation of CREB. Moreover, pSTAT3 was enhanced in the combination of PGE1 and sorafenib-treated cells but not in the combination of PGE1, sorafenib, and CREB-I-treated cells and sorafenib alone-treated cells for 24 h (Fig.3B and 3D). Therefore, we considered that pSTAT3 is enhanced by activating CREB and not by PGE1 itself. And also, sorafenib-mediated inhibition of cell proliferation cannot be rescued by CREB activation alone and the sustained activation of STAT3 is important for maintaining homeostasis in the skin.

CREB is a transcription factor that promotes the transcription of target genes by activating upstream receptors (e.g., EP2) and enhancing intracellular cAMP and Ca^{2+} levels [26]. We demonstrated that PGE_1 enhances cell proliferation via the transcription of CREB-associated proteins

(e.g., Arc, c-fos, c-jun, and brain derived neurotropic factor [BDNF]) [27,28]. A role for CREB in STAT3 signaling is further supported by the observation that CREB contributes to lymphoma cell proliferation by promoting formation of the c-jun/STAT3/ILS-1 complex [29]. Therefore, we considered that the CREB-STAT3 interaction is an indirect action. It may take a reasonable amount of time that the activated CREB phosphorylates STAT3, but the present study could not clarify the mechanism for phosphorylating STAT3 by CREB. Precise experiments are need in a future.

Our findings suggest that the thickness between the granular and basal layers of a human skin 3D culture model treated with sorafenib decreased by approximately 30%. In contrast, the thickness between the granular and basal layers was similar in the sorafenib + PGE_1 and control groups (Fig. 4A and 4B). And, there are no changes of pCREB in the experiment that 3D skin model was exposed to sorafenib for 1 week, because activation of CREB induced by sorafenib is temporary as shown in Fig. 1B. As the pathology of sorafenib-associated HFSR is characterized by the thinning of the spinous layer and parakeratotic cells [30], the human skin 3D culture model might be a valuable model for investigating HFSR. p-STAT3 and Mcl-1 levels increased in human skin 3D cultures and single-layer HaCaT cells treated with sorafenib + PGE_1 (Fig. 4B), suggesting that PGE_1 might have therapeutic value in HFSR prophylaxis and treatment.

Cornification in the epidermis helps maintain its barrier function. The cells of the granular layer

are primed to develop into the cornified layer. The histology of cornification is characterized by cell flattening and enucleating, and these morphological changes are mediated by the cross-linking of involucrin and the fragmentation of filaggrin, which forms a source of the natural moisturizing factor (NMF) [31]; however, the nuclei of sorafenib-treated cells in the granular layer appeared round and unfit for enucleating (Fig. 4A); PGE_1 blocked this effect. A recent study reported that, in addition to its anti-apoptotic role, Mcl-1 plays a role in the cornification of skin keratinocytes. Mc1-1 knockdown induced a thinning of the tissue between the spinous and granular layers, and a reduction in levels of the differentiation markers loricrin, keratin 1, keratin 10, filaggrin, and fragmented Caspase [12,14]. These findings indicate that the pathogenesis of sorafenib-induced HFSR is due to not only the inhibition of cell growth but also the inhibition of the development of immature skin during parakeratosis. Furthermore, studies focusing on skin cornification are required to understand the pathogenesis of this effect and develop prevention methods. In addition, the effects of PGE_1 on parakeratosis, another hallmark of the pathology of HFSR, remain unclear.

In conclusion, we demonstrated that PGE_1 alleviated sorafenib-induced toxicity by enhancing cell viability according to activation of the CREB and STAT3 signaling pathways. Therefore, PGE_1 may have therapeutic potential in the prevention of sorafenib-induced HFSR.

Conflict of authors

Dr. Takahiro Ishida is employee of Momotani Juntenkan LTD, Japan.

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Figure legends

Figure 1. The effects of PGE_1 on sorafenib-mediated inhibition of STAT3 signaling.

(A) HaCaT cells were incubated with sorafenib at the indicated concentration in combination with 1 μM PGE_1 for 24 h. Total cell lysates were evaluated using western blot assays. (B) Quantitative analysis of (A). Each bar represented mean ± S.E. (n=3). (C) HaCaT cells were incubated with 5 μM sorafenib at the indicated time in combination with 1 μM PGE_1. Total cell lysates were evaluated using western blot assays. (D) Quantitative analysis of (C). Each bar represented mean ± S.E. (n=3, $*P < 0.05$).

Figure 2. The effects of PGE_1 and CREB-I on the sorafenib-induced cell growth inhibition.

(A) HaCaT cells were incubated with sorafenib at the indicated concentration in combination with 1

μM PGE_1 for 48 h. (B) HaCaT cells transfected with nonspecific control (NC) siRNA or STAT3 siRNA were treated with the indicated reagents for 48 h. Each bar represented mean ± S.D. (n=4, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Figure 3. The effects of PGE_1 on STAT3 signaling in the presence of a CREB-I.

(A) HaCaT cells were treated with 5 μM sorafenib and 1 μM PGE_1. Cells were cultured in medium supplemented with the indicated reagent for 1 h and (B) 24 h. Fsk is an activator of CREB signaling and was used as a positive control. Total cell lysates were analyzed using western blot assays. (C) HaCaT cells transfected with nonspecific control (NC) siRNA or STAT3 siRNA were treated with 5 μM sorafenib and 1 μM PGE_1. Total cell lysates were analyzed using western blot assays. (D) Quantitative analysis of (B). (E) Quantitative analysis of (C). Each bar represented mean ± S.E. (n=3, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Figure 4. The effect of PGE_1 in human epidermis 3D cultures treated with sorafenib.

(A) The hematoxylin and eosin (H & E)-stained 3D human epidermis model. Sorafenib was administered under the basal layer and PGE_1 was administered on the cornified layer. PBS was used as the control treatment. The tissues were precultured for 3 days and subsequently incubated with the

indicated reagent for 1 week.

(B) The thickness between the basal and granular layer in live cells was measured using ImageJ. Nine randomly selected regions from each image were measured and the average was calculated. The values are presented as percentages of control thickness ± S.D.

(C) After the drug treatments, the human epidermis 3D cultures were washed with PBS and homogenized using CHAPS Buffer. The insoluble fraction of the tissue suspension was removed. Total tissue lysates were analyzed using western blot assays.

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Figure 1

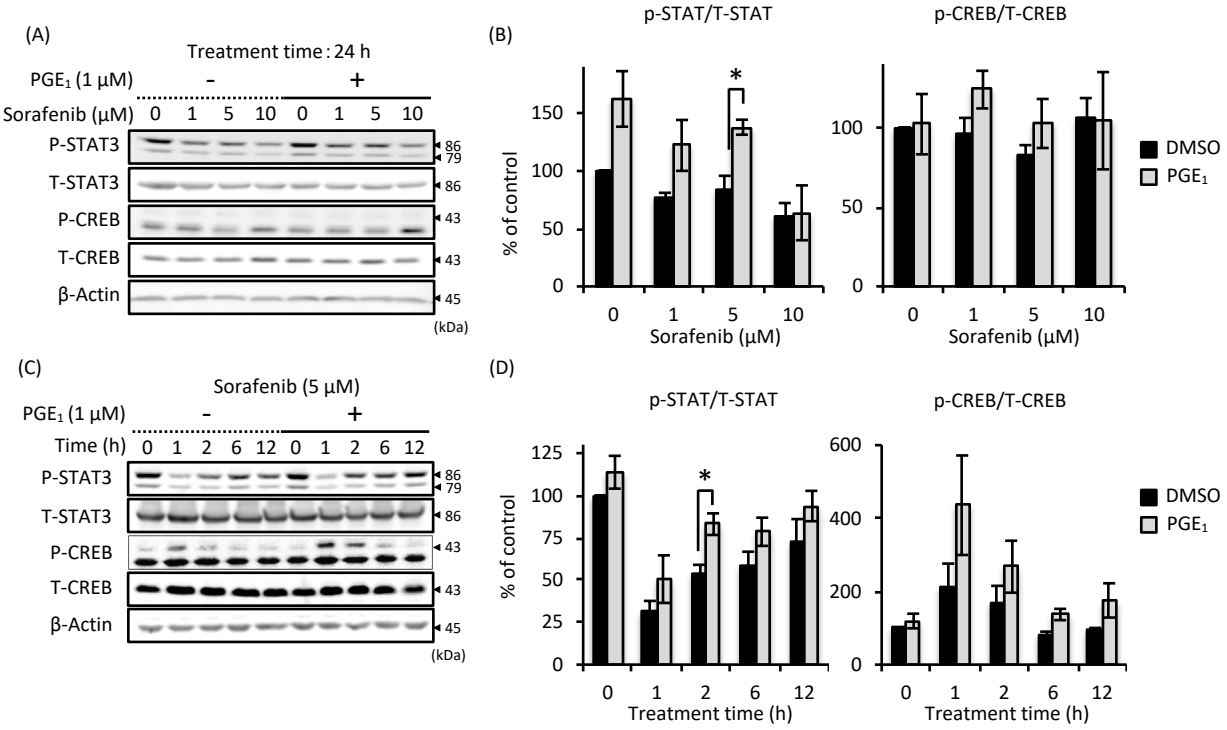

(A)
Treatment time : 24 h
PGE1 (1 μM) - +
Sorafenib (μM) 0 1 5 10 0 1 5 10
P-STAT3
T-STAT3
P-CREB
T-CREB
β-Actin
86
79
86
43
43
45
(kDa)
(B)
p-STAT/T-STAT
p-CREB/T-CREB
% of control
Sorafenib (μM)
DMSO
PGE1
(C)
Sorafenib (5 μM)
PGE1 (1 μM) - +
Time (h) 0 1 2 6 12 0 1 2 6 12
P-STAT3
T-STAT3
P-CREB
T-CREB
β-Actin
86
79
86
43
43
45
(kDa)
(D)
p-STAT/T-STAT
p-CREB/T-CREB
% of control
Treatment time (h)
DMSO
PGE1

Figure 2

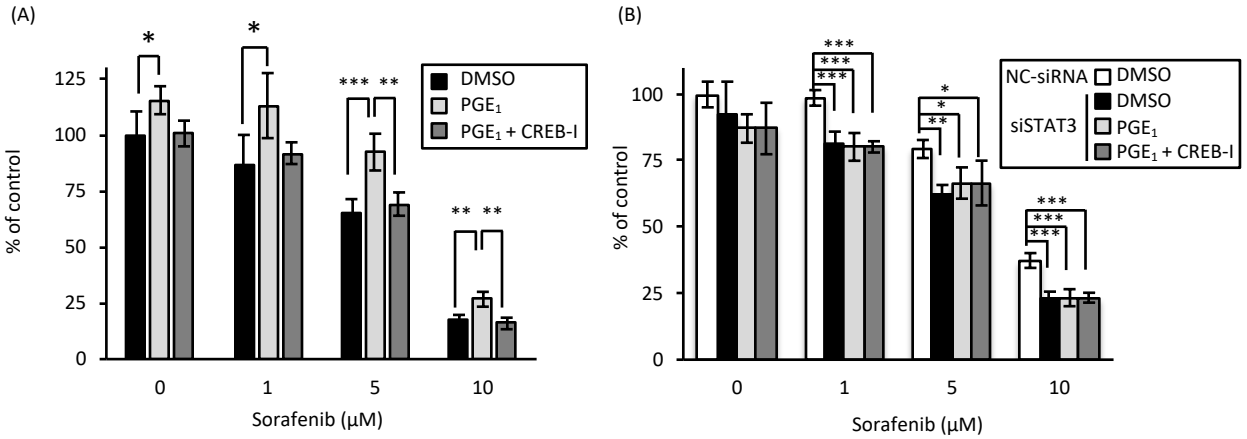

(A)
% of control
125
100
75
50
25
0
0
1
5
10
Sorafenib (μM)
DMSO
PGE1
PGE1 + CREB-I
(B)
% of control
100
75
50
25
0
0
1
5
10
Sorafenib (μM)
NC-siRNA
DMSO
siSTAT3
DMSO
PGE1
PGE1 + CREB-I

Figure 3

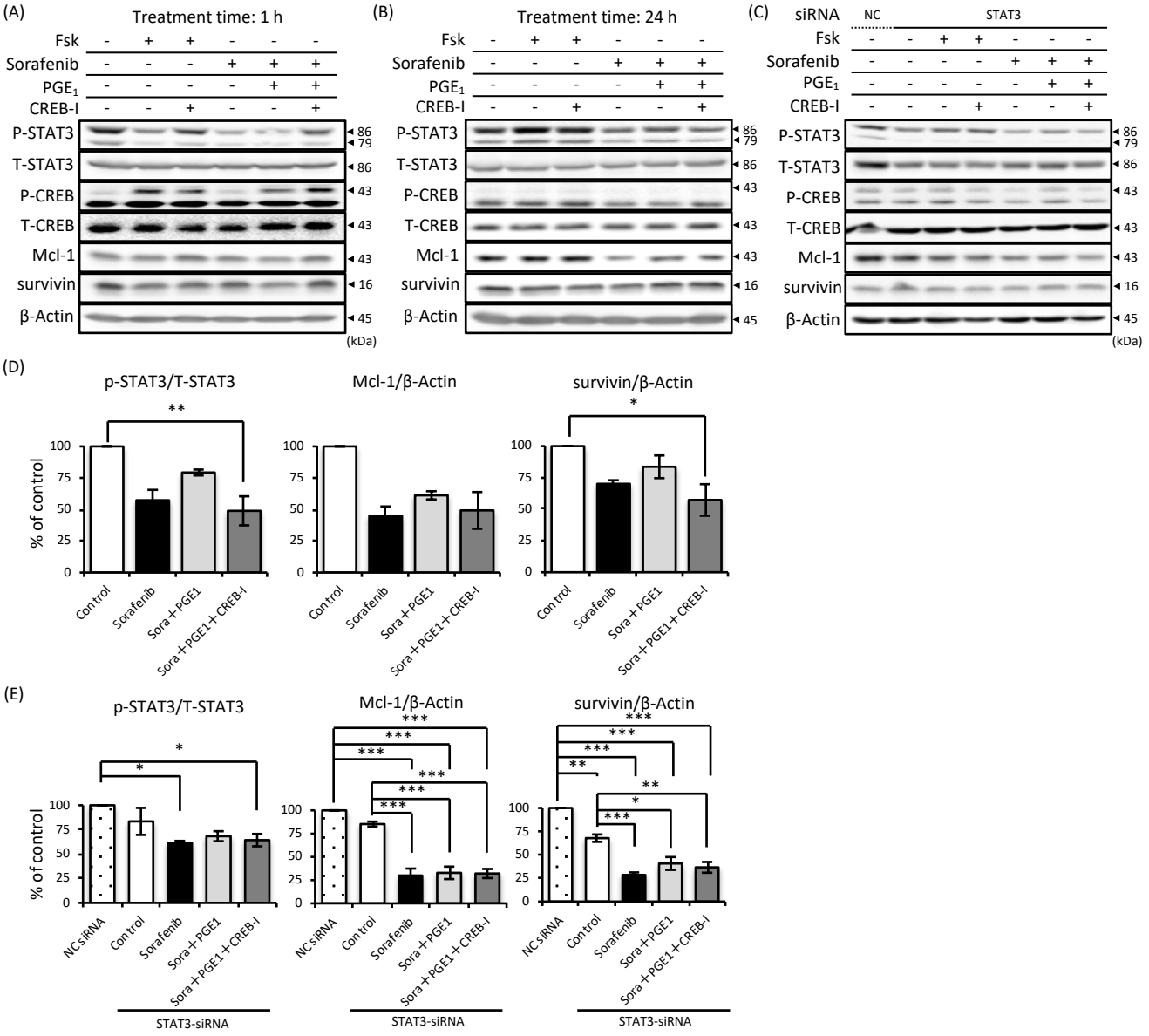
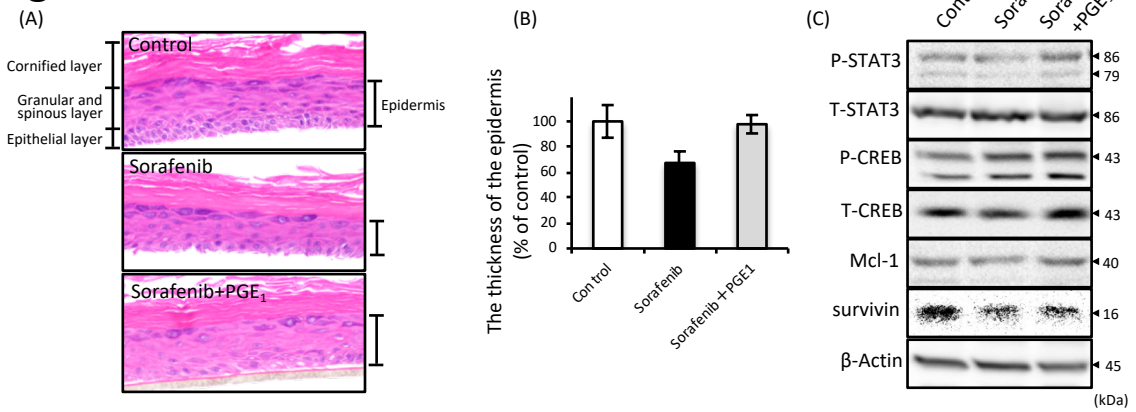

(A)
Treatment time: 1 h
Fsk
Sorafenib
PGE1
CREB-I
P-STAT3
T-STAT3
P-CREB
T-CREB
Mcl-1
survivin
β-Actin
86
79
43
16
45
(kDa)
(B)
Treatment time: 24 h
(C)
siRNA
NC
STAT3
(D)
p-STAT3/T-STAT3
Mcl-1/β-Actin
survivin/β-Actin
% of control
Control
Sorafenib
Sora+PGE1
Sora+PGE1+CREB-I
(E)
NC siRNA
STAT3-siRNA

Figure 4


(A)
Control
Cornified layer
Granular and spinous layer
Epithelial layer
Epidermis
Sorafenib
Sorafenib+PGE1
(B)
The thickness of the epidermis (% of control)
100
80
60
40
20
0
Control
Sorafenib
Sorafenib+PGE1
(C)
Control
Sorafenib
Sorafenib +PGE1
P-STAT3
T-STAT3
P-CREB
T-CREB
Mcl-1
survivin
β-Actin
86
79
86
43
43
40
16
45
(kDa)