Isosakuranetin, a 4'-O-methylated flavonoid, stimulates melanogenesis in B16BL6 murine melanoma cells

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Abstract

Aims

The beneficial effects of 4'-O-methylated flavonoids on induction of melanogenesis are well established. Here, we report the effect of isosakuranetin (Iso) on melanogenesis in B16BL6 melanoma cells and an analysis of the signaling pathways involved in this activity.

Methods

B16BL6 melanoma cells were treated with several concentration of Iso and melanin content was measured. Activation and expression of factors involved in melanogenesis were assessed via western blotting.

Key findings

Iso (15 and 30 μmol/L) strongly stimulated melanogenesis in a dose-dependent manner. Iso increased tyrosinase activity and up-regulated tyrosinase (*Tyr*), tyrosinase related protein 1 (*TRP1*), and tyrosinase related protein 2 (*TRP2*) in a time-dependent manner. Iso decreased B16 cell proliferation at a concentration above 45 μmol/L, and had no effect on cell viability as revealed by MTT and trypan blue assays. Iso up-regulated expression of microphthalmia transcription factor (*MITF*), with a maximum effect after 12 h. H89, a specific inhibitor of PKA, showed that MITF up-regulation is mediated through PKA/CREB activation. Furthermore, Iso decreased phosphorylation of MITF at Ser73 after 24 h and 48 h of exposure, activating MITF and leading to up-regulation of *Tyr*, *TRP1*, and *TRP2*. Iso inhibited phosphorylation and activation of ERK1/2 after 12 h, while no significant effects on p38 and JNK phosphorylation were observed. Iso inhibited AKT phosphorylation and led to activation of GSK3β.

Significance

Iso stimulates melanogenesis in B16 melanoma cells via up-regulation of *MITF*. Furthermore, Iso-induced inhibition of ERK1/2 and PI3K/AKT signaling pathways activate MITF and subsequent expression of *Tyr*, *TRP1*, and *TRP2*.

Keywords: melanogenesis; isosakuranetin; flavonoid; MITF; Tyr; TRP1; TRP2

Introduction

Flavonoids, including isosakuranetin (Iso), are consumed daily in the diet as constituents of a variety of plant components. In addition, flavonoids are available in a wide range of natural products. Some flavonoids have been developed into therapeutic products due to their pharmacological activities. Previous investigations identified Iso as an important component of propolis, *Baccharis dracunculifolia*, *Terminalia fagifolia*, and *Citrus sinensis* [1-4]. Iso possesses a wide range of biochemical and pharmacological properties (Fig. 1), including antioxidative, neuroprotective, anticarcinogenic, and antiallergic properties [5, 6, 2].

Melanogenesis is the biosynthetic pathway for melanin production, which operates in dendritic melanocytes located in the lowest layer of the epidermis, as well as in hair and the eyes [7]. Melanin synthesis is mediated by at least 3 important enzymes: tyrosinase (Tyr), tyrosinase-related protein 1 (TRP1), and tyrosinase-related protein 2 (TRP2) [8]. Tyr is the key enzyme involved in melanin synthesis and catalyzes hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), oxidation of DOPA to produce dopaquinone, and oxidation of 5,6-dihydroxyindole (DHI) to indole-quinone [9]. TRP2 acts as a dopachrome tautomerase and functions together with TRP1 in melanin biosynthesis downstream of tyrosinase [10]. Two types of melanin are produced in mammals: red/yellow pheomelanin and black/brown eumelanin. Whereas tyrosinase is a common enzyme required for both eumelanin and pheomelanin synthesis, TRP1 and TRP2 seem to be primarily involved in eumelanin production [11, 12].

When the melanocortin-1 receptor (MC1R) is stimulated upon release of alpha-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) by keratinocytes after UV irradiation, the G-protein coupled to MC1R activates adenylate cyclase (AC), which in turn leads to cyclic AMP (cAMP) production. Activation of protein kinase A (PKA) by cAMP results in binding of CRE-binding protein (CREB) to the cAMP regulatory element (CRE) promoter, leading to transcription of the gene encoding microphthalmia transcription factor (MITF). Increased expression of MITF leads to elevated expression of genes in the melanogenic enzyme family, *Tyr*, *TRP1*, and *TRP2*, which stimulate melanin synthesis [13]. Activation of Ras by cAMP leads to activation of ERK1/2 through MEK. Activated ERK1/2 phosphorylates MITF at Ser73, which is followed by MITF ubiquitination and degradation. Furthermore, p38 has been shown to be involved in UVR-induced melanogenesis via activation of MITF and subsequent up-regulation of *Tyr* expression [14].

In addition to transcriptional regulation, MITF can be regulated by posttranslational modification. Inhibition of PI3K and AKT by cAMP induces activation of GSK3β, which phosphorylates MITF at Ser289 and increases its binding to the M-box of the *Tyr* promoter, leading to expression of melanogenesis-related enzymes [15, 16].

This study was conducted to understand the signaling pathways underlying Iso-induced melanogenesis in B16BL6 melanoma cells. First, we determined whether Iso enhanced melanogenesis via up-regulation of *Tyr*, *TRP1*, and *TRP2*. Next, we assessed the effects of Iso on MITF expression and phosphorylation. Finally, we determined whether changes in MITF activation induced by Iso were mediated via inhibition of ERK1/2 and PI3K/AKT signaling pathways.

Materials and methods

Materials. The B16BL6 murine melanoma cell line was provided by RIKEN (Institute of Physical and Chemical Research Cell Bank, Tsukuba, Ibaraki, Japan). Iso was purchased from Extrasynthese Company (Genay, France). H89, and U0126 were obtained from Cayman Chemical Company (Michigan, USA). RPMI1640, L-DOPA, α-MSH, and anti-pMITF ser73 were purchased from Sigma-Aldrich (St. Louis, MO, USA). LY294002 and anti-MITF, anti-ERK1/2, anti-p-ERK1/2, anti-JNK, anti-p-JNK, anti-p38, anti-p-p38, anti-AKT, anti-p-AKT (Ser473), anti-p-AKT (Thr308), anti-p-GSK3β (Ser9), and anti-β-actin antibodies were obtained from Cell Signaling Technology (Tokyo, Japan). Anti-Tyr, anti-TRP1, and anti-TRP2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. B16 melanoma cells were cultured in RPMI1640 medium containing 10% FBS at 37 °C and 5% CO_2. The cells were plated at a density of 3×10^5 cells/dish in 100-mm culture dishes and at a density of 4.5×10^4 cells/well in 6-well plates. The cells were cultured and allowed to attach for 24 h, followed by treatment with Iso at the indicated concentrations and for the indicated periods of time.

MTT assay. B16 melanoma cells were cultured in 24-well plates at a density of 1×10^4 cells/well. After 24 h, the cells were incubated for 72 hours in the presence of Iso at the indicated concentrations. The culture medium was removed and replaced with 500 μL of fresh culture medium containing 10% sterile filtered MTT (Sigma-Aldrich). After 3 hours, the formazan crystals were dissolved in 500 μL/well isopropanol and absorbance was measured at

570 nm against 630 nm. Inhibition of proliferation (%) was expressed as the percentage of viable treated cells in comparison with control cells.

Trypan blue assay. B16 melanoma cells were seeded in 6-well plates at a density of 4.5×10^4 cells/well. After 24 h, the cells were incubated for a further 72 h in the presence of Iso at the indicated concentrations and cell viability was quantified by the trypan blue assay. After washing the cells twice with PBS, they were trypsinized, immediately stained with 0.5% trypan blue dye (Trypan Blue, Sigma-Aldrich) for 3 min, and observed under an optical microscope. Cell viability was calculated as the percentage (%) of unstained cells relative to the total number of counted cells.

Measurement of melanin content. Melanin content was measured as described previously with some modifications [17]. B16 cells were seeded in 6-well plates at a density of 4.5×10^4 cells/well. After a 24-h incubation, the medium was replaced with a fresh medium containing Iso at 15 and 30 μmol/L and the cells were incubated for a further 72 h. The cells were washed twice in phosphate-buffered saline (PBS) and detached with 0.25% trypsin-EDTA solution, after which the solution containing the cells was transferred to a 1.5-mL tube. After centrifugation for 10 min at 14000 rpm, the pellets were dissolved in 1N NaOH (100 μL) for 60 min at 80 °C. The absorbance of the sample at 405 nm was measured with a microplate reader.

Western blot analysis. B16 melanoma cells were cultured in 100-mm culture dishes at a density of 3×10^5 cells/dish. After treatment, cells were harvested in RIPA buffer (50 *m*mol/L Tris-HCl (pH 7.4), 150 *m*mol/L NaCl, 0.1% SDS, 0.5% Na-deoxycholate, 1% NP40, 1 *m*mol/L EDTA, 2 *m*mol/L NaF, and 1 *m*mol/L Na_3VO_4). Cells lysates were separated with SDS-PAGE and blotted on a PVDF membrane. After blocking in 2% BSA, the membrane was incubated with one of the selected primary antibodies (described in materials section) in TBS-T buffer overnight at 4 °C. Next, the membrane was incubated with the appropriate secondary antibodies for 1 h at RT and treated with the LuminoGLO reagent (Cell Signaling Technology). Protein bands were visualized using chemiluminescence (EZ-Capture MG, ATTO, Tokyo, Japan). The band intensity was analyzed using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

Intracellular tyrosinase activity assay. An intracellular tyrosinase activity assay was performed as previously described [18]. After 72 h, the cultured cells were washed with PBS and scraped in phosphate buffer (100 *m*mol/L, pH 6.8) supplemented with 1% Triton X100 and a protease inhibitor. The cell lysate was obtained after centrifugation at 14000 rpm for 10 min. The protein concentration was determined using a BCA protein assay kit and the concentrations of the samples were equilibrated. The cell lysate supernatants (containing 50 μg of protein) were transferred to 96-well plates and mixed with 100 μL of L-DOPA (0.2% in phosphate buffer). After incubation at 37 ºC for 1 h, the absorbance of each sample was measured at 475 nm using a microplate reader. Cellular tyrosinase activity was determined as the ratio of tyrosinase content to total protein content.

Statistical analysis. Results are expressed as mean ± S.D. Comparisons between groups were analyzed with Student's *t*-test. Differences were considered significant at a P-value < 0.05.

Results

Iso activates melanin synthesis in B16 melanoma cells

B16 melanocytes were cultured for 48 h and 72 h in the presence of the indicated concentrations of Iso. As shown in Fig. 2A, Iso stimulated melanin accumulation in a dose-dependent manner. In addition, Iso at 30 μmol/L gradually increased the expression of *Tyr*, *TRP1*, and *TRP2* after 48 and 72 h (Fig. 2B).

Iso decreases the proliferation rate of B16 melanoma cells without affecting viability

To investigate whether Iso is cytotoxic to B16 melanocytes, cells were cultured for 72 h in the presence of several concentrations of Iso (0–75 μmol/L). The MTT assay showed that Iso decreased the number of cells at concentrations greater than 45 μmol/L (Fig. 3A). Further analysis of trypan blue staining demonstrated that Iso did not affect the viability of B16 melanocytes, but showed that Iso decreased their proliferation rate (Fig. 3B).

Iso increases tyrosinase activity in a dose-dependent manner

Cellular B16 tyrosinase activity was assayed using L-DOPA as a substrate. After Iso treatment, cells were lysed and tyrosinase activity was assessed. The tyrosinase activity of B16 melanocytes treated with 15 and 30 μmol/L Iso for

72 h was significantly increased by 2- and 3.2-fold, respectively, in comparison with that of the control cells (Fig. 4). Treatment with positive control MSH increased tyrosinase activity by 1.6-fold.

Iso up-regulated MITF expression through PKA/CREB pathway activation, and decreased MITF phosphorylation in B16 melanoma cells

To investigate the effect of Iso on melanogenesis-related protein expression and phosphorylation, B16 melanocytes were treated with 30 μmol/L Iso for 0, 3, 6, 12, 24, and 48 h. As shown in Fig. 5A, MITF expression increased rapidly after Iso treatment and reached a maximum level after 12 h. MITF expression was slightly decreased after 24 and 48 h of Iso treatment, but protein expression remained elevated in comparison with that measured at 0 h. Similarly, phosphorylation of MITF at Ser73 was increased after 3, 6 and 12 h of Iso exposure, decreased after 24 h, and almost completely abolished at 48 h (Fig. 5A). To examine whether Iso increased MITF expression via PKA/CREB activation, a PKA specific inhibitor H89 was used. Preincubation of B16 melanocytes for 1 h with H89 (5 μmol/L) and then the addition of Iso (30 μmol/L) for further 72 h in the continued presence of the inhibitor significantly reduced the Iso-induced melanogenesis (Fig. 5B). These findings indicate that Iso stimulates melanogenesis through PKA/CREB activation and subsequent MITF up-regulation.

Effects of Iso on MAP kinases in B16 melanoma cells

To elucidate the mechanisms underlying Iso-induced melanogenesis, we examined the influence of Iso on ERK, p38, and JNK expression and phosphorylation after 0, 3, 6, 12, 24, and 48 h. Although Iso did not affect the expression of ERK, p38, or JNK, Iso markedly decreased phosphorylation of ERK after 3 h of exposure in a time-dependent manner. Iso did not affect phosphorylation of p38 or JNK (Fig. 6A). Exposure to 10 μmol/L U0126, a selective ERK inhibitor, increased melanin accumulation in B16 melanocytes by 3.5 times in comparison with that of the control group, demonstrating the capability of ERK to modulate melanin accumulation (Fig. 6B).

Effect of Iso on AKT phosphorylation in B16 melanoma cells.

Several previous studies have described the relationship between PI3K/AKT signaling and melanogenesis in melanocytes. Therefore, we determined the effect of Iso on AKT activation after 0, 3, 6, 12, 24, and 48 h. As shown in Fig. 7A, 30 μmol/L Iso gradually decreased AKT phosphorylation at Ser473 and Thr308. AKT inhibition by Iso

was accompanied by decreased phosphorylation of GSK3β, a direct target of AKT, at Ser9. Decreased GSK3β phosphorylation leads to its activation and subsequent activation of MITF transcriptional activity. LY294002, a direct inhibitor of PI3K/AKT signaling via selective PI3K inhibition, was used as a positive control to confirm the involvement of PI3K/AKT inhibition for activation of melanogenesis. At a concentration of 10 μmol/L, LY294002 increased B16 melanin content by 4.2-fold in comparison with that of the control group (Fig. 7B).

Discussion

Flavonoids are attractive natural compounds in health care applications due to their combination of beneficial bioactivities and low toxicity. Many flavonoids have been reported to control melanogenesis. However, these different flavonoids exert controversial effects on melanin synthesis in spite of their structural similarity [19-22]. Recent studies have documented the crucial role of 4'-O-methyl group on the B-ring of flavonoids in the promotion of melanin synthesis despite the alteration of functionalities of the other position. In fact, 4'-O-methylated flavonoids highly induce melanogenesis in B16F10 melanoma cells as well as in Sik2+/- mice model [23, 24]. Nevertheless, the pathways underlying elevation of melanin content by 4'-O-methylated flavonoids are not well understood. In this study, we explored the effect of Iso, a 4'-O-methylated flavonoid, on melanogenesis in B16 melanoma cells and used molecular approaches to understand the pathways involved in its effect.

Melanogenesis is a complex mechanism that is related to at least 125 genetic loci [25]. Among the genes involved in melanogenesis, the tyrosinase family, which consists of *Tyr*, *TRP1*, and *TRP2*, has been recognized as containing the most critical regulators of melanin biosynthesis. Indeed, tyrosinase family enzymes catalyze the rate-limiting steps in melanogenesis [26, 27]. As expected, our results showed that Iso, a 4'-O-methylated flavonoid, dose-dependently induced melanogenesis in B16 melanoma cells. MTT and trypan blue assays showed that Iso decreased the proliferation rate of B16 melanoma cells at a concentration above 45 μmol/L, but did not affect their viability at all tested concentrations. In addition, we found that Iso-induced melanogenesis was accompanied by increased expression of *Tyr*, *TRP1*, and *TRP2*. Moreover, treatment with Iso at 15 and 30 μmol/L increased tyrosinase activity by 2- and 3.2-fold, respectively. Taken together, our results demonstrate that Iso-induced melanogenesis is mediated mainly by elevated tyrosinase expression and activity.

MITF is the key transcriptional regulator of melanogenic enzymes [28]. In response to UV light, α-MSH increases MITF expression by activating cAMP signaling [13]. Activation of protein kinase A (PKA) by cAMP results in

binding of CRE-binding protein (CREB) to the cAMP regulatory element (CRE) promoter, leading to transcription of the gene encoding microphthalmia transcription factor (MITF). MITF specifically binds to the M-box and E-box motifs in the promoter regions of Tyr, TRP1, and TRP2 and up-regulates their expression [29]. Our results showed an increase in MITF expression upon Iso treatment that reached a maximum level after 12 h. Furthermore, H89, a specific PKA inhibitor, significantly abolished Iso-stimulated melanogenesis, showing that MITF up-regulation is mediated by PKA/CREB pathway activation.

Previous research indicated that MAPK signaling pathways (ERK, JNK, and p38) regulate MITF activity [30-32]. Activation of ERK MAP kinases induces phosphorylation of MITF at Ser73, and, together with recruitment of the transcriptional coactivator p300, this process leads to ubiquitination and proteasome-mediated degradation of MITF, finally diminishing tyrosinase synthesis and melanin production [33, 34]. Our western blotting data revealed that Iso decreased phosphorylation and activation of ERK after 12 h of exposure. Additionally, Iso did not produce significant differences in phosphorylation of p38 or JNK. The decrease in ERK phosphorylation induced by Iso led to inhibition of MITF phosphorylation at Ser73 as detected by our experiments after 24 h of exposure, thereby activating MITF and up-regulating expression of tyrosinase family genes. Furthermore, specific ERK inhibitor U0126 induced melanin accumulation in B16 melanoma cells. Thus, the results described above suggest that inhibition of ERK signaling might play an important role in Iso-induced melanogenesis.

Another important signaling pathway involving PI3K/AKT/GSK3β has been suggested to modulate the transcriptional activity of MITF [15, 16]. Indeed, α-MSH-induced cAMP can inhibit phosphorylation of AKT at Thr308 and Ser473 through a PI3K-dependent mechanism, thus inhibiting AKT activity [35, 36]. Therefore, unphosphorylated GSK3β, a molecule downstream of AKT, was activated and phosphorylated MITF at Ser289, thereby up-regulating tyrosinase family genes, consequently inducing melanogenesis [37]. In this study, we found that Iso reduced the AKT activity and phosphorylation of AKT at Thr308 and Ser473, which was followed by inhibition of GSK3β phosphorylation. Among other effects, GSK3β activation leads to activation of MITF and increased tyrosinase expression. PI3K-specific inhibitor LY294004 was used as a positive control to confirm the importance of the PI3K/AKT/GSK-3β signaling pathway as an activator of melanogenesis. Exposure to LY294004 increased melanin secretion by 4.2 times in comparison with that of the control group.

Conclusion

In this study, we showed that the 4'-O-methylated flavonoid Iso induced melanin synthesis in B16 melanoma cells. Iso triggered up-regulation of tyrosinase family protein via up-regulation of MITF expression and enhanced MITF activation. Iso-induced MITF expression and activation was clearly mediated through activation of PKA/CREB pathway, and inhibition of ERK1/2 and PI3K/AKT signaling pathways. Taken together, the results of this study demonstrate that Iso is a promising therapeutic compound that could be applied as a treatment for patients with hypo-pigmentation disorders.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research and Education from University of Tsukuba, Japan. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s).

Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Figures and legends

Fig. 1 Chemical structure of Isosakuranetin

Fig. 2 Effect of Iso on melanogenesis and expression of *Tyr*, *TRP1*, and *TRP2*. B16 melanoma cells were cultured for 48 and 72 h in the presence of several doses of Iso (0–30 μmol/L). Melanin was extracted and measured (A). Cells were sonicated in a lysis buffer and cellular proteins were subjected to western blotting with antibodies against Tyr, TRP1, and TRP2 (B). The final DMSO concentration in all samples was 0.1%. Values represent mean ± SD. Results are representative of 2 different experiments (n = 3). $^{**}P < 0.01$, $^{\#\#}P < 0.01$, versus the control of each group.

Fig. 3 Effect of Iso on B16 melanoma cell proliferation and viability. B16 melanoma cells were cultured for 72 h in the presence of Iso (0–75 μmol/L). Cell proliferation was measured by the MTT assay (A). Cells were trypsinized and their viability was determined by trypan blue staining (B). The final DMSO concentration in all samples treated with Iso was 0.1%. Values represent mean ± SD. Results are representative of 3 different experiments (n = 3). $^{**}P < 0.01$, versus the control group.

Fig. 4 Effect of Iso on tyrosinase activity in B16 melanoma cells. B16 melanoma cells were cultured in the presence of Iso (0–30 μmol/L) for 72 h. Cells were harvested and subjected to assessment of tyrosinase activity. Cellular tyrosinase activity was determined as the ratio of tyrosinase content to total protein content. α-MSH was used in this experiment as a positive control. The final DMSO concentration in all samples treated with Iso was 0.1%. Values represent mean ± SD. Results are representative of 3 different experiments (n = 3). $^{**}P < 0.01$, versus the control group.

Fig. 5 Effect of Iso on MITF expression and phosphorylation (A), and PKA inhibitor H89 on Iso-stimulated melanogeneis (B). B16 melanoma cells were cultured in the presence of Iso (30 μmol/L) for 0, 3, 6, 12, 24, and 48 h. Cells were harvested in lysis buffer and cellular proteins were subjected to western blotting with antibodies against MITF and p-MITF Ser73. Band densities were quantified with ImageJ software. Values represent mean ± SD. Results are representative of 3 different experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, versus the control of each group (A).

B16 melanoma cells were preincubated for 1h in the presence or absence of 5 μmol/L H89, and then Iso (30 μmol/L) was added for an additional 72 h. Melanin was extracted and measured. Values represent mean ± SD. Results are representative of 2 different experiments (n = 3). $^{**}P < 0.01$, versus Iso (30 μmol/L) treated group (B).

Fig. 6 Effect of Iso on ERK1/2, p38, and JNK expression and phosphorylation (A), as well as stimulation of melanogenesis by ERK1/2 specific inhibitor U0126 in B16 melanoma cells (B). B16 melanoma cells were cultured in the presence of Iso (30 μmol/L) for 0, 3, 6, 12, 24, and 48 h. Cells were harvested and cellular proteins were subjected to western blotting with antibodies against ERK1/2, p-ERK1/2, p38, p-p38, JNK, and p-JNK. Band densities were quantified with ImageJ software. Values represent mean ± SD. Results are representative of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, versus the control group (A). B16 cells were cultured in the presence of ERK specific inhibitor U0126 (10 μmol/L) for 72 h. Cells were harvested and melanin content was measured. Values represent mean ± SD. Results are representative of 2 different experiments (n = 3). $^{**}P < 0.01$, versus the control group (B).

Fig. 7 Effect of Iso on GSK3β phosphorylation, Akt expression, Akt phosphorylation (A), and stimulation of melanogenesis by PI3K inhibitor LY294002 in B16 melanoma cells (B). B16 melanoma cells were cultured in the presence of Iso (30 μmol/L) for 0, 3, 6, 12, 24, and 48 h. Cells were harvested and proteins were subjected to western blotting with antibodies against p-GSK3β, AKT and p-AKT. Band densities were quantified with ImageJ software. Values represent mean ± SD. Results are representative of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus the control of each group (A). B16 cells were cultured in the presence of PI3K inhibitor LY294002 (10 μmol/L) for 72 h. Cells were harvested and melanin content was measured. Values represent mean ± SD. Results are representative of 2 different experiments (n = 3). $^{**}P < 0.01$, versus the control group (B).

Figure 1.

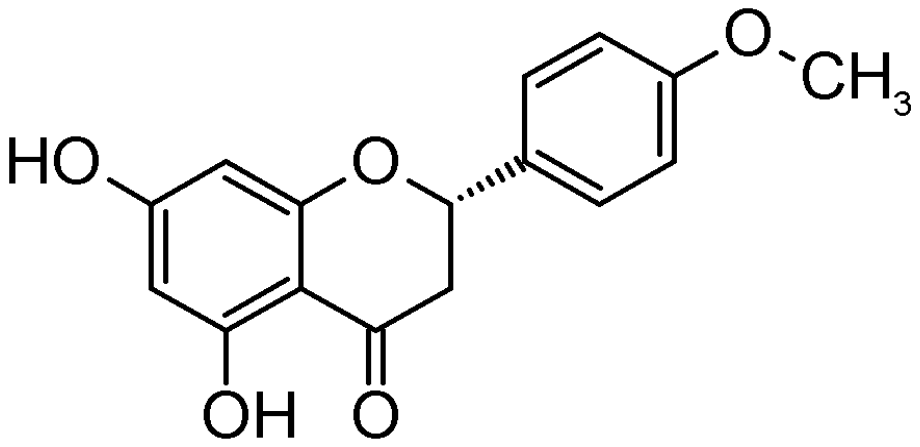
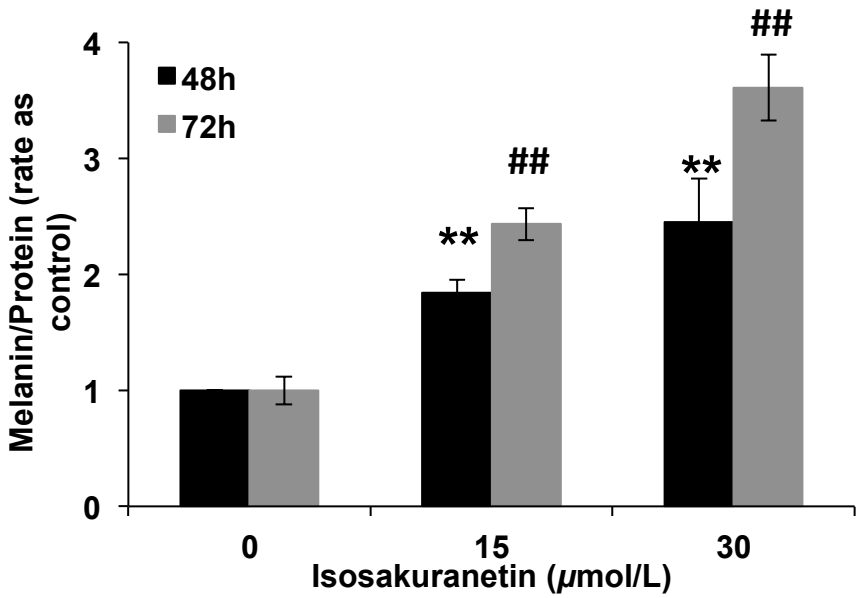


Figure 2.

A


4
3
2
1
0
48h
72h
Melanin/Protein (rate as control)
**
##
**
##
0
15
30
Isosakuranetin (μmol/L)

B

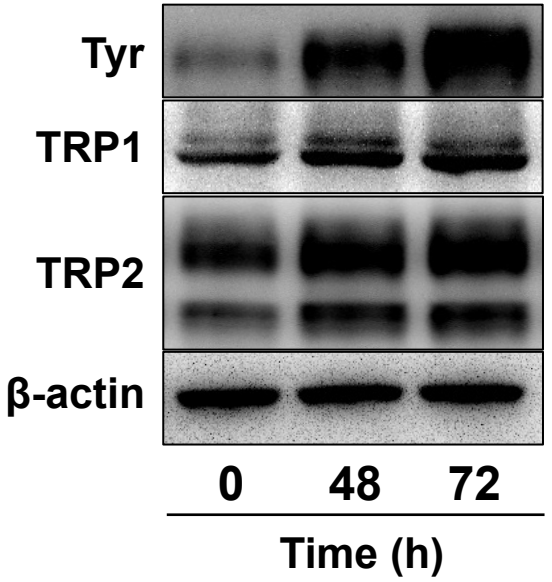
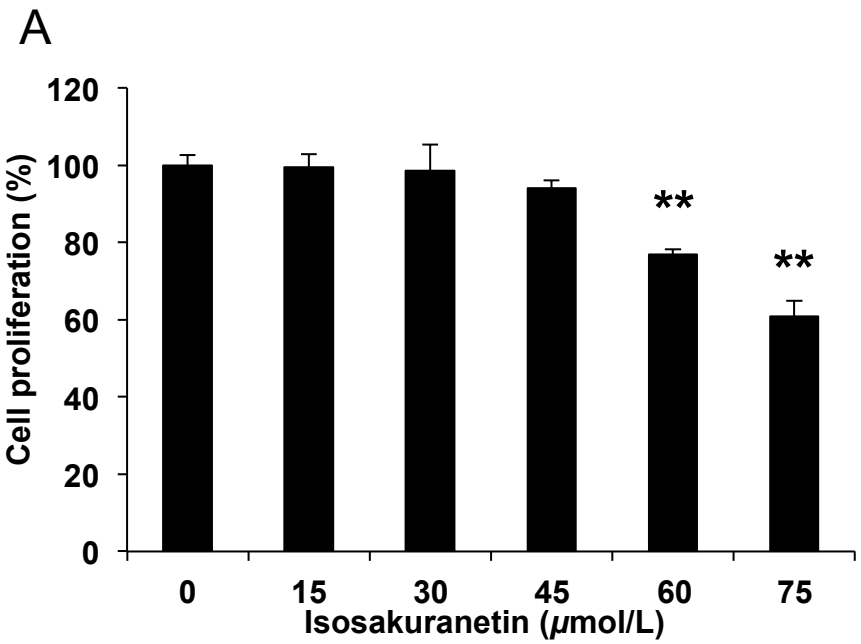

Tyr
TRP1
TRP2
β-actin
0
48
72
Time (h)

Figure 3.


A
120
100
80
60
40
20
0
Cell proliferation (%)
**
**
0
15
30
45
60
75
Isosakuranetin (μmol/L)

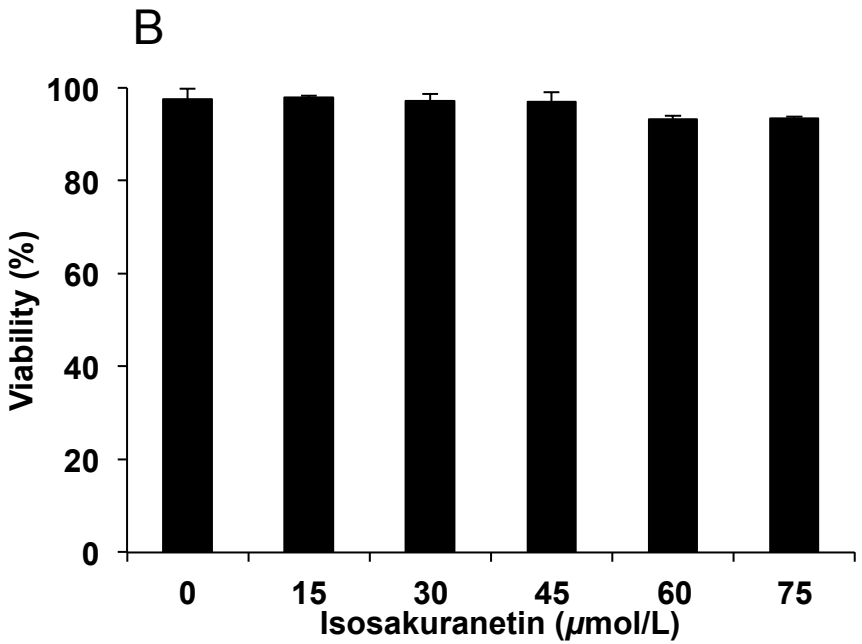

B
100
80
60
40
20
0
Viability (%)
0
15
30
45
60
75
Isosakuranetin (μmol/L)

Figure 4.

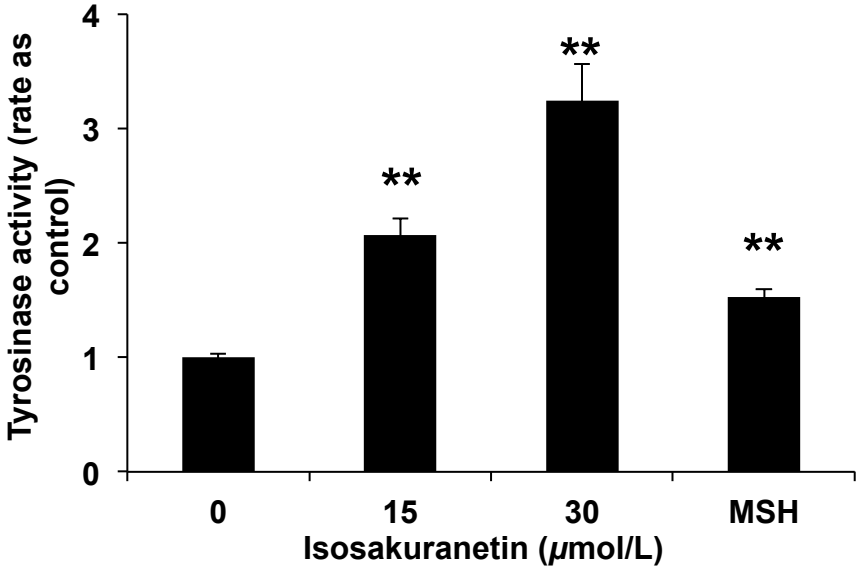
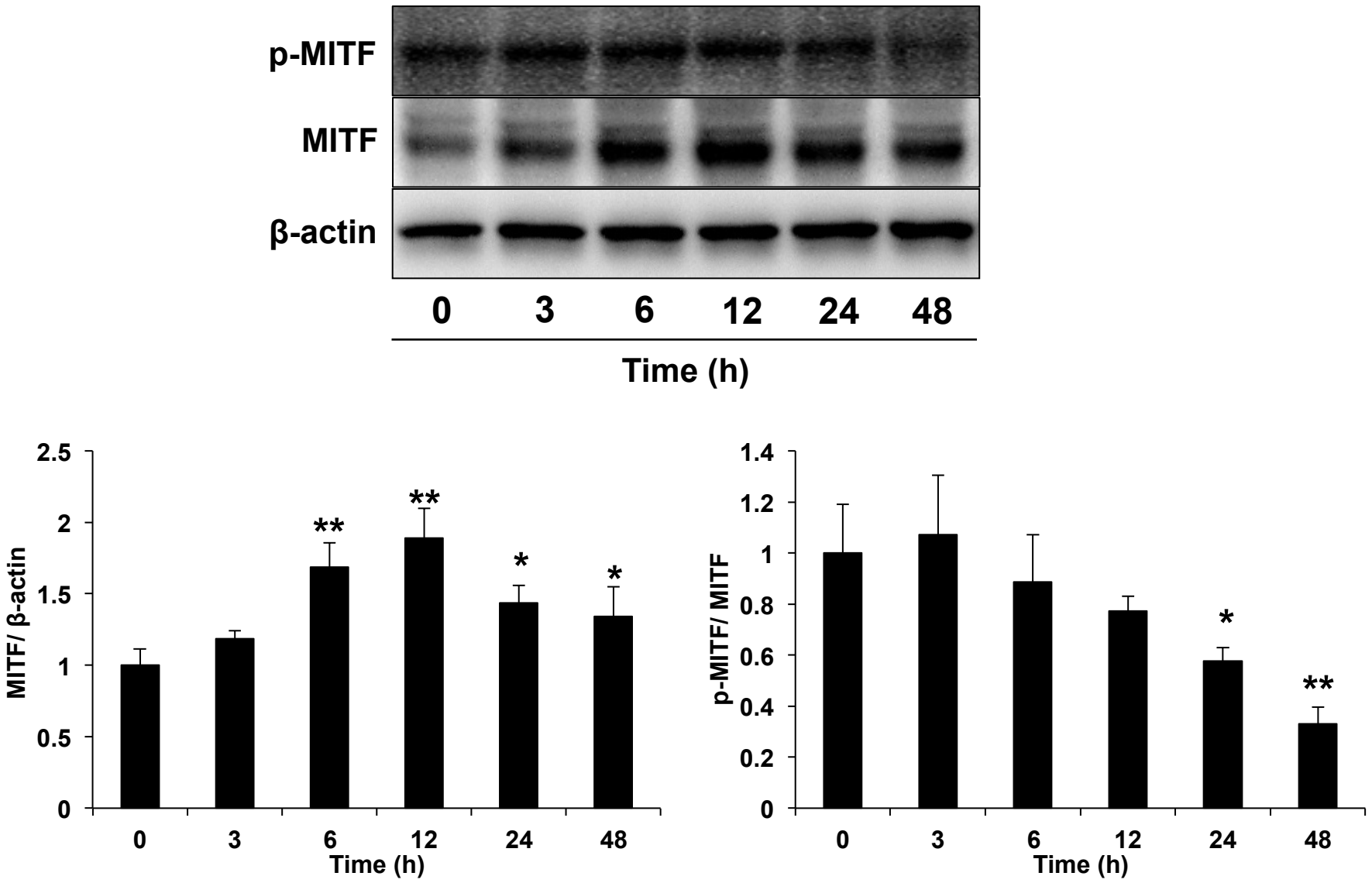

Tyrosinase activity (rate as control)
4
3
2
1
0
**
**
**
0
15
30
MSH
Isosakuranetin (μmol/L)

Figure 5.

A


p-MITF
MITF
β-actin
0
3
6
12
24
48
Time (h)
MITF/ β-actin
2.5
2
1.5
1
0.5
0
**
**
*
*
0
3
6
12
24
48
Time (h)
p-MITF/ MITF
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
0
3
6
12
24
48
Time (h)

B

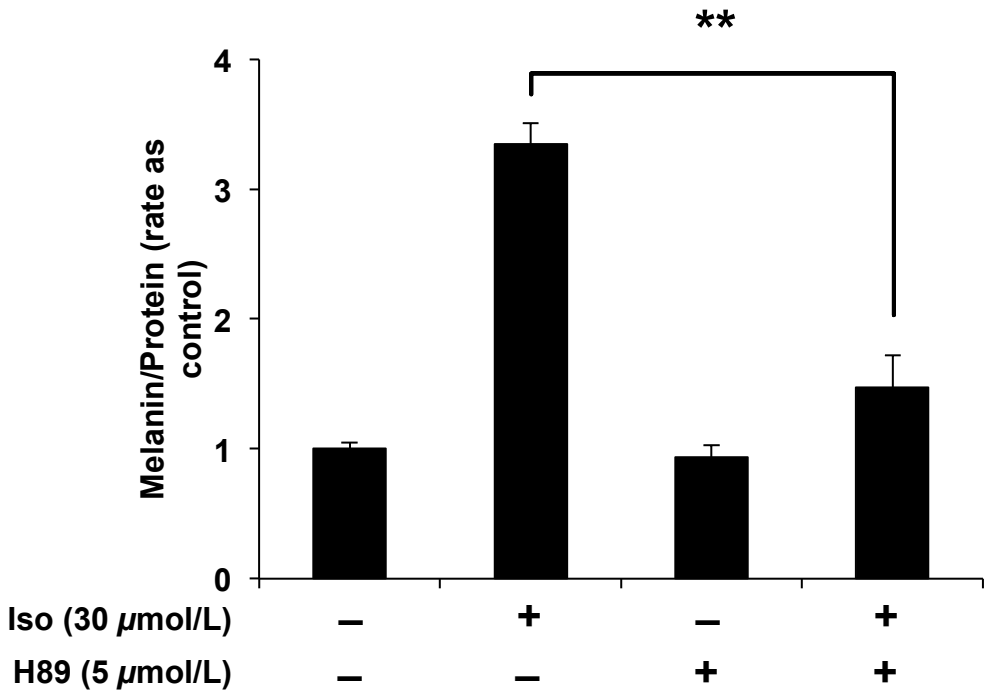
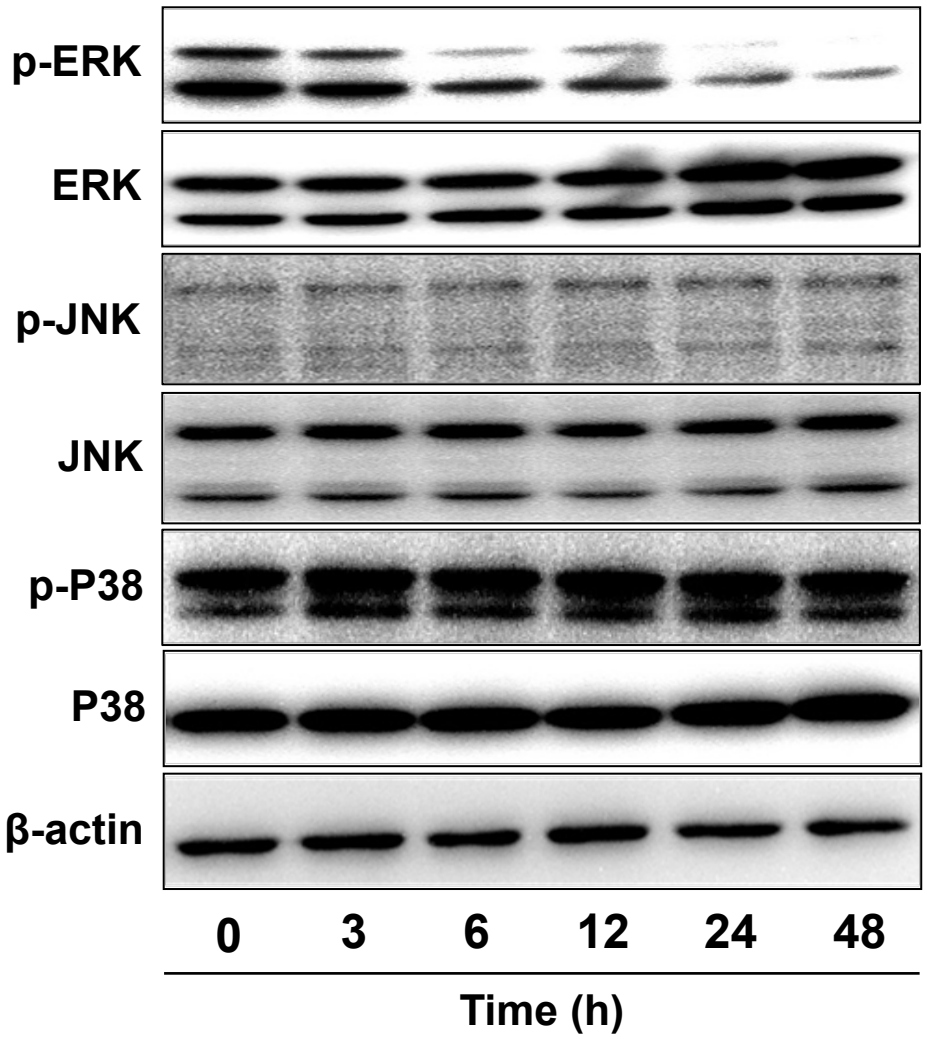
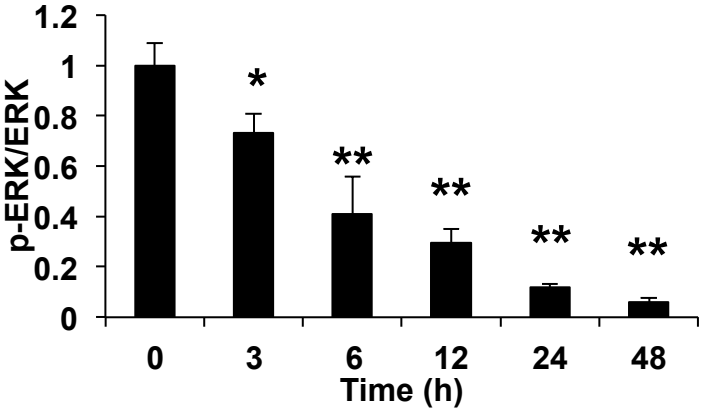
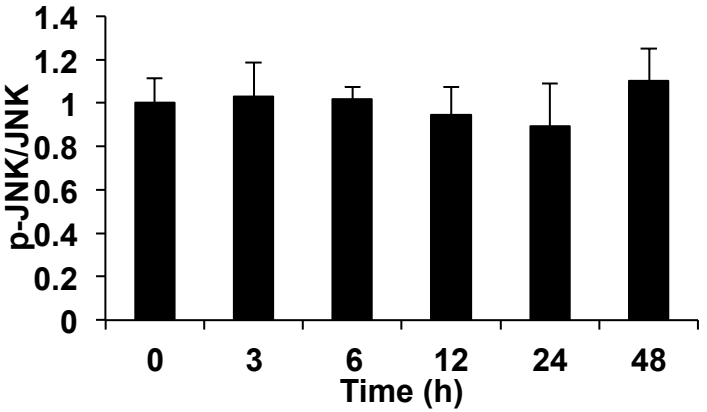

**
Melanin/Protein (rate as control)
4
3
2
1
0
Iso (30 μmol/L)
H89 (5 μmol/L)
–
+
–
+
–
–
+
+

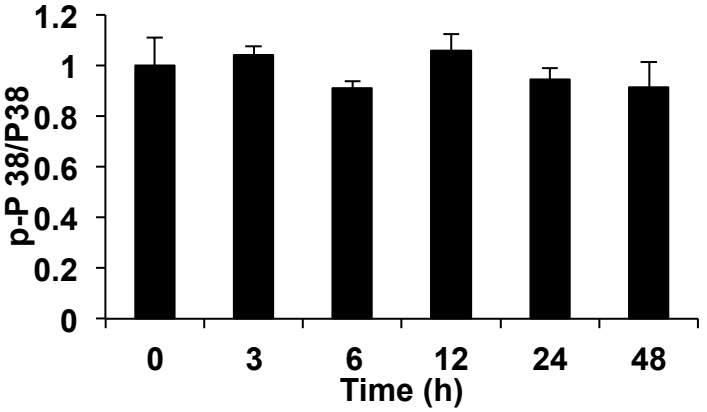
Figure 6.

A


p-ERK
ERK
p-JNK
JNK
p-P38
P38
β-actin
0
3
6
12
24
48
Time (h)


p-ERK/ERK
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
**
**
0
3
6
12
24
48
Time (h)


p-JNK/JNK
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
3
6
12
24
48
Time (h)


p-P 38/P38
1.2
1
0.8
0.6
0.4
0.2
0
0
3
6
12
24
48
Time (h)

B

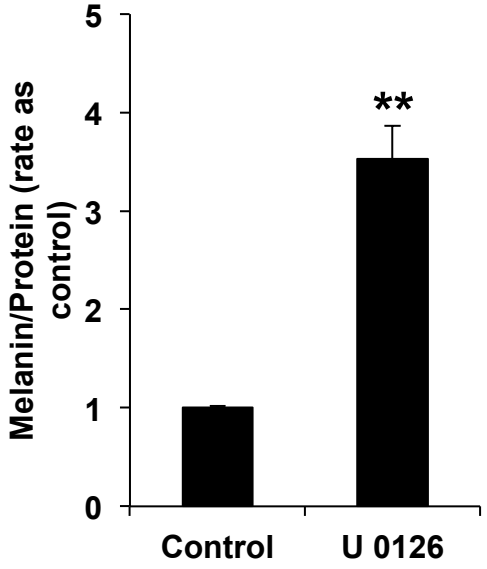
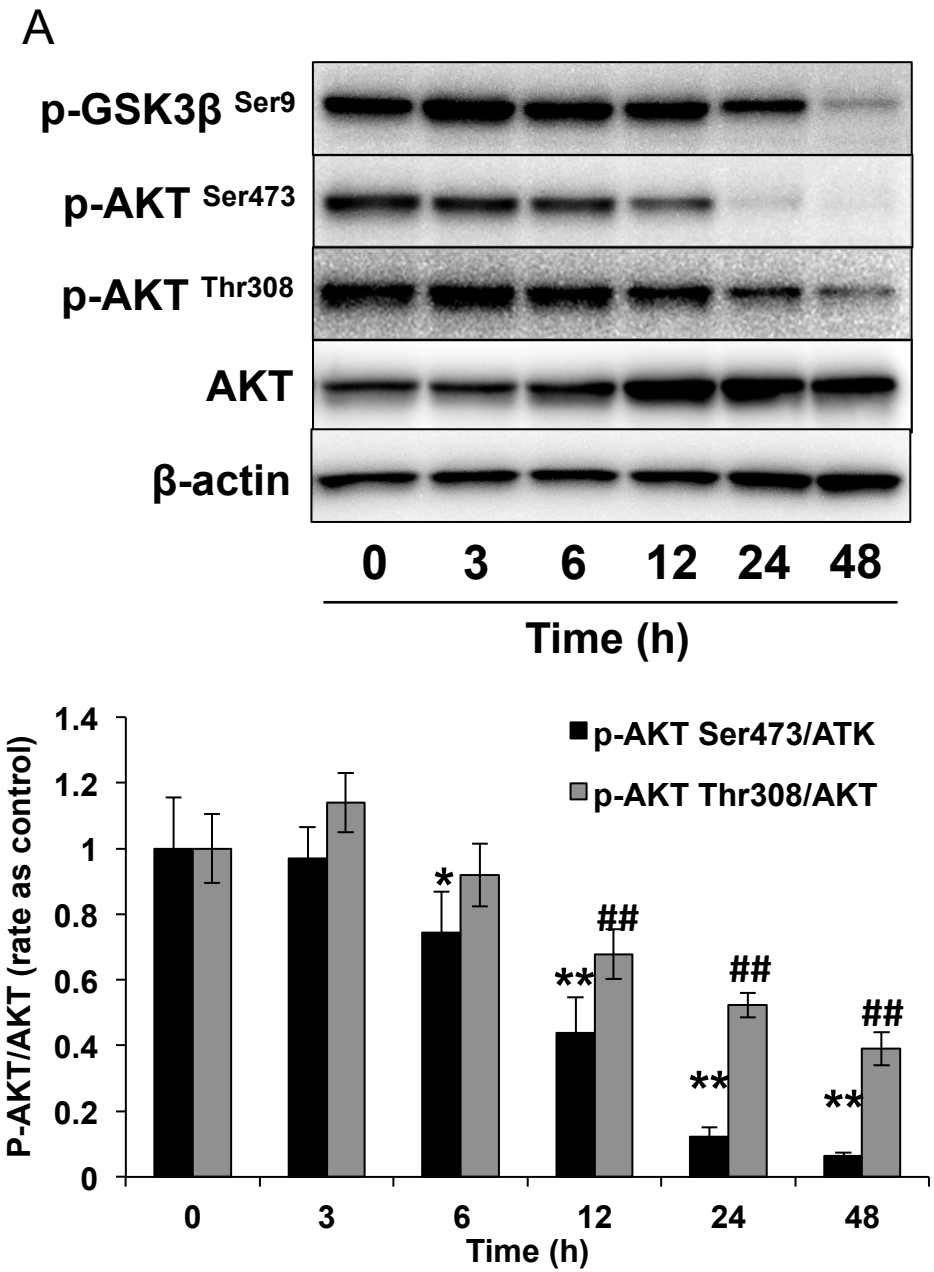


Figure 7.


A
p-GSK3β Ser9
p-AKT Ser473
p-AKT Thr308
AKT
β-actin
0 3 6 12 24 48
Time (h)
p-AKT Ser473/ATK
p-AKT Thr308/AKT
P-AKT/AKT (rate as control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
##
0 3 6 12 24 48
Time (h)
B
Melanin/Protein (rate as control)
5
4
3
2
1
0
**
Control
LY294002