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FULL LENGTH ARTICLE

Sulfuretin exerts diversified functions in the processing of amyloid precursor protein

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Abstract Sulfuretin is a flavonoid that protects cell from damage induced by reactive oxygen species and inflammation. In this study, we investigated the role of sulfuretin in the processing of amyloid precursor protein (APP), in association with the two catalytic enzymes the α-secretase a disintegrin and metalloproteinase (ADAM10), and the beta-site APP cleaving enzyme 1 (BACE1) that play important roles in the generation of β amyloid protein (Aβ) in Alzheimer's disease (AD). We found that sulfuretin increased the levels of the immature but not the mature form of ADAM10 protein. The enhanced ADAM10 transcription by sulfuretin was mediated by the nucleotides −444 to −300 in the promoter region, and was attenuated by silencing or mutation of transcription factor retinoid X receptor (RXR) and by GW6471, a specific inhibitor of peroxisome proliferator-activated receptor α (PPAR-α). We further found that sulfuretin preferentially increased protein levels of the immature form of APP (im-APP) but significantly reduced those of BACE1, sAPPβ and β-CTF, whereas Aβ1-42 levels were slightly increased. Finally, the effect of sulfuretin on BACE1 and im-APP was selectively attenuated by the translation inhibitor cycloheximide and by lysosomal inhibitor chloroquine, respectively. Taken together, (1) RXR/PPAR-α signaling was involved in sulfuretin-mediated ADAM10 transcription. (2) Alteration of Aβ protein level by sulfuretin was not consistent with that of ADAM10 and BACE1 protein levels, but was consistent with the elevated level of im-APP protein, suggesting that im-APP, an isoform mainly localized to trans-Golgi network, plays an important role in Aβ generation.

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Introduction

Amyloid precursor protein (APP) is the primary source of β amyloid protein (Aβ) generation, which plays a key role in Alzheimer's disease (AD).[1] The proteolytic processing of APP is balanced by the amyloidogenic and non-amyloidogenic pathways.[2] In amyloidogenic pathway, APP can be sequentially cleaved by beta-site APP cleaving enzyme 1 (BACE1) and γ-secretase, leading to the increased levels of β—COOH—terminal fragment (β-CTF), soluble APPβ (sAPPβ) and Aβ. On the contrary, the non-amyloidogenic pathway is promoted by a disintegrin and metalloproteinase 10 (ADAM10), which results in the increased levels of α-CTF, soluble APPα (sAPPα) and thus precludes Aβ generation.[3,4] Evidences have suggested that pharmacological inhibition of BACE1 or enhancement of ADAM10 activity alleviates Aβ deposition, thus represents therapeutic strategy for AD.[5,6] In addition, APP may function as a cell surface receptor and signaling protein in the pathogenesis of AD,[7] highlighting the importance of APP in the development of therapeutic approaches.

Sulfuretin (3′, 4′, 6-trihydroxyaurone) is a compound extracted from Rhus verniciflua stokes.[8] It belongs to a member of flavonoids[9] that are shown to reverse age-related cognitive declines through a variety of molecular and network mechanisms.[10] In addition to inhibiting the platelet aggregation[11] and preventing tumor invasion,[12] sulfuretin reduces palmitate-induced reactive oxygen species (ROS) in hepatic cells[13] and inhibits LPS-induced inflammatory responses in murine macrophage cells.[14] In neuronal cells, sulfuretin protects against Aβ-induced neurotoxicity and drug-induced cell death.[15,16] It is reported that ROS and neuroinflammation contribute to the pathogenesis of AD including the altered APP processing.[17] These observations lead to the hypothesis that sulfuretin might also regulate APP processing mainly by affecting the activity of ADAM10 or BACE1.

We have previously performed high-throughput small-molecule screening in SH-SY5Y cells that stably expressed ADAM10 promoter sequence fused with a luciferase reporter gene. Among the positive hits, the histone deacetylase inhibitor apicidin and a flavonoid cosmosiin were found to promote ADAM10 transcription and translation, respectively[18,19]; and sulfuretin also enhanced the ADAM10-luciferase activity. In the present study, we found that sulfuretin increased ADAM10 transcription leading to an augmentation of the immature form of ADAM10 protein levels. Sulfuretin decreased the protein levels of BACE1 but dramatically increased those of the immature form of APP, with the concomitant increase of Aβ1-42 levels. The potential mechanisms were also investigated, respectively.

Results

Sulfuretin up-regulated ADAM10 expression in multiple cell lines

To determine whether sulfuretin may affect the expression of ADAM10, we first assessed ADAM10 protein levels in SH-SY5Y cells treated with different concentrations of sulfuretin (1, 5, 10, 20, 40 μM) for 24 h. Western blots of ADAM10 exhibited two bands near 80 KD and 60 KD, which were considered as immature and mature forms of ADAM10, respectively.[19,20] As shown in Fig. 1A and B, sulfuretin starting at 5 μM significantly increased the immature form of ADAM10 protein (im-ADAM10) levels in SH-SY5Y cells without altering the mature form of ADAM10 (m-ADAM10) protein levels. Similar to SH-SY5Y cells, HEK-293 and HT22 cells also showed an increased level of im-ADAM10, while the m-ADAM10 levels were not altered (Fig. 1C—F). Time-course experiments showed that the enhanced expression of im-ADAM10 protein levels mediated by sulfuretin (20 μM) began at 12 h and lasted for up to 48 h in SH-SY5Y cells (Fig. 1G and H). The effect of sulfuretin on ADAM10 protein levels was further confirmed by immunofluorescent study. As shown in Fig. 1I and J, an increased immunofluorescence intensity of ADAM10 was seen in sulfuretin-treated SH-SY5Y cells. The effect of sulfuretin on im-ADAM10 expression seemed to be mediated by transcription, as ADAM10 mRNA was significantly increased in cells treated with sulfuretin (Fig. 1K). As a positive control, similar augmentation of ADAM10 mRNA was also seen in apicidin-treated cells (Fig. 1K). We further showed that sulfuretin at 20 and 40 μM did not affect the viability of HT22 and SH-SY5Y cells (Fig. 1L). Thus, 20 μM sulfuretin was chosen for next experiments.

Regulation of ADAM10 transcription by sulfuretin involved promoter sequence −444 to −300

The enhanced ADAM10 mRNA levels suggested that sulfuretin might promote ADAM10 transcription. Thus, we assessed the effect of sulfuretin on ADAM10 protein level in the presence of transcription inhibitor ActD (0.1 μM) or translation inhibitor CHX (5 μM) in SH-SY5Y cells. As shown in Fig. 2A and B, although both ActD and CHX significantly decreased the basal level of ADAM10 protein levels, ActD but not CHX prevented sulfuretin-mediated enhancement of ADAM10 protein levels. To further identify the potential effecter sites of sulfuretin in ADAM10 promoter, we assessed the effect of sulfuretin on a hybrid ADAM10 promoter/firefly luciferase reporter gene stably expressed in

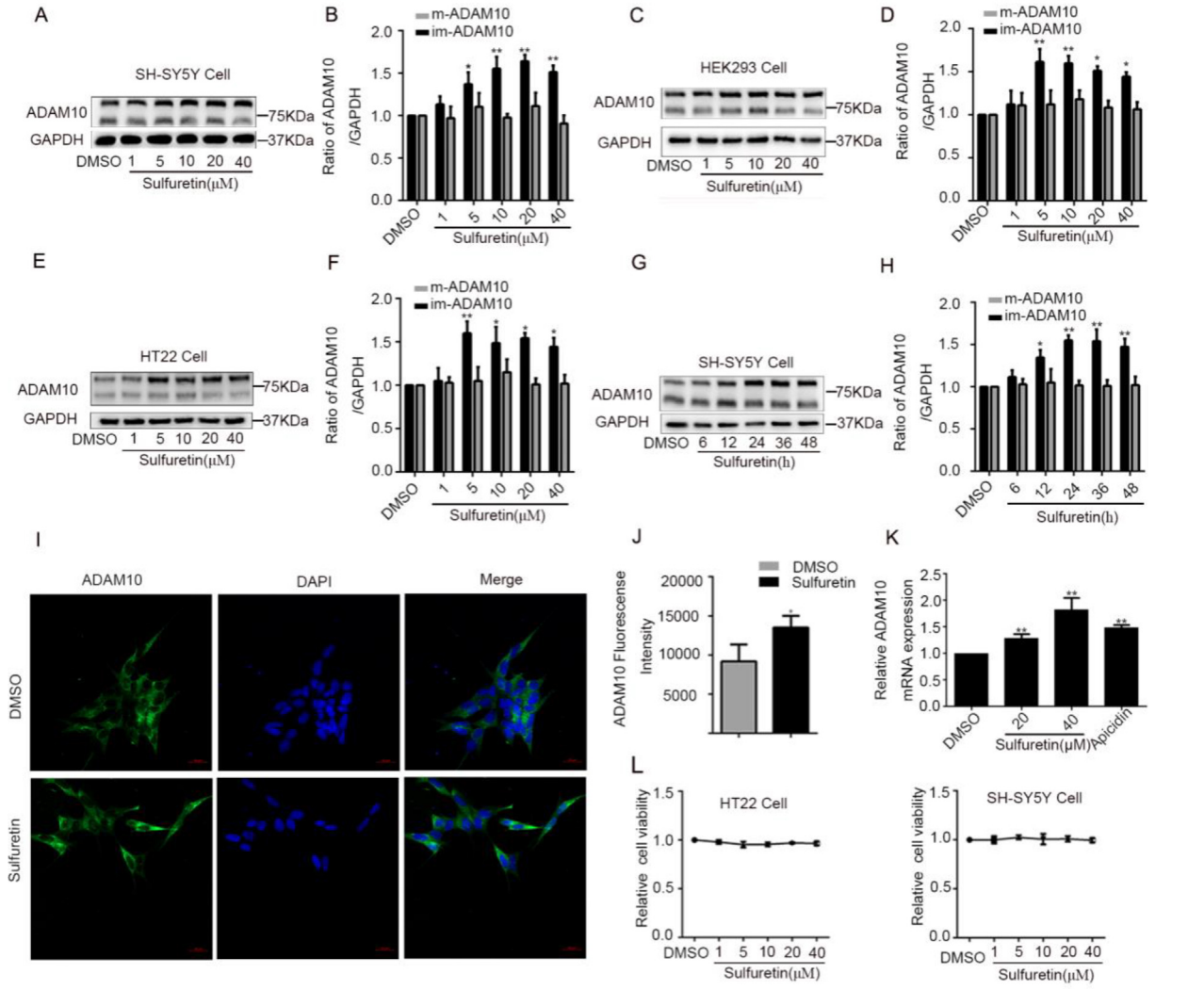

Figure 1 Sulfuretin enhances ADAM10 expression in multiple cell lines. **(A&B)** Representative Western blots **(A)** and bar plot summaries **(B)** of ADAM10 protein in SH-SY5Y cells treated with sulfuretin (1, 5, 10, 20 and 40 μM) for 24 h. The two bands in (A) near 80 KD and 60 KD represent the immature (im) and mature (m) forms of ADAM10 ($n = 3$). **(C—F)** Dose-response effect of sulfuretin on ADAM10 proteins in HEK293 (C&D) and HT22 cells (E&F) ($n = 3$) **(G&H)** Time-course effect of sulfuretin on ADAM10 protein. Representative Western blots (G) and bar plot summaries (H) of the m-/im-ADAM10 in SH-SY5Y cells treated with 20 μM sulfuretin for indicated times (6, 12, 24, 36 and 48 h, $n = 3$). **(I)** Immunofluorescent images show ADAM10 protein intensity in SH-SY5Y cells treated with sulfuretin at 20 μM for 24 h, the control cells were treated DMSO. Green: ADAM10; Blue: DAPI. **(J)** Quantification of immunofluorescence intensity of ADAM10 (green) in human SH-SY5Y cells. Scale bar, 20 μm. ($n = 30$). **(K)** Relative ADAM10 mRNA levels in SH-SY5Y cells incubated with sulfuretin (20 and 40 μM) and a positive control apicidin (0.25 μM) for 24 h ($n = 3$). **(L)** Relative cell viability of SH-SY5Y and HT22 cells treated with sulfuretin at indicated concentrations for 24 h ($n = 3$). Data are expressed as means ± SD, $^{*}P < 0.05$; $^{**}P < 0.01$.

SH-SY5Y cells. Different constructs with sequentially deleted 5′-flanking regions (−467/−30, −444/−30, −508/−300, −467/−300, and −444/−300) were generated (Fig. 2C). According to the JASPAR database (http://jaspar.binf.ku.dk), the corresponding transcription factors were also included (Fig. 2C). Among all the fragments tested, the luciferase construct (PGL-4.17-ADAM10-E2) that contains the minimal sequence −444/−300 was sufficient to show enhanced luciferase activity in cells treated with sulfuretin, indicating that this promoter region was critical (Fig. 2D).

Transcription factor RXR was involved in the regulation of ADAM10 by sulfuretin

It is reported that the −444 to −300 region of the ADAM10 promoter contains the following binding sites for transcription factors: upstream transcription factor 1 (USF1), specificity protein 1 (SP1) and retinoid X receptor (RXR).[21] To test whether these transcription factors might play a role in sulfuretin-mediated regulation of ADAM10, we assessed the effect of sulfuretin in cells transiently transfected with the small interfering RNA against SP1,

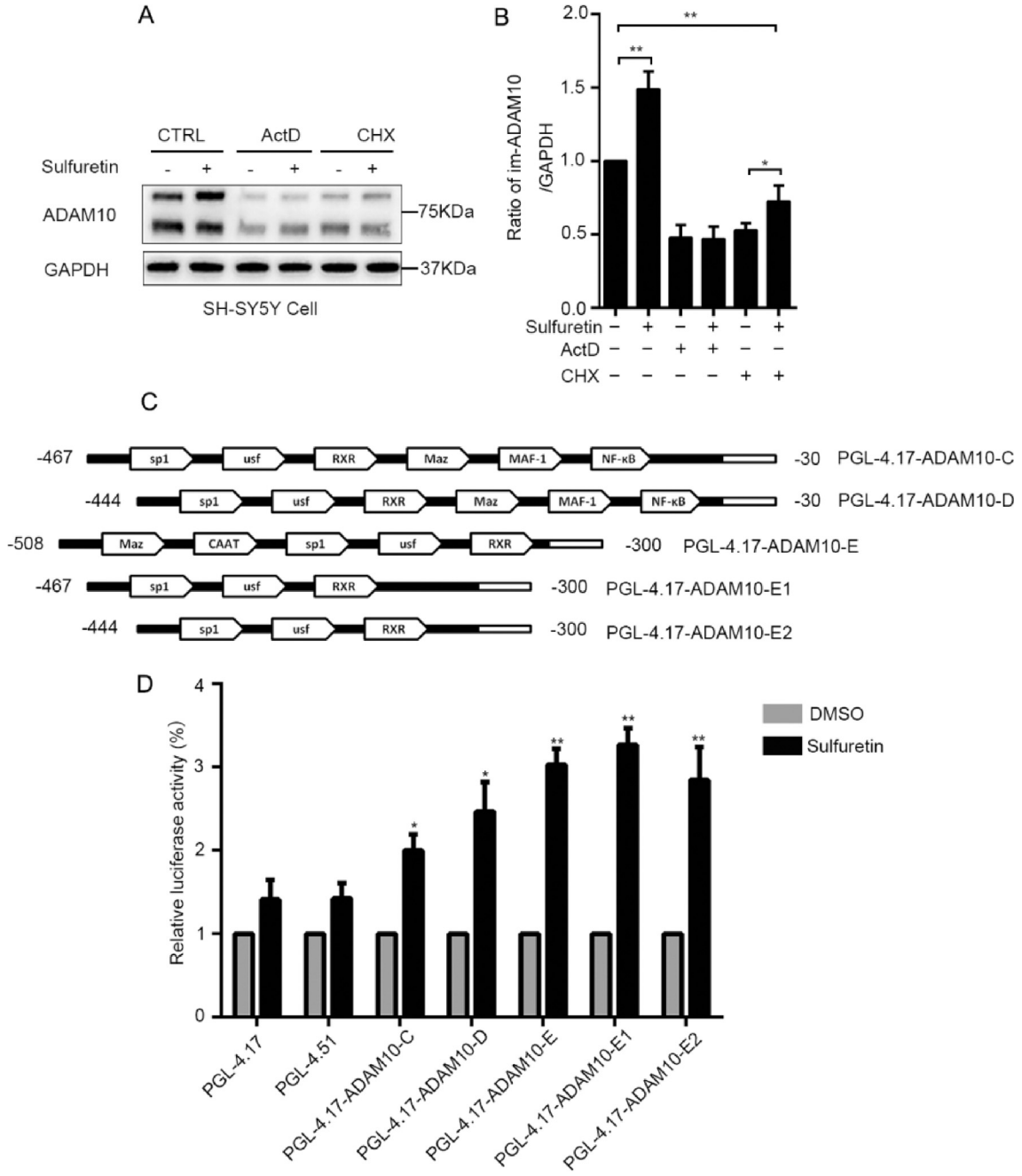

Figure 2 The nucleotides -444 to -300 in ADAM10 promoter are involved in the regulation of ADAM10 by sulfuretin. **(A&B)** Representative Western blots (A) and quantification (B) of ADAM10 protein in SH-SY5Y cells pretreated with transcription inhibitor actinomycin D (ActD, 0.1 μM) and translation inhibitor cycloheximide (CHX, 5 μM) for 1 h, then incubated with or without sulfuretin at 20 μM for 24 h. **(C)** The ADAM10 promoter was truncated into different fragments and subcloned into a pGL4.17 vector to construct luciferase reporter plasmids: pGL4.17-ADAM10-C, pGL4.17-ADAM10-D, pGL4.17-ADAM10-E, pGL4.17-ADAM10-E1 and pGL4.17-ADAM10-E2. Numbers indicate the relative positions relative to the start codon; individual transcription factor names in promoter fragments are shown in white boxes. **(D)** Relative luciferase activities in SH-SY5Y cells transiently transfected different pGL4.17ADAM10 plasmids for 24 h and were then treated with sulfuretin at 20 μM for 24 h. Luciferase activity was normalized to that of the internal control plasmid pGL4.17. Data are expressed as means ± SD, $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 3$–4).

USF1 or RXR, respectively. As shown in Fig. 3A and B, knockdown of SP1 did not affect basal level of ADAM10 protein, nor did it prevent sulfuretin-mediated enhancement of ADAM10 protein levels. Similarly, USF1 silencing slightly decreased the basal protein level of ADAM10, but failed to abolish sulfuretin-mediated enhancement of ADAM10 protein levels (Fig. 3C and D). Although RXR silencing also failed to alter basal ADAM10 protein levels, the effect of sulfuretin on ADAM10 was diminished (Fig. 3E and F). To further confirm that RXR response element was

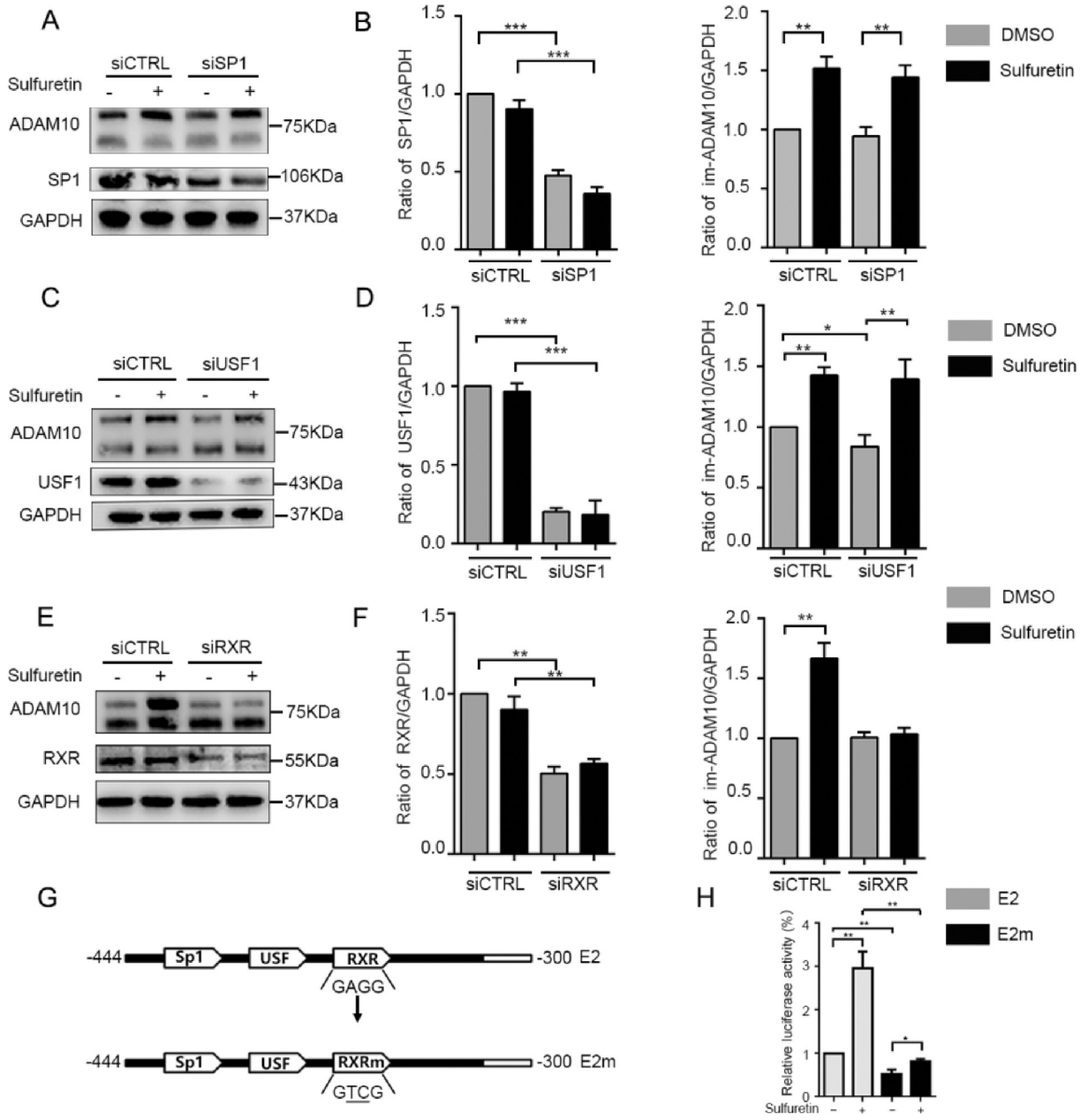

Figure 3 RXR knockdown abolishes sulfuretin-mediated enhancement of ADAM10 expression. **(A&B)** Representative Western blot images (A) and bar plot summary (B) in SH-SY5Y cells transiently transfected with SP1 siRNA (siSP1, 100 nM) or control siRNA (siCTRL) for further 24 h, in the absence or presence of sulfuretin at 20 μM for 24 h. **(C&D)** Representative Western blot images (C) and bar plot summary (D) of ADAM10 in SH-SY5Y cells transiently transfected with USF1 siRNA (siUSF1, 100 nM) and siCTRL for 24 h, in the absence or presence of sulfuretin at 20 μM for 24 h. **(E&F)** Representative Western blot images (E) and bar plot summary (F) in SH-SY5Ycells transiently transfected with RXR siRNA (100 nM) or siCTRL for 24 h, in the absence or presence of sulfuretin at 20 μM for 24 h. $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 3$). **(G)** Diagram shows the site-directed mutagenesis of RXR (−302) in pGL4.17-ADAM10-E2. **(H)** Relative luciferase activities in SH-SY5Y cells transiently transfected E2 or E2m plasmids for 24 h and were then treated with DMSO or sulfuretin at 20 μM for 24 h. Luciferase activity was normalized to E2 treated with DMSO. Data are expressed as means ± SD, $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 3$).

involved in the regulation of ADAM10 transcription by sulfuretin, we measured the luciferase activity using pGL4.17-ADAM10-E2 construct, in which the GAGG sequence of RXR element was mutated to GTCG, resulting in E2m (mutated) construct relative to E2 (wild-type) (Fig. 3G).[21] Compared with E2, E2m showed significantly reduced luciferase activity by ~50%, suggesting that RXR controlled ADAM10 expression. Whereas sulfuretin increased the luciferase activity by ~3-fold in E2, an increase by ~1.5 fold was found in E2m (Fig. 3H), indicating that RXR mutation significantly attenuated sulfuretin-mediated augmentation of ADAM10 expression.

PPAR-α inhibitor attenuated the effect of sulfuretin on ADAM10

It is reported that sulfuretin promotes the activity of ERK and PI3K,[22] which also regulates ADAM10 protein levels.[18,19] To determine whether these signaling pathways might be involved in sulfuretin regulation of ADAM10, we assessed the effect of sulfuretin in cells treated with PI3K inhibitors LY294002 (10 μM) and 3-MA (50 μM), PKA inhibitor H89 (0.04 μM), ERK inhibitor U0126 (20 μM) and mTOR inhibitor rapamycin (RAPA, 5 μM). The concentrations of these inhibitors did not cause overt cellular toxicity.[23–27] As shown in Fig. 4A and B, although the selected inhibitors used alone caused a decreased level of ADAM10 protein to a varied extent, they did not prevent sulfuretin-mediated enhancement of ADAM10 protein levels. These results thus indicated that ERK/PI3K/PKA signaling was not involved in sulfuretin regulation of ADAM10.

The peroxisome proliferator-activated receptor-α (PPAR-α) is a transcription factor that interacts with RXR and regulates ADAM10 transcription.[28] Thus, we assessed the effect of sulfuretin on ADAM10 protein levels in cells treated with the selective PPAR-α inhibitor GW6471.[29,30] As shown in Fig. 4C and D, the PPAR-α inhibitor GW6471 did not alter the basal level of ADAM10 compared with control, but significantly inhibited the enhancement of ADAM10 by sulfuretin. Cell viability assay revealed that GW6471 at 12.5 μM for 24 h did not result in significant toxic effect in SH-SY5Y cells, whereas GW6471 at 25 and 50 μM was indeed toxic (Fig. 4E). These results suggested that PPAR-α might cooperate with RXR to mediate ADAM10 regulation by sulfuretin.

Sulfuretin also affected protein levels of APP, BACE1 and Aβ

We have established that RXR/PPARα signaling contributes to sulfuretin-mediated regulation of ADAM10 transcription. To better understand the effect of sulfuretin in AD-like pathology, we assessed the protein levels of APP and BACE1, with the associated catalytic products of APP including sAPPα/β, α/β-CTFs and Aβ.[31] APP has two isoforms: the mature (m-APP, N- and O-glycosylated, and tyrosyl-sulfated) and immature form (im-APP, N-glycosylated).[32] We found that sulfuretin preferentially increased protein levels of im-APP by two-folds without affecting those of m-APP in SH-SY5Y-APP cells (Fig. 5A and B). However, BACE1 protein levels were significant decreased by sulfuretin, which was accompanied by the

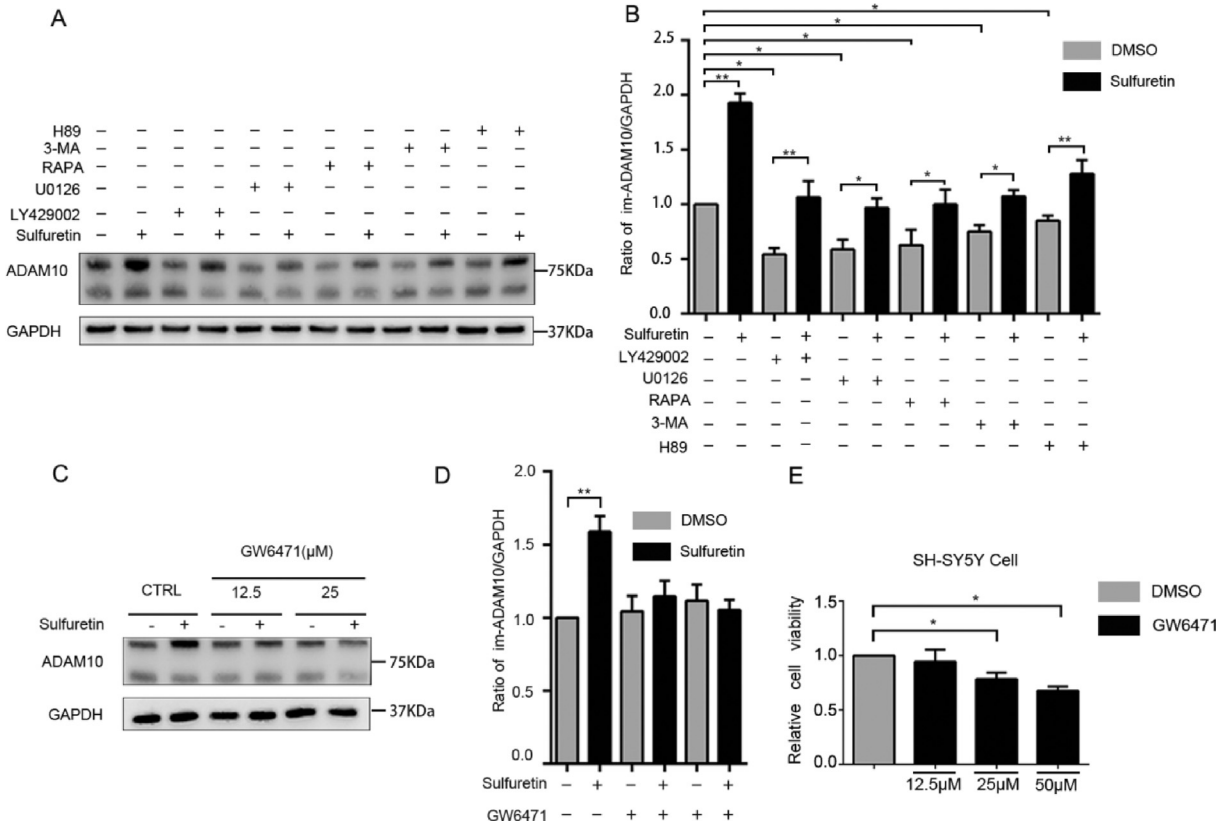

Figure 4 Sulfuretin-mediated enhancement of ADAM10 is prevented by PPAR-α inhibitor GW6471. **(A&B)** Representative Western blot images (A) and bar plot summary (B) of ADAM10 protein in SH-SY5Y cells pretreated with PI3K inhibitors LY294002 (10 μM), 3-MA (50 μM); ERK inhibitor U0126 (20 μM); PKA inhibitor H89 (0.04 μM) and mTOR inhibitor rapamycin (RAPA, 5 μM) for 1 h, in the absence and presence of sulfuretin (20 μM) for further 24 h. **(C&D)** Representative Western blot images (C) and bar plot summary (D) of ADAM10 protein levels in SH-SY5Y cells pretreated with PPAR-α inhibitor GW6471 at 12.5 and 25 μM for 1 h, in the absence or presence of sulfuretin (20 μM) for further 24 h. **(E)** Relative cell viability of SH-SY5Y cells treated with GW6471 at indicated concentrations for 24 h ($n = 3$). Data are expressed as means ± SD, $^*P < 0.05$; $^{**}P < 0.01$ ($n = 3–4$).

decreased level of sAPPβ and the increased level of sAPPα (Fig. 5A and B). We further assessed the effect of sulfuretin on α/β-CTFs in cells treated with γ-secretase inhibitor DAPT,[33] which is also supposed to inhibit the production of APP intracellular domain (AICD).[34] As shown in Fig. 5C—F, the Western blot bands of β-CTF probed by three antibodies (A8717, 6E10 and 4G8) were similarly around 17 KD. In comparison, α-CTF band that was presented between 11 and 14 KD could be clearly probed by A8717 and 4G8 but not 6E10 in our experimental conditions. While the levels of β-CTF was significantly reduced by sulfuretin, those of α-CTF were not altered (Fig. 5G). To further clarify the identity of α/β-CTFs, we compared the effect of sulfuretin in the presence of β-secretase inhibitor LY2811376 or α-secretase inhibitor GI254023X[35—38] in cells incubated with DAPT. As show in Fig. 5H—J, LY2811376 significantly reduced β-CTF levels whereas GI254023X abolished the generation of α-CTF. Interestingly, sulfuretin-mediated reduction of β-CTF levels was not blocked by LY2811376. We also found that although Aβ1-40 levels were not significantly altered, Aβ1-42 levels

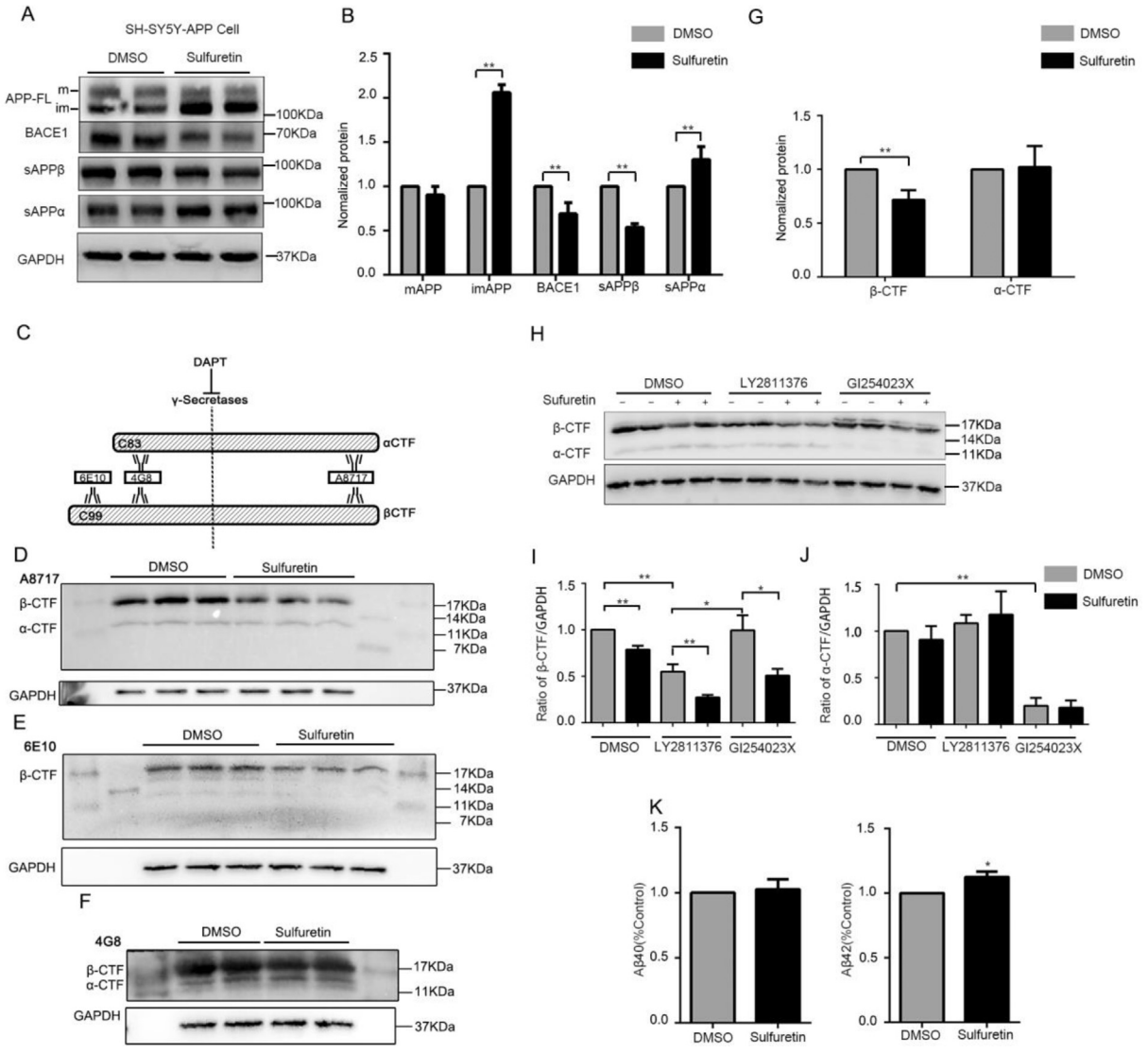

Figure 5 Sulfuretin regulates protein levels of APP, BACE1 and Aβ. **(A&B)** Representative Western blots (A) and quantification (B) of protein levels of mature and immature form of APP (m-APP and im-APP), BACE1 and sAPPα/β in SH-SY5Y-APP cells in the absence and presence of sulfuretin (20 μM) for 24 h. **(C)** Schematic diagram shows that CTFs could be probed by antibodies 6E10, 4G8 and A8717. γ-secretase activity can be inhibited by DAPT. **(D—F)** Representative Western blots of α- or β-CTF probed by A8717 (D), 6E10 (E) and 4G8 (F), with the molecular markers flanking on both sides. SH-SY5Y-APP cells were treated with DAPT (250 nM) in the presence and absence of sulfuretin (20 μM) for 24 h. **(G)** Quantification of α/β-CTF levels in samples relative to (D) using A8717 antibody. **(H—J)** Representative Western blots (H) and bar plot summaries (I&J) of CTF proteins in SH-SY5Y-APP cells pretreated with vehicle (DMSO), β-secretases inhibitor (LY2811376, 50 μM) or α-secretases inhibitor (GI254023X, 10 μM) in combination with DAPT (250 nM) for 1 h, then incubated with or without sulfuretin at 20 μM for 24 h. **(K)** Levels of Aβ1-40 and Aβ1-42 measured by enzyme-linked immunosorbent assay (ELISA) in SH-SY5Y-APP cells treated with or without sulfuretin at 20 μM for 36 h. Data are expressed as means ± SD, $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 3—4$).

were slightly but significantly increased in sulfuretin-treated cells (Fig. 5K). These results indicated that the function of sulfuretin in AD-like pathology was diversified. On the one hand, sulfuretin decreased BACE1 protein level with the concomitant reduction of sAPPβ and β-CTF; on the other hand, sulfuretin increased im-APP and Aβ protein levels, suggesting that sulfuretin might employ different mechanisms to regulate im-APP and BACE1.

Sulfuretin-mediated reduction of BACE1 protein was attenuated by translation inhibitor

BACE1 mRNA levels were not altered in sulfuretin treated cells (Fig. 6A), suggesting that post-transcriptional mechanisms may be involved in this regulation. We thus assessed the effect of sulfuretin on BACE1 protein in the presence of the ActD, CHX, the proteasome inhibitor MG132 and the lysosomal inhibitor chloroquine (CQ). As expected, the basal BACE1 protein levels were decreased in cells treated with ActD or CHX alone, but were increased in the presence of MG132 or CQ alone (Fig. 6B and C). However, the reduction of BACE1 protein by sulfuretin was selectively inhibited only by CHX (Fig. 6B and C). Further does-response analysis of CQ revealed that at concentrations ranging from 20 μM to 80 μM, CQ significantly increased the basal protein levels of BACE1. However, sulfuretin-mediated reduction of BACE1 protein levels was not attenuated by CQ at these concentrations (Fig. 6D and E). These results suggested that a translational mechanism might be involved in BACE1 regulation by sulfuretin.

Sulfuretin-mediated enhancement of im-APP protein level was prevented by CQ

As shown in Fig. 7A, APP mRNA levels were not altered in sulfuretin treated cells. To further identify the potential mechanisms that im-APP was increased by sulfuretin, we assessed the effect of sulfuretin in the presence of the transcription inhibitor ActD, the translation inhibitor CHX, the proteasome inhibitor MG132 and the lysosomal

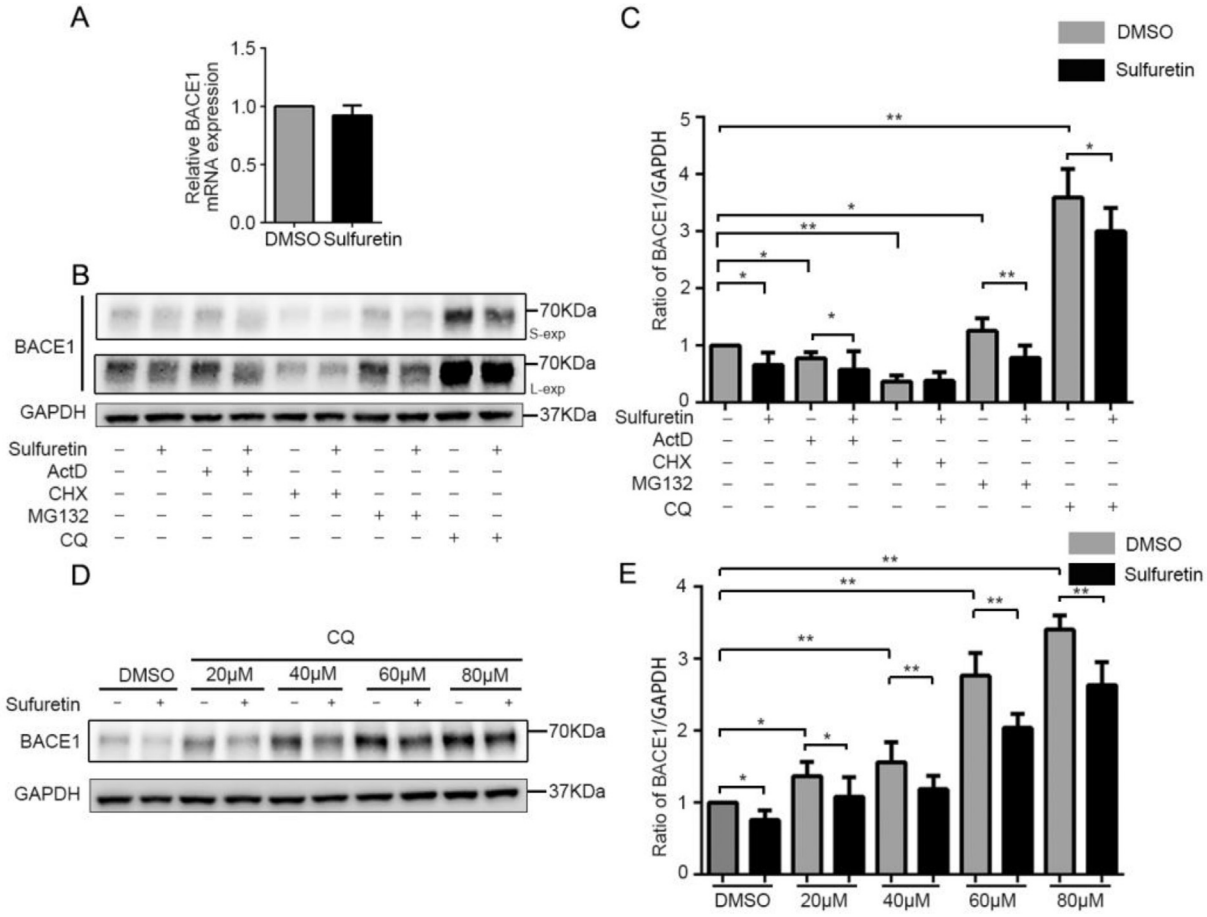

Figure 6 Sulfuretin-mediated regulation of BACE1 is attenuated by translation inhibitor. **(A)** The relative BACE1 mRNA levels in SH-SY5Y-APP cells treated with sulfuretin at 20 μM for 24 h. **(B&C)** Representative Western blots (B) and bar plot summary (C) of BACE1 in SH-SY5Y-APP cells that were pretreated with transcription inhibitor actinomycin D (ActD, 0.1 μM), translation inhibitor cycloheximide (CHX, 5 μM), proteasome inhibitor (MG132, 1 μM), and lysosome inhibitor (CQ, 60 μM) for 1 h, followed by further incubation in the absence or presence sulfuretin at 20 μM or vehicle DMSO (1:10000) for 24 h. BACE1 bands are shown with short (S-exp) and long exposure times (L-exp). **(D&E)** Representative Western blots (D) and quantification (E) of BACE1 protein in SH-SY5Y-APP cells that were pretreated with CQ (20, 40, 60 and 80 μM) for 1 h, and then incubated with or without sulfuretin at 20 μM for 24 h. Data are expressed as means ± SD, $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 3$).

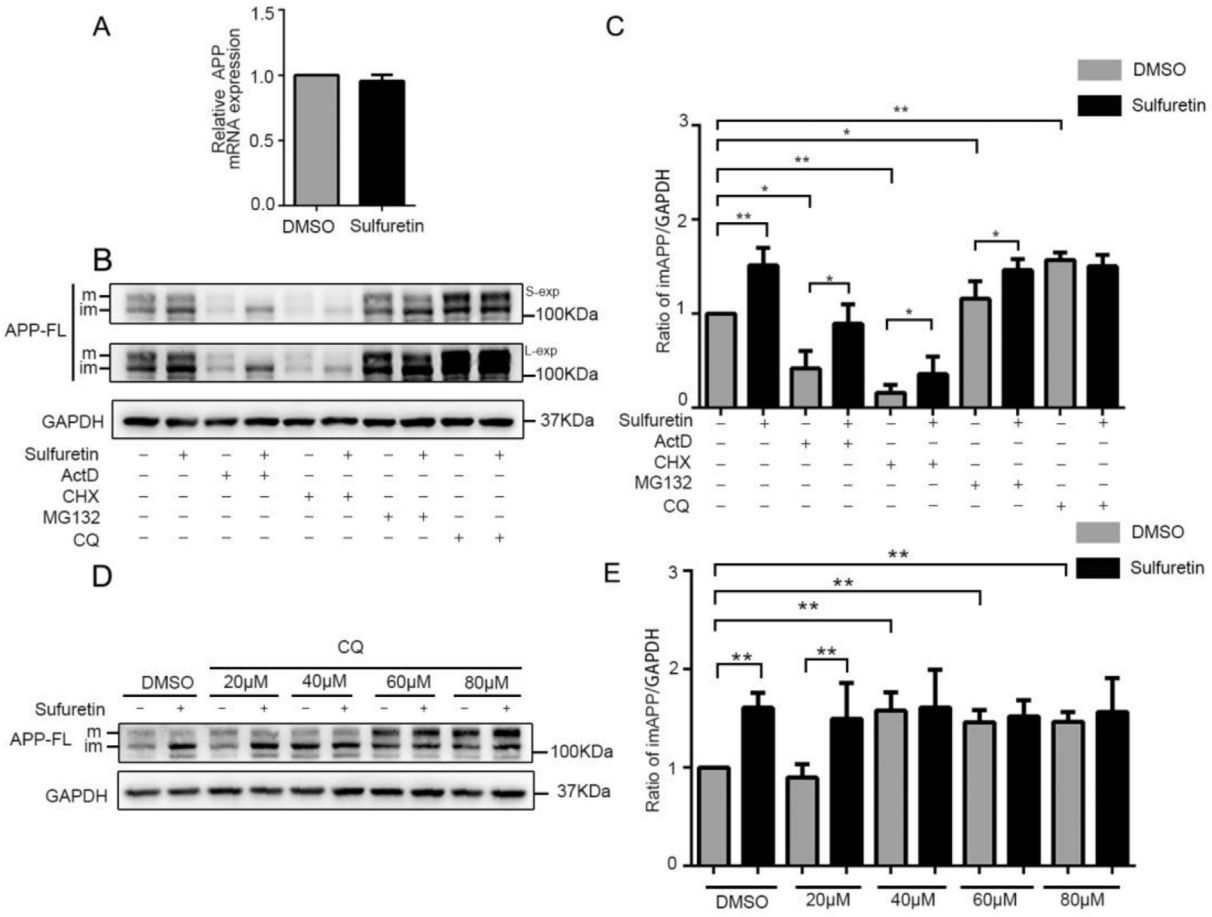

Figure 7 Sulfuretin-mediated enhancement of im-APP protein is abolished by lysosomal inhibitor chloroquine. **(A)** The relative APP mRNA levels in SH-SY5Y-APP cells treated with sulfuretin at 20 μM for 24 h. **(B&C)** Representative Western blots (B) and quantification (C) of im-APP in SH-SY5Y-APP cells that were pretreated with transcription inhibitor actinomycin D (ActD, 0.1 μM), translation inhibitor cycloheximide (CHX, 5 μM), proteasome inhibitor (MG132, 1 μM) or lysosome inhibitor chloroquine (CQ, 60 μM) for 1 h, in the absence and presence of 20 μM sulfuretin for further 24 h. APP bands are shown with short (S-exp) and long exposure times (L-exp). **(D&E)** Representative Western blot images (D) and bar plot summary (E) of im-APP isoforms in SH-SY5Y cells pretreated with CQ (20, 40, 60 and 80 μM) for 1 h, and then incubated with or without sulfuretin at 20 μM for 24 h. Data are expressed as means ± SD, $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 3-4$).

inhibitor CQ. The basal protein levels of im-APP were decreased by ActD and CHX and increased by MG132 and CQ as expected. However, only CQ significantly attenuated sulfuretin-mediated enhancement of im-APP (Fig. 7B and C). Does-response analysis revealed that CQ significantly increased the basal protein levels of im-APP at 40 μM and above, which further prevented the effect of sulfuretin on im-APP (Fig. 7D and E). These results suggested that lysosomal mechanism was involved in sulfuretin regulation of im-APP, which might account for the increased Aβ1-42 levels.

Discussion

The diversified function of sulfuretin in APP processing

The current study has revealed that the major function of sulfuretin in APP processing includes: (1) sulfuretin increases ADAM10 transcription via RXR/PPAR-α signaling; (2) sulfuretin reduces BACE1 protein levels with the concomitant reduction of β-CTF and sAPPβ levels; (3) sulfuretin increases im-APP and Aβ1-42 protein levels; (4) different mechanisms are involved in BACE1 and im-APP regulation by sulfuretin, as the reduction of BACE1 protein is inhibited by CHX and the enhancement of im-APP is prevented by CQ. These results indicate that the small molecule sulfuretin might have variety of cellular functions beyond its anti-oxidant activity.

Sulfuretin-mediated augmentation of ADAM10 involves RXR/PPAR-α signaling

Our study reveals that sulfuretin increases im-ADAM10 but not m-ADAM10 levels, suggesting sulfuretin preferentially influences ADAM10 transcription. Maturation of ADAM10 requires proprotein convertases (PCs) such as furin or PC7 to eliminate the inhibitory prodomain.[39] Enhanced ADAM10

transcription usually leads to increased im- and m-forms of ADAM10 proteins, as revealed by studies demonstrating that deletion of PPAR-α decreased and activation of transcription factors USF1 and RXR increased both forms of ADAM10, respectively.[18,28,40] Interestingly, overexpression of ADAM10 by plasmid transfection only leads to the predominant enhancement of im-ADAM10,[41] suggesting an intrinsic mechanism that might play a role in preventing ADAM10 maturation. It is worth noting that the im-ADAM10 might be functional, as missense mutations (Q170H and R181G) in the prodomain of ADAM10 enhances Aβ plaque load by shifting APP processing toward BACE1-mediated cleavage.[42]

The nucleotides −508 to −300 are located in the core promoter region of ADAM10 containing the recognition sites for SP1, USF1 and RXR.[21] SP1-mediated transcription is controlled by insulin and estrogen signaling,[43] USF1 that plays an important role in hepatic lipase regulation mediates ADAM10 transcription by apicidin.[18,44] It is reported that RXR is critical in mediating acitretin-mediated upregulation of ADAM10.[40] RXR and PPAR-α form a heterodimer which are recruited to ADAM10 promoter, by the PPAR-α agonist gemfibrozil.[28] In line with this, silencing RXR or PPAR-α inhibitor attenuates sulfuretin-mediated enhancement of ADAM10 transcription (Fig. 3 and 4). Our study has excluded the involvement of SP1 and USF1 in sulfuretin regulation of ADAM10 based on the following results: (1) Silencing SP1 or USF1 does not prevent the effect of sulfuretin on ADAM10 (Fig. 3); (2) PI3K or ERK inhibitor does not diminish the enhancement of ADAM10 by sulfuretin (Fig. 4A and B). Evidences suggest that PI3K or ERK control SP1[45] and USF1 phosphorylation and activity,[46] which in turn regulate ADAM10 expression involved in insulin[47] or apicidin[18] signaling pathways. Although sulfuretin protects cell from oxidative stress and apoptosis by PI3K/ERK signaling pathway,[22,48] this pathway might not be involved in sulfuretin-mediated regulation of ADAM10.

RXR/PPAR-α associated mechanisms might suggest a physiological significance.[49] Whereas RXR is implicated in a variety of cellular functions including cell proliferation, macrophage biology and lipid metabolism,[50] PPAR-α plays an important role in lipid metabolism and atherosclerosis.[51,52] In this respect, sulfuretin relative to RXR/PPAR-α signaling might deserve further study.[53,54]

The inconsistency between the altered protein levels of BACE1/ADAM10 and Aβ induced by sulfuretin

It is generally conceived that the enzymatic activity of BACE1 and ADAM10 contributes to APP processing with respect to levels of CTFs and sAPPs, which are consistent with Aβ alterations. Evidence has suggested that BACE1 overexpression in cells that overexpress wild-type APP increases β-CTF and Aβ level,[55,56] and BACE1 inhibitor in SH-SY5Y-APP cells reduces Aβ1-40.[57] On the other hand, ADAM10 silencing decreases α-CTF and sAPPα levels with the slight increase of Aβ level in SH-SY5Y cells and murine cultured neurons where APP was not overexpressed.[58] Overexpression of ADAM10 in mice promotes the generation of α-CTF and sAPPα and reduces Aβ load.[59]

In our study, the decreased BACE1 protein levels are consistent with the alteration of β-CTF and sAPPβ. In addition, the unaltered levels of α-CTF could be explained by m-ADAM10 that is not affected by sulfuretin, whereas the increased level of sAPPα induced by sulfuretin may not be attributed to alteration of im-ADAM10, as mutation of prodomain of ADAM10 does not result in altered sAPPα levels.[42] However, Aβ levels are not decreased accordingly. Instead, the slight but significant increase of Aβ1-42 is observed, which might be indispensable, because Aβ is slowly accumulated as early as 15—20 years before AD symptoms occur.[60] Thus, we speculate that additional mechanisms might contribute to the inconsistency (described below).

BACE1 protein level can be up-regulated by ROS, which is known to be reduced by sulfuretin.[13,31] In the present study, sulfuretin down-regulates BACE1 protein levels which is abolished by CHX, suggesting a translational mechanism. It might be unlikely that this effect is attributed to ROS or PI3K activation, because ROS directly enhances BACE1 mRNA level,[61,62] which is not altered by sulfuretin in our study; and PI3K activation should promote rather than inhibit BACE1 translation.[63]

Enhancement of im-APP levels might be associated with Aβ generation by sulfuretin

The association between Aβ generation and APP intracellular trafficking has been recently intensively investigated.[64] Both isoforms of APP (im- and m-APP) can be found in endoplasmic reticulum (ER) and trans-Golgi network (TGN).[65,66] It is known that im-APP is localized to TGN before secretion,[32,66] and APP that is re-directed from other subcellular compartments to TGN leads to an increased level of Aβ.[67—69] Importantly, im-APP is capable of being catalyzed by α- and β-secretase,[70] suggesting that im-APP contributes to the generation of Aβ. In our study, sulfuretin-mediated enhancement of im-APP is attenuated by CQ, which dramatically increases the basal protein levels of APP.[71,72] In addition to its role in APP degradation,[73] evidence suggests that CQ might also influence subcellular localization of APP that is associated with endosome, ER, TGN and lysosome.[74] CQ is shown to disseminate the puncta of EEA1 (early endosome), GOLGA2 (Golgi apparatus) and M6PR (transporting lysosomal enzymes) labeled compartments, and reduce EEA1 but increases GOLGA2 and M6PR puncta intensity.[75] We speculate that the slightly increased Aβ level could be partially explained by the increased level of im-APP that is closely associated with TGN localization, which could be inhibited by CQ.

The enzymatic activity of BACE1 is dramatically increased in cells overexpressing APP[76,77]; and co-localization of BACE1 and im-APP in TGN could account for a large body of Aβ generation.[66,78] It is likely that the enhanced generation of Aβ in TGN by sulfuretin might be neutralized by the reduction of Aβ in other subcellular compartments associated with the decreased level of BACE1, β-CTF and sAPPβ. Similarly, APP accumulation in TGN could increase α-CTF level by ~4-folds,[79] which might partially explain the increased level of sAPPα by sulfuretin in the secretory pathway of APP.[69]

In summary, our study highlights the following: (1) RXR/PPAR-α signaling contributes to the regulation of ADAM10 transcription by sulfuretin. In the peripheral organs such as liver or skeletal muscle, or when APP/Aβ is not a prominent issue, the RXR/PPAR signaling involved in sulfuretin function might deserve future study in lipid metabolism and macrophage biology.[50,80] (2) The inconsistency between ADAM10/BACE1 protein level and Aβ generation implies that in addition to protein expression, subcellular compartmentation involved in APP processing might play an important role in the pathogenesis of AD; and (3) The elevated level of TGN-localized im-APP could partially explain sulfuretin-mediated enhancement of Aβ level. However, whether sulfuretin might finally influence synaptic function and other cellular and molecular signaling associated with learning and memory remains unknown, which requires future study.

Materials and methods

Chemicals and antibodies

Sulfuretin was purchased from Xili (Kunming, Yunnan, China), dimethyl sulfoxide (DMSO) was purchased from Dinguo (Beijing, China). Actinomycin D (ActD), cycloheximide (CHX), chloroquine (CQ), PKA inhibitor H89, mTOR inhibitor rapamycin (RAPA), PPAR-α inhibitor GW6471, ERK inhibitor U0126, β-secretase inhibitor LY2811376, α-secretase inhibitor GI254023X, γ-secretase inhibitor DAPT, PKA inhibitor H89 and PI3K inhibitor LY429002, 3-MA were purchased from MCE. All chemicals were dissolved in DMSO to generate a 20 mM stock solution. Subsequent dilutions were performed by culture medium. The final concentration of DMSO was no more than 1:1000.

Antibody information was as follows: ADAM10 (ab1997, Abcam, 1:1000); BACE1 (ab2077, Abcam, 1:1000); APP-full length (A8717, Sigma, 1:3000); α-CTF and/or β-CTF: A8717 (Sigma; 1:3000), 4G8 (BioLegend, 1:1000) and 6E10 (BioLegend, 1:1000); SP1 (Santa Cruz Biotechnology, Dallas, TX, USA, 1:500); USF1 (Santa Cruz Biotechnology, 1:500); RXR-α (Santa Cruz Biotechnology, 1:500); sAPPα (2B3, IBL, 1:500); sAPPβ (SIG-39138, Biolegend, 1:500); GAPDH (Proteintech, 1:10000). Horseradish peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat secondary antibodies were purchased from Proteintech (Wuhan, China).

Cell culture

SH-SY5Y (Human neuroblastoma) cells were cultured under standard conditions (37 °C, 5% CO2, 95% air) in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific), 100 mg/ml streptomycin and 100 U/ml penicillin G. HEK-293 (Human embryonic kidney) and HT22 (a mouse hippocampal cell line) were cultured under standard conditions (37 °C, 5% CO2, 95% air) in DMEM/F12 supplemented with 10% FBS. SH-SY5Y-APP (SH-SY5Y cells stably expressing full-length human APP) cells was generated as described previously.[18] All of cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Plasmid constructs and luciferase assay

Human genomic DNA was extracted from cultured cells by using Mammalian Genomic DNA Miniprep kits (Dinguo, Beijing, China) and was used as a template for amplification of nucleotides −467 to −30 (pGL4.17-ADAM10-D), −444 to −30 (pGL4.17-ADAM10-C), −508 to −300 (pGL4.17-ADAM10-E), −467 to −300 (pGL4.17-ADAM10-E1), −444 to −300 (pGL4.17-ADAM10-E2). PCR-amplified fragments were subsequently cloned into the firefly luciferase reporter vector pGL4.17 (Promega, Madison, WI, USA). PCR primers used for amplification as previously described.[18] SH-SY5Y cells were transfected using Lipofectamine 3000 (Invitrogen). Luciferase assays were detected with a GloMax 96 microplate luminometer (Promega). pGL4.17-ADAM10-E2 was used for mutations of the RXR response element (GAGG → GTCG) using Mut Express MultiS Fast Mutagenesis Kit (Vazyme, china). Primer sequences were: 5′- GGCGGTCGTCTGAGTTTCGCTCGA GGATATCAAGATCTG-3′(forward) and 5′- CGAAACTCA-GACGACCGCCTCCTTCCTCACCACGTGA-3′(reverse). Mutation was confirmed by DNA sequencing (Sangon Biotech, china).

Western blotting

Cultured cells were washed with ice-cold PBS followed by RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 50 mM Tris) containing protease inhibitors and phosphatase inhibitors. Protein concentrations were determined using a BCA Protein Assay Kit (P0011, Beyotime, Haimen, China) and Western blotting was performed as previously described.[18] For better detection of α/β-CTF, cells were treated with a γ-secretase inhibitor DAPT (250 nM) for 24 h, and proteins were separated on 16.5% Tris-tricine gels.[81,82] Membranes were treated using ECL reagent (GE healthcare, UK) and the Fusion FX5 image analysis system (Vilber Lourmat, Marne-la-Vallee, France), visualized and quantified by Quantity One software (Bio-Rad, Hercules, CA) normalized to GAPDH.

Immunofluorescence

Cells incubated on coverslips were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 30 min at 37 °C. Cells were then permeabilized with 0.3% Triton X-100 for 20 min at 24 °C and incubated with the primary antibody against ADAM10 (1: 200) in 4% BSA for 3 h. Coverslips were washed with PBS three times and incubated with fluorescence-labeled secondary goat anti-rabbit antibody Alexa Fluor 488 (1:200) for 1.5 h at 24 °C. After washing, the coverslips were mounted with DAPI Fluoromount-G (Southern Biotech, Birmingham, Alabama, USA). Images were acquired using a laser scanning confocal microscope (Leica TCS SP8 X, Germany). Quantification of immunofluorescence intensity was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD, USA).

Enzyme-linked immunosorbent assay

Human Aβ1-40 and Aβ1-42 in the cultural media were measured by ELISA kits (Cusabio, China) after drug treatment

for 36 h, according to the manufacturer's instructions. All samples were assayed in 3 duplicates, and each experiment was repeated 3 times.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from SH-SY5Y cells using RNAiso plus (Takara, Dalian, Liaoning, China), and cDNA was generated by the 5 × HiScript II Select qRT Super Mix II (R233-01-AC, Vazyme, Nanjing, China) according to the manufacturer's instructions. All qRT-PCRs were run and analyzed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The reaction mixture (20 μl total) contained 10 μl SYBR mix, 5.2 μl nuclease-freewater, 0.4 μl of each primer, and 4 μl diluted cDNA. Reactions were performed using the following steps: 1 cycles of 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, and the melting curve was run after RT-PCR. Total human APP, BACE1, ADAM10 mRNA levels were normalized to human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. The results presented are based on fold change using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: ADAM10: 5′-TTATGTGCCCCGTGTTCCCTGTTCT-3′ (forward) and 5′-GGTCGAGCCTCCTAGCCTTGATTGG-3′ (reverse); BACE1: 5′-GCAAGGAGTACAACTATGAC-3′ (forward) and 5′-AGCTTCAAACACTTTCTTGG-3' (reverse); APP: 5′-TGGTGGGCGGTGTTGTCATA-3′ (forward) and 5′-TGGATTTTCGTAGCCGTTCTGC-3′ (reverse); GAPDH: 5′-AGAAGGCTGGGGCTCATTTG-3′ (forward) and 5′-TGCTGATGATCTTGAGGCTGTTG-3′ (reverse).

Small interfering RNA transfection

The small interfering RNA (siRNA) oligonucleotides for human SP1, USF1 and RXR-α were synthesized by Shanghai GenePharma (Shanghai, China), and a non-targeting control siRNA (siControl) was used as the negative control. Transfection of SH-SY5Y cells with siRNA was performed using Lipofectamine 3000 transfection reagent according to the manufacturer's instructions. Briefly, Lipofectamine 3000 transfection reagent (4 μl) was added to 200 ml of serum-free Opti-MEM containing each siRNA (100 nM for siSP1, siUSF1, siRXR-α) followed by 20-min incubation at room temperature. The siRNA sequences were 5′-UUCUCCGAACGUGUCACGUTT-3′ for SP1,[83] 5′-GCUGGACAAUGACGUGCUUTT-3′ for USF1,[18] and 5′-GCUGUACACUCCAUAGUGCUU-3′ for RXR.[84]

Cell viability assay

Cell viability was measured with a CCK-8 Cell Counting Kit (Vazyme, Nanjing, China) according to manufacturer's protocol. HT22 or SH-SY5Y cells were seeded on 96-well plates at a density of 2×10^3 or 4×10^3 and cultured for 24 h. The cells were then treated with different concentrations of sulforetin or same volume of DMSO for 24h. Optical density values were determined at 450 nm by using a Spectra Max 340 PC (Molecular Devices, Sunnyvale, CA, USA).

Statistical analyses

All data were performed with Prism software (GraphPad Software, La Jolla, CA, USA) and expressed as mean ± SD. Data were analyzed by independent Student's *t* test or one-way analyses of variance (ANOVA) with a Dunnett's multiple comparison test. Differences were considered as significant when $P < 0.05$.

Authors contribution

Guo-Jun Chen designed the study. Jian Chen and Biao Luo performed the experiments. Bi-Rou Zhong, Kun-Yi Li, Qi-Xin Wen, Li Song, Xiao-Jiao Xiang, Gui-Feng Zhou, Li-Tian Hu, Xiao-Juan Deng and Yuan-Lin Ma provided assistance with research. Jian Chen and Guo-Jun Chen wrote the paper.

Conflict of Interests

The authors declare no competing interests.

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