Sulfuretin Inhibits Ultraviolet B-induced MMP Expression in Human Dermal Fibroblasts

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Sulfuretin is one of the main flavonoids produced by Rhusverniciflua. Sulfuretin has been shown to exhibit many pharmacological activities including anti-oxidant, anti-obesity, anti-inflammatory and anti-mutagenic activities. However, the anti-skin photoaging effects of sulfuretin has not yet been reported. In the present study, we investigated the inhibitory effect of sulfuretin on the expression levels of MMP-1 and -3 in the human dermal fibroblast cells. Western blot analysis and real-time PCR revealed sulfuretin inhibited UVB-induced MMP-1 and -3 expressions in a dose-dependent manner. UVB-induced MAPK/NF-κB/p50 activation and MMP expression were completely blocked by pretreatment of sulfuretin. Taken together, sulfuretin could prevent UVB-induced MMP expressions through inhibition of MAPK/NF-κB/p50 activation.

Key words : UVB, MMP, NF-kB, HDFs, Sulfuretin

Introduction

Skin change is one of the most prominent signs of aging. Skin aging can be divided into intrinsic or chronologic aging, which is the process of senescence that affects all body organs, and extrinsic aging (photoaging), which occurs as a consequence of exposure to environmental factors. One of themost important extrinsic aging factors is sunlight, particularly exposure to ultraviolet (UV)B irradiation, which causes skin photoaging. It has been well known that chronic exposure of human skin to UVB radiation results in photoaging and induces the production of matrix metalloproteinases (MMPs)[1]. In this study, we demonstrated that sulfuretin inhibits UVB-induced expression of MMP-1 and -3 in cultured human dermal fibroblasts. Our results also showed that sulfuretin blocked UVB-induced activation of NF-κB, which has an important role in MMP-1 and -3 expressions.

MMPs are responsible for the degradation or synthesis inhibition of collagenous extracellular matrix in connective tissues[2]. Collagen represents the main component of the extracellular matrix of dermal connective tissue, and its concentration decreases in chronoaging and photoaging. MMP-1 preferentially degrades fibrillar collagens, which maintain the tensile strength of fetal membranes, whereas MMP-3 degrades an extremely broad array of extracellular matrix (ECM) substrates and can activate the secreted, zymogenic form of other MMPs[3]. UVB is known to induce the expressions of interstitial collatinase (MMP-1) and stromelysin-1 (MMP-3) in human dermal fibroblasts (HDF) and normal human epidermis in vivo[4,5]. Among those MMPs, MMP-1 is the most important MMP in the degradation of the extracellular matrix by skin photoaging[6].

NF-κB is a crucial factor for the immunoinflammatory responses and is also implicated in various skin diseases including allergic dermatitis, psoriasis vulgaris, and skin cancer[7]. Hence, although NF-κB is involved in maintaining the skin homeostasis[8,9], excessive activation is pathogenic. NF-κB is initially located in the cytoplasm in an inactive form complexed with IκB, an inhibitory factor of NF-κB. Various inducers such as IL-1, TNF-α and UV can cause dissociation of this complex, presumably by phosphorylation of IκB, resulting in NF-κB being released from the complex. NF-κB then translocates to the nucleus, where it interacts with specific DNA recognition sites to mediate gene transcription.

Sulfuretin is a major flavonoid isolated from the heartwood of Rhus verniciflua, which has been used to reduce oxidative stress[10], platelet aggregation[11], inflammatory

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response[12], and mutagenesis[13]. A recent study has shown that sulfuretin inhibits the cytokine-induced β-cell damage and prevents streptozotocin-induced diabetes by suppressing the NF-κB pathway[14]. In the present study, we evaluated the preventive effects of sulfuretin on UVB-induced MMPs expression in HDF. Sulfuretin was found to block UVB-induced MAPK/NF-κB/p50 pathway, thereby inhibiting the MMPs expression. These results indicate that sulfuretin may be useful as an anti-skin photoaging agent.

Materials and Methods

1. Materials

Sulfuretin obtained from R&D chemicals. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and anti-β-actin were obtained from Sigma (St. Louis, MO. USA). Primary antibodies for MMP-1 and -3 were obtained from R&D Systems (Minneapolis, MN. USA). High glucose-containing Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were obtained from Gibco-BRL (Gaithersburg, ME. USA). Antibody against for p-p38 ,p-JNK and p-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody of p50, p65, and p-I κBα, PCNA, and Horseradish peroxidase (HRP)-conjuga-ted IgG was from Santa Cruz Biochemi-cals (SantaCruz, CA. USA).

2. Isolation and culture of HDF

HDF were aseptically isolated from a circumcised neonatal foreskin. The epidermis and dermis were separated by incubation in 0.9 units/ml dispase in medium for 16 hours at 4℃. After the epidermis and dermis were mechanically separated, the dermis was minced and attached on the surface of tissue culture flask and fed with DMEM containing 10% fetal bovine serum (FBS) for 1~2 weeks. The dermal fibroblasts spreading as radial outgrowth from attached pieces of dermis, cultured in DMEM with 10% FBS.

3. UV Irradiation

For a UVB irradiation, we used UVB cross-linker (6×8 W, 312 nm, Model CL-508M, Vilber lourmat, Paris, France). In brief, serum-starved confluent cells were rinsed twice with PBS, and all irradiations were performed under a thin layer of PBS. Immediately after irradiation, fresh serum-free medium was added to the cells. Responses were measured after an incubation period of 24 h. Mock-irradiated controls followed the same schedule of medium changes without UVB irradiation.

4. MTT assay for UV-induced cytotoxicity

A protective effect of sulfuretin against UV-induced cytotoxicity of HDFs was determined using a MTT assay. Briefly, cells were seeded to 3×10^4 cells/well, allowed to attach. After 24 h, cells were treated with various concentrations of sulfuretin (1, 5, 10 20, and 50 μM). After incubation for 24 h, cells were washed twice with PBS, and then MTT (0.5 mg/ml PBS) was added to each well. The plates were incubated at 37℃ for 30 min. Formazan crystals formed were dissolved by adding DMSO (100 μl/well) and then measured at 570 nm using a microplate reader (Model 3550, BIO-RAD, Richmond, CA. USA).

5. Trypan blue exclusion test for sulfuretin-induced cytotoxicity

Cells were seeded onto 10 cm dish and allowed to attach for 24 h. They were then treated with UVB at 25 mJ/ cm^2. After 24 h, they were detached by treatment of trypsin and, stained with trypan blue. Stained cells were counted under an optical microscope using a hemocytometer.

6. Western blot analysis

HDF (2×10^6 cells) were irradiated with UVB(25 mJ/cm^2) and then treated with sulfuretin for 24 h. Cells were lysed with 40 ml of ice-cold M-PER®Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL). In lysates, the protein concentration was determined using the Bradford method[15]. Samples were separated by SDS-PAGE with 10% acrylamide running and 3% acrylamide stacking gels, and then transferred to hybondTM-PVDF membranes using a Western blot apparatus. The PVDF membranes were blotted with 1 μg/ml of primary antibodies. HRP-conjugated IgG was used as a secondary antibody. The protein expression levels were then determined by analyzing the signals captured on the PVDF membranes using an image analyzer (Las-1000, fuji-film, Japan).

7. Quantitative real-time PCR assay

Total RNA was extracted from the cells using FastPureTM RNA Kit (TaKaRa, Shiga, Japan). The concentration and purity of RNA were determined by absorbance at 260/280 nm. cDNA was synthesized from 1 μg total RNA using PrimeScriptTM RT reagent Kit (TaKaRa, Shiga, Japan). The expressions of MMP-1 and -3 mRNA was determined by real-time-PCR using the ABI PRISM 7900 sequence detection system and the SYBRÒ Green (Applied Biosystems, Foster City, CA, USA). The primers were: MMP-1 (NM002424.2) sense, AGTGACTGGGAAACCAGATGCTGA; antisense, GCTCTTGGCAAATCTGGCCTGTAA and MMP-3

(NM002422) sense, ATTCCATGGAGCCAGGCTTTC; antisense, CATTTGGGTCAAACTCCAACTGTG and GAPDH (NM002046) sense, ATGGAAATCCCATCACCATCTT; antisense, CGCCCCACTTGATTTTGG. To control variationin mRNA concentr-ation, all results were normalized to the house keeping gene, GAPDH. Relative quantitation was performed using the comparative △△Ct method according to the manufacture' sinstructions.

8. Determination of MMP-1 and -3 secretions by ELISA

HDF were seeded in 100 mm culture dishes at density of 2×10^6 cells perdish, and then irradiated with UVB (25 mJ/cm^2) and then treated with sulfuretin. Following 24 h of incubation, the culture supernatant was collected and centrifuged at 10,000 × g for 5 min to remove the particulate matter, and stored at -80℃ in fresh tubes. The active MMP-1 in culture supernatants were quantified by fluorescent assay, using the Fluorokine E Human Active MMP-1 Fluorescent Assay Kit (R&D Systems) and MMP-3 in the cell culture supernatants were then determined using QuantikineELISA kits (R&D Systems), according to the manufacturer's protocol.

9. Preparation of nuclear extract

HDF (2×10^6 cells) were irradiated with UVB (25 mJ/cm^2) and then treated with sulfuretin for 24 h. Cells were immediately washed twice, scraped in to 1.5 ml of ice-cold PBS (pH 7.9), and then pelleted at 12,000 × g for 30s. Cytoplasmic and nuclear extracts were prepared from cells using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL).

10. Electrophoretic mobility shift assay (EMSA)

The activation of NF-κB was assayed by a gel mobility shift assay using nuclear extracts. An oligonucleotide containing the κ-chain binding site (κB, 5'-CCGGTTAACAGAGGGGGCTTTCGAG-3') was synthesized and used as a probe for the gel retardation assay. The two complementary strands were then annealed and labeled with [α-^{32}P]dCTP. Labeled oligonucleotides (10,000 cpm), 10 μg of nuclear extracts, and binding buffer (10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 10 0ngpoly (dI·dC), 1 mM DTT) were then incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixtures were analyzed by electrophoresison 4% polyacrylamide gels in 0.5X Tris-borate buffer, and the gels were then dried and examined by autoradiography. Specific binding was controlled by competition with a 50-fold excess of cold κB oligonucleotide.

11. Statistical analysis

Statistical analysis of the data was performed using ANOVA and Duncan's test. Differences with a $p<0.05$ were considered statistically significant.

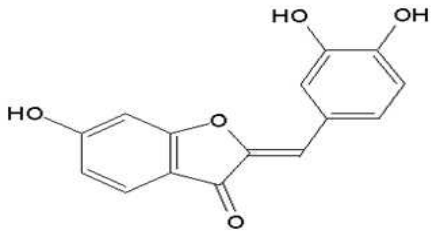

Fig. 1. Chemical structure of sulfuretin

Results

1. Effect of sulfuretin on cell viability of HDF

The structure of sulfuretin is shown in Fig. 1. In order to investigate the Cytotoxicity of sulfuretin on HDF, the cells were seeded into 96-well culture plates at a density of 1×10^5cells/well. Effect of sulfuretin on cellular toxicity of HDF was analyzed using the MTT assay. Treatment of HDF with indicated concentrations of sulfuretin for 24 h did not cause any significant change in cell viability(Fig. 2A). Therefore, we performed experiments in optimal non-toxic concentration (10 mM and 30 mM) of sulfuretin with no change in morphology. To investigate the cell viability of sulfuretin in the presence of UVB, Cells were incubated with the indicated concentration of sulfuretin for 24 h in the presence of UVB. Cell viability determined by cell counting. Sulfuretin (10 mM and 30 mM) significantly inhibited the cell toxicity by UVB irradiation(Fig. 2B).

2. Effect of sulfuretin on UVB-induced MMP-1 and -3 expressions in HDF

In this study, to investigate UVB-induced MMP-1 and -3 expressions, we performed Western blot analysis and real-time PCR in HDF. Treatment with sulfuretin (10 mM and 30 mM) completely blocked the up-regulation of MMP-1 and -3 induced by UVB irradiation(Fig. 3A). Real-time PCR revealed that UVB irradiation increased the level of MMP-1 and -3 in HDF and sulfuretin blocked UVB-induced up-regulation of MMP-1 and -3 in a dose-dependent manner(Fig. 3B). To determine the effect on UVB-induced MMP-1 and -3 secretions by sulfuretin, we used ELISA to investigate the effect of sulfuretin on UVB-induced MMP secretion. Irradiation of HDF with UVB (25 mJ/cm^2) resulted in an increase in the secretion

of MMP-1 and -3, respectively. However, sulfuretin (10 mM or 30 mM) significantly diminished the UVB-induced MMP-1 and -3 secretions, respectively(Fig. 3C). As shown, Sulfuretin itself had no effect on either MMP-1 or -3 in HDF. These results indicate that sulfuretin is a potent inhibitor of UVB-induced MMP-1 and -3 expressions in HDF.

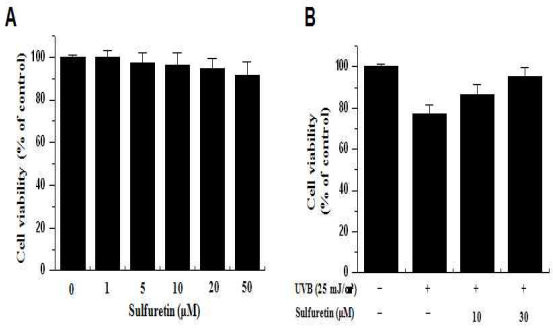

Fig. 2. Effect of sulfuretin on cell viability in HDF. Cells were cultured in 96-well plates until 70% confluence, then incubated with the indicated concentration of sulfuretin for 24 h. MTT assay was used to detect the viability of the cells as detailed in Materials and methods (A). Cells were cultured in 100 mm culture dishes until 70% confluence, then incubated with the indicated concentration of sulfuretin for 24 h in the presence of UVB. Cell viability determined by cell count method (B). The optical density value of control was regarded as 100%. Data points are the mean±SE of three independent experiments.

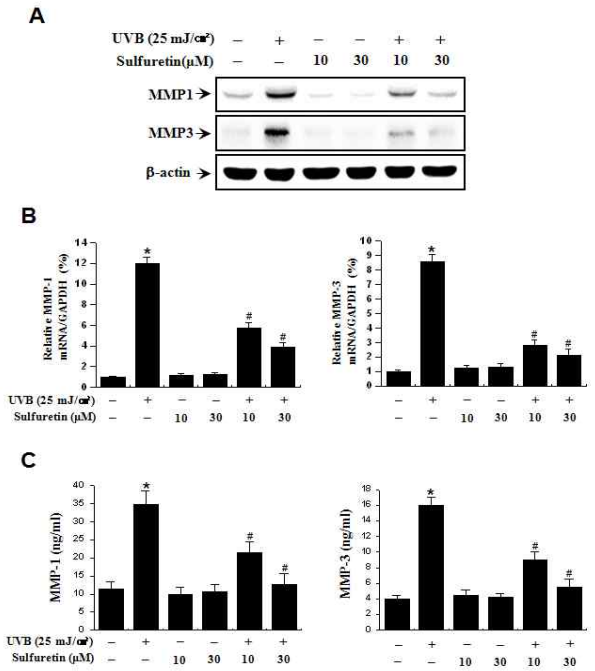

Fig. 3. Effect of sulfuretin on UVB-induced MMP-1 and MMP-3 expressions in HDF. To investigate effect of sulfuretin, The cell lysates were analyzed by Western blotting with anti-MMP-1 and-3. The blot was reprobed with anti-β-actin to confirm equal loading. Cells were stimulated with UVB (25 mJ/cm^2) and the indicated concentrations of sulfuretin for 24 h (A). Total cellular RNA was analyzed by real-time PCR for MMP-1 and -3 (B). Level of MMP-1 and -3 secretions in culture media was measured using a commercially available ELISA kit as described in Materials and Methods. Each value represents the mean±the SEM of three independent experiments. *p<0.01 vs. untreatedcontrol; #p<0.01 vs. UVB

3. MAPK signaling pathways are involved in the inhibition of UVB-induced MMP-1 and -3 expression and secretion by sulfuretin

The role of MAPKs (ERK, p 38 and JNK) in the activation of MMP-9 expression is well-known as upstream modulators of NF-κB[16,17]. An experiment was devised to elucidate which of the three signal transduction pathways was involved in UVB-induced MMP-9 expression and to investigate the inhibitory effect of MAPK on suppression of MMP-1 and -3 expression of sulfuretin in HDF. The effect of sulfuretin on UVB-induced phosphorylation of the MAPKs was investigated. sulfuretin displayed inhibitory effects on the phosphorylation of p 38 MAPK, 15 min after UVB treatment(Fig. 4). These results suggest that the specific inhibition of the p-38 pathway is directly involved in the regulation of UVB-induced MMPs expression by sulfuretin.

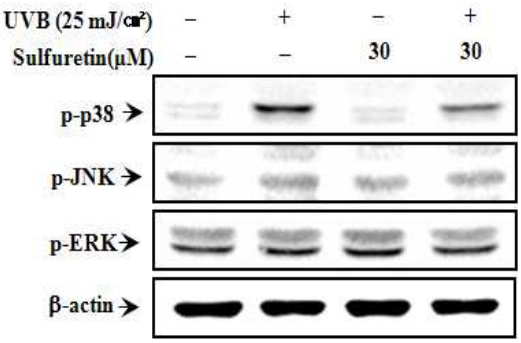

Fig. 4. Sulfuretin inhibits UVB-induced activation of MAPK signaling pathway in HDF. Cells were pretreated with UVB (25 mJ/cm^2) for 15 min in the presence or absence of sulfuretin. Cell lysates were prepared for Western blotting with specific p-p38, p-JNK and p-ERK antibodies

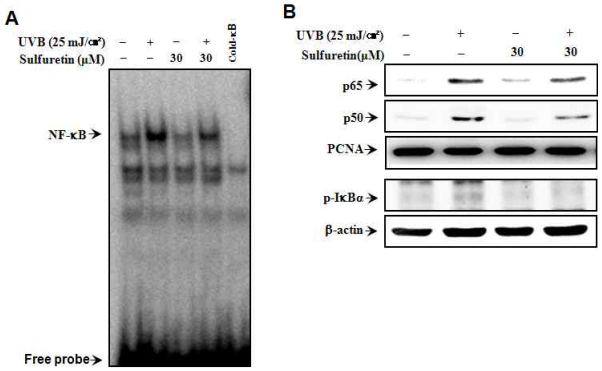

Fig. 5. Suppression of UVB-induced DNA binding of NF-κB, translocation of p65 and p50 to the nucleus, and IκBα phospholylation by sulfuretin. Cells were stimulated with UVB (25 mJ/cm^2) and the indicated concentrations of sulfuertin. Following 3 h of incubation, DNA binding of NF- κB was analyzed by electrophoretic mobility shift analysis (A), and the translocation of p65 and p50 to the nucleus and IκBα phospholylation the cytoplasm (B) were determined by Western blotting.

4. Suppressive effect of sulfuretin on UVB-induced NF-κB activation

We studied the effect of sulfuretin on UVB-stimulated translocation of NF-κB from the cytoplasmic compartment to the nucleus and on DNA binding in HDFs. UVB irradiation of HDFs increased binding activity of an NF-κB consensus sequence(Fig. 5A), and nuclear levels of p65 and p50 subunit (Fig. 5B) compared to those in control cells. The UVB-induced NF-κB activation was significantly suppressed by treatment with sulfuretin(Figs. 5A and 5B). To ascertain the observation that sulfuretin inhibits UVB-induced NF-kB activation, we determined p-IκBα levels. UVB irradiation of HDFs showed a decreased level of p-IκBα protein as compared to the levels in control cells. The decrease of p-IκBα levels was significantly suppressed by treatment with sulfuretin(Fig. 5B).

Discussion

In this study, we demonstrated that sulfuretin inhibits UVB-induced expression of MMP-1 and -3 in cultured human dermal fibroblasts. Our results also showed that sulfuretin blocked UVB-induced activation of NF-κB, which has an important role in MMP-1 and -3 expressions.

Skin aging can be attributed to extrinsic aging (photoaging) and intrinsic (chronological) aging. Photoaging concerns premature skin aging caused by repeated sun exposure[18,19]. UV irradiation of cultured HDF invitro or human skin *in vivo* induces the expression of MMPs which play important roles in the degradation of extracellular matrix components during skinaging[4,20,21]. Varani et al reported that with increasing age, MMP levels rise and collagen synthes is decline for sun-protected human skin in vivo[22]. Recently, it was suggested that excessive matrix degradation by UV-induced MMPs secreted by various cells (e.g., keratinocytes, fibroblasts, and inflammatory cells) contributes substantially to the connective tissue damage that occurs during photoaging[19,23,24]. The following mechanism of photoaging has been proposed. Initially, AP-1 and NF-κB are activated by UV light, and AP-1 and NF-κB driven MMPs such as MMP-1 and -3 are induced[23-25]. These MMPs then degrade collagen, which results in a collagen deficiency in photodamaged skin and eventually causes skin wrinkling[26].

The MAPK pathway is involved in the regulation of cell proliferation, apoptosis, cytokine expression and production of MMPs. The three major MAPK families, JNK, ERK, and p38 kinase, are expressed and the active phosphorylated forms can be detected in MCF-7 cells[27,28]. The present results suggest that DHAvD inhibits MAPK activation in TPA-mediated signaling pathways. MAPK signaling pathways are important for NF-κB activation, which requires I-κB kinase, phosphoinositide 3 kinase (PI3K)-Akt or p38 MAPK, depending on the cell type[29-31].

In this study, we observed an increase in the activation of MAPK/NF-κB in the UVB-irradiated HDF, and the ability of sulfuretin to protect against the UVB-induced MMPs expressions, suggesting that sulfuretin inhibits UVB-induced expression of MMPs by suppressing the MAPK/NF-κB pathway in HDF. It is well established that a nuclear transcription factor, NF-κB, is activated upon UV irradiation[6,32,33]. In previous study, it is also reported that UVB-mediated skin photoaging is prevented by suppression of NF-κB activation[34,35]. Thus, inhibition of the NF-κB activation pathway is important the UVB-mediated skin damage. In fact, NF-κB is known to increase MMP-1 in dermis[36,37]. NF-κB is a crucial factor for the immunoinflammatory responses and is also implicated in various skin diseases including allergic dermatitis, psoriasis vulgaris, and skin cancer[7]. Hence, although NF-κB is involved in maintaining the skin homeostasis[8,9], excessive activation is pathogenic. NF-κB is an inducible dimeric transcription factor that belongs to the Rel/ NF-κB family of transcription factors. NF-κB consists of two major polypeptides, p65 and p50[38]. NF-κB is initially located in the cytoplasm in an inactive form complexed with IκB, an inhibitory factor of NF-κB. Various inducers such as IL-1, TNF-α and UV can cause dissociation of this complex, presumably by phosphorylation of IκB, resulting in NF-κB being released from the complex. NF-κB then translocates to the nucleus, where it interacts with specific DNA recognition sites to mediate gene transcription. In this study, we observed an increase in the activation of NF-κB in the UVB-irradiated HDFs, and the ability of sulfuretin to protect against the UVB-induced MMPs expressions, suggesting that sulfuretin inhibits UVB-induced expression of MMPs by suppressing the NF-κB/p50 pathway in HDFs.

In conclusion, the development of MMP inhibitors is considered to be a promising strategy for skin cancer therapy and photoaging. In recent years, the development of a compound with MMP inhibition activities from natural plants has received a great deal of attention. This study demonstrates the inhibitory effect of sulfuretin on the MMPs expression via mRNA assay. Also, our results have demonstrated that sulfuretin is a potent inhibitor of UVB-induced MMPs expressions and blocks strongly the ability of MAPK/NF-κB signaling pathway in HDF. The dose of sulfuretin that can inhibit UVB-induced MMPs expressions are below the toxicity limit. Therefore, we suggest that sulfuretin should be viewed as a potential therapeutic candidate for preventing and treating skin photoaging.

Acknowledgements

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