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Research Article

Natural Compound Isoliensinine Inhibits Stress-Induced Hair Greying by Blocking β2-Adrenoceptor

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Chronic and acute stress caused by emotional or physical insults can affect the function of other organs via the brain-body axis. As one of the smallest organs in mammalian, hair follicles are highly susceptible to stress. Under stress, the sympathetic nerves release norepinephrine (NA), which acts directly on the β-2 adrenergic receptors on melanocyte stem cells (MeSCs) within the hair follicles, causing the MeSCs to lose quiescence and enter a rapid proliferation state followed by differentiation and migration, leading to rapid loss of MeSCs and, ultimately, grey hair. Here, we screened out β-2 blockers forming the ZINC 15 compound database, found a natural product isoliensinine, and was effective in preventing stress-induced hair greying in mice. The study sheds light on the development of products that use natural compounds to prevent stress-induced hair greying.

1. Introduction

Hair follicle is an accessory organ of the skin, which plays an important role in temperature regulation, appearance modification, and more. Over the course of evolution, humans in different regions have evolved different hair colors. The effect of hair on a person's appearance is unique. However, hair follicle is fragile; it is extremely susceptible to oxidative, inflammatory, nutritional, and psych emotional stressors [1]. Greying hair is an unwelcome appearance for most people, especially young people. However, efficient grey hair prevention product or method is still lacking.

Hair greying is one of the earliest and most visible signs of aging in human beings, but it can also occur at a younger age. Stress is a condition of mental or emotional strain or suspense. It almost affects people of all ages and can cause body dysfunction and a variety of diseases in serious cases [2]. There are many famous figures and stories associated with hair greying with stress such as Wu Zixu and Marie Antoinette; under great stress, their hairs turned grey overnight[3]. Although we do not have such acute stress, various stress affects people of all ages and occupations; living under prolonged stress certainly affects our hair follicles [4].

Stress has been anecdotally related to a variety of changes in tissues, including hair greying. The mechanism by which stress causes hair greying has been dissected [4]. Hair pigmentation is controlled by melanocytes, which are derived from melanocyte stem cells (MeSCs) in hair follicles [5]. Hair follicles have the characteristic of periodic growth

and undergo three phases: growth phase (anagen), regression phase (catagen), and resting phase (telogen). During the hair growth cycle, MeSCs remain in a quiescent state for most of the time until they become activated during the early growth phase [5–8]. At anagen, a tiny number of MeSCs are activated and differentiated into melanocytes that migrate downward to the hair bulb and synthesize melanin which colors the newly regenerated hair. The other MeSCs remain in the stem cell niche of hair follicle and are activated in subsequent cycles. At catagen, mature melanocytes are destroyed [4, 9]. Moreover, under stress, the body's sympathetic nervous system releases noradrenaline (NA), which acts directly on the β2-adrenoceptor of hair follicle bulge, causes almost all MeSCs to lose quiescence and undergo rapid proliferation followed by differentiation and migration, leading to their loss in the niche, which eventually leads to hair greying in subsequent cycles [10]. Even so, there is still no product available to treat and prevent hair greying currently. Therefore, blocking NA action on the β2-adrenoceptor is a key to preventing melanin stem cell depletion and hair greying. β2-adrenoceptor blockers have been used for many years and are widely used to treat diseases such as hypertension and asthma, and their safety is guaranteed [11]. Propranolol is one of the most classic β2-adrenoceptor blockers, and we selected it as the positive control for subsequent operations and experiments.

2. Results

2.1. Isoliensinine Is a Potent β2 Adrenergic Receptor Inhibitor. Using computer-aided drug design software MOE, we screened out some compounds that can bind β-adrenoceptor, one is the natural compound isoliensinine (Figure 1(a)). The docking score "S" of propranolol and isoliensinine are −6.5223 and −6.83178616; the more negative the value of S, the more closely the ligand binds to the receptor, and other docking parameters are also excellent (Figure 1(b)). Isoliensinine is derived from the traditional Chinese medicine lotus plumule, which has antiarrhythmia and antihypertensive properties and improves cardiac and cerebrovascular function and other biological activities [12]. To evaluate transdermal absorption, we performed in vitro penetration assays using a transdermal diffusion cell and mouse skin (Figure 1(c)) [13]. The permeation curves showed that both propranolol and isoliensinine had good transdermal ability, and in which isoliensinine had faster transdermal speed and higher efficiency (Figure 1(d)).

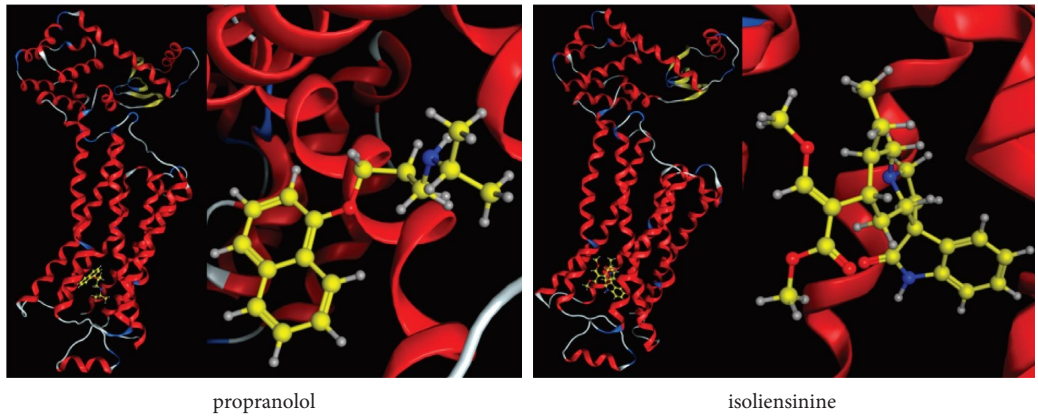
2.2. Isoliensinine Blocks NA-Induced Ca^{2+} Spark. To test the efficiency of isoliensinine in blocking β2-adrenoceptor, we tried to establish an in vitro cell model. We firstly select cells that are reactive to the NA/β2 signaling with easy detecting response. Given, NA could induce a rapid increase of intracellular Ca^{2+} concentration, which was termed as "Ca^{2+} spark" in cardiomyocytes [14] (Figure 2(a)), and NA/β2 signaling could activate cAMP/PKA signal pathway, and induce the increase of P-PKA substrate [15]. Both human and mouse melanoma cell predominantly express β2-adrenoceptor in cell surface, and their gene expression is similar to that of homologous melanocytes [16, 17]. Therefore, we selected human melanoma cell to explore the influence of NA on it. The result showed that NA was able to dramatically induce fluorescence signals in melanoma cells loaded with Fluo4, a fluorescence probe of calcium (Figures 2(c) and 2(d)), and increased the P-PKA substrate levels (Figure 2(b)). Thus, melanoma cell is an ideal model to test the β2 inhibition efficiency of isoliensinine. After treated with propranolol or isoliensinine, no fluorescence signal was detected in the melanoma cell (Figures 2(c) and 2(d)), and P-PKA substrate levels caused by NA reduced obviously (Figure 2(b)). The results suggest that isoliensinine can efficiently block NA/β2 signaling.

2.3. Isoliensinine Prevented Stress-Induced Hair Greying. To further evaluate the hair grey preventing effect of isoliensinine, additional animal study was performed. Injection of resiniferatoxin (RTX) is an efficient and reliable stress-induced hair greying mice model [4]. The mice were firstly depilated, and a single dose of RTX was injected in the flank daily for 7 days, and 30 days after RTX injection, 29.2% of the black hairs on the dorsal of the mice turned grey [9] (Figure 3(a)). To test the hair grey preventing effect of isoliensinine, the mice were injected with RTX and topically applied with isoliensinine or propranolol solution for 7 consecutive days (Figure 3(c)). Mice treated with propranolol and isoliensinine showed a significant reduction in the area of white hair on the dorsal skin. Statistical analysis result showed that propranolol and isoliensinine significantly reduced the grey hair ratios to 7.5% and 9.6%, respectively, ($P < 0.05$) (Figures 3(a) and 3(b)). Abovementioned data suggested that isoliensinine effectively inhibit the stress-induced hair greying.

3. Materials and Methods

3.1. Molecular Docking. Virtual screening for novel β2-adrenoceptor inhibitors was performed using the ZINC database. The total number of natural compounds in the database is 642129, and carrying out the docking with β2-adrenoceptor by computer-aided drug design software Molecular Operating Environment (MOE 2019.01) [18]. The docking results are represented by the following parameters: S, rmsd_refine, and E_conf. "S" means the docking score; the more negative the value, the more the ligand molecule binds to the receptor. "rmsd_refine" means the root-mean-squared-deviation (RMSD) between the refined predicted pose and those of the unrefined crystal structure. "E_conf" means the energy released after the docking. β2-adrenoceptor's 3D structure was downloaded from RCSB PDB (rcsb.org, number 6PS5); compounds and propranolol's 3D structures were downloaded from ZINC 15 (https://www.zinc15.docking.org/) or ChemicalBook (https://www.chemicalbook.com/).

3.2. Transdermal Permeation Experiment. Transdermal permeation experiments were performed employing vertical

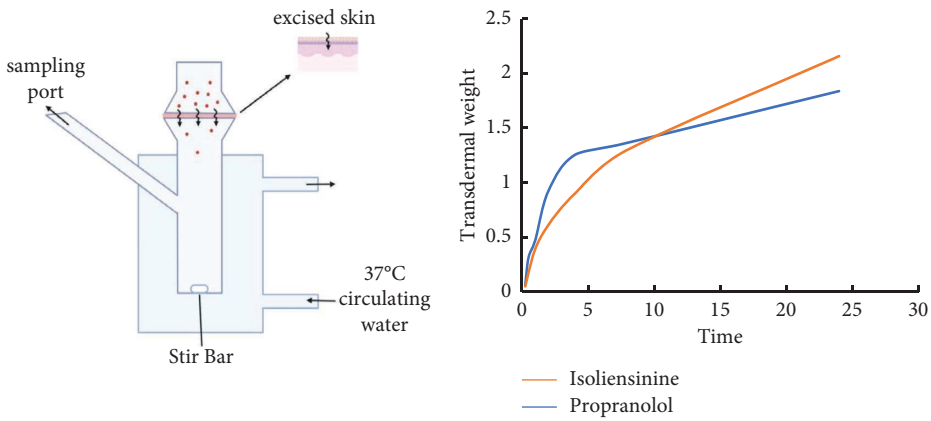


propranolol isoliensinine

(a)

	S	rmsd_refine	E_conf
propranolol	-6.5223	1.5712	9.4502
Isoliensinine	-6.83178616	1.51732719	75.2706146

(b)



(c) (d)

FIGURE 1: Isoliensinine is a potent β2 adrenergic receptor inhibitor: (a) schematic diagram of propranolol and isoliensinine docking with β2-adrenoceptor. (b) The docking parameters of propranolol and isoliensinine to β2-adrenoceptor. (c) Schematic diagram of transdermal diffusion cell and transdermal permeation experiment. (d) The curves of transdermal permeation.

Franz-type diffusion cells (DISA, Milan, Italy). The supply liquid was a 4 ml volume transdermal preparation of the blocker propranolol (Sigma-Aldrich, PHR1308) of 1 mg/ml isoliensinine (YuanYe, B21537) of 1 mg/ml, the solvent was a mixture of 1, 2-propylene glycol, absolute ethanol, peppermint ketone and, ddH2O in a ratio of 50 : 32 : 5 : 13. The acceptance liquid is 1% PBS solution with a volume of 50 ml. The osmotic membranes between the supply liquid and the acceptance liquid were the dorsal skins (4 cm × 4 cm) excised from C57BL/6 donor mice, and the skins were immediately processed to remove the underlying fat and connective tissue. The supply liquid was a 4 ml volume transdermal preparation of the blocker propranolol (Sigma-Aldrich, PHR1308) of 1 mg/ml or isoliensinine (YuanYe, B21537) of 1 mg/ml, the solvent was a mixture of 1, 2-propylene glycol, absolute ethanol, peppermint ketone, and ddH2O in a ratio of 50 : 32 : 5 : 13. The acceptance liquid is 1% PBS solution with a volume of 50 ml. Take 1 ml of the acceptance solution for each test and refill, at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h, take the samples and measure the absorbance by ultra-micro UV spectrophotometer (Nanodrop N60).

3.3. Calcium Influx Experiment. Melanoma cells into confocal culture dishes were incubated with Fluo-4, AM fluorescent calcium probe (Invitrogen, F14201, 2 μM) for 30 minutes at 37°C. After washing with PBS for 3 times, the cells were replaced with Hanks' solution with calcium and magnesium, and the cells were incubated for 20–30 minutes. NA (Sigma-Aldrich, 489350, 100 μmol/L) was added to the Petri dish, and the confocal microscope was adjusted to the "time series" mode, the laser frequency was 488, the time interval was 10 s, and the continuous photographing was performed for 300 s. Inhibitor propranolol (50 μmol/L) or isoliensinine (20 μmol/L) was added 30 minutes before NA addition. NA was prepared in DPBS and propranolol/isoliensinine was prepared in DMSO.

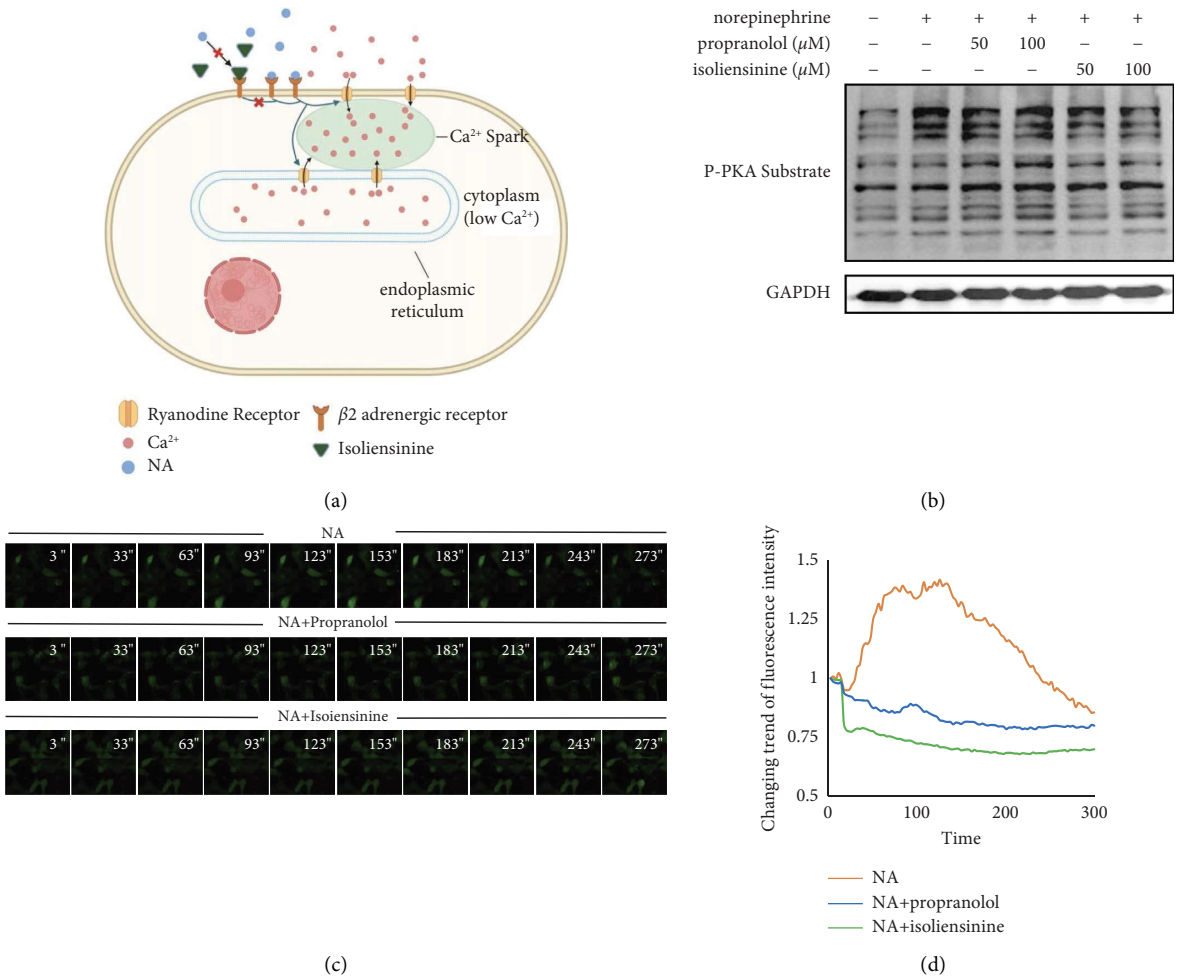


Figure 2: Isoliensinine could efficiently block NA/β2 signaling: (a) schematic diagram of NA-induced rise in intracellular Ca^{2+} concentration. (b) Propranolol and isoliensinine reduce the NA-induced P-PKA substrate levels rise ($n \geq 3$ independent experiments are performed and representative data are shown). (c) Propranolol and isoliensinine abolish the NA-induced Ca2+ signal rise ($n \geq 3$ independent experiments are performed and representative data are shown). (d) Statistical plot of calcium signal as a function of time.

3.4. Western Blotting. The treated cells were lysed with a RIPA buffer (Beyotime, P0013) containing the protease (CWBIO and CW2200S) and phosphatase inhibitors (NCM and PO03) on ice. Then, the protein was extracted by ultrasonic disintegration for three times and centrifuged at 4°C, 13,000 rpm for 15 min. Followed by the extraction, the protein concentration in the supernatants was measured using a BCA Protein Assay Kit (Beyotime, P0009) according to the manufacturer's instructions. After that, the prepared sample was separated by polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and incubated with primary antibodies overnight at 4°C: anti-P-PKA substrate (Cell Signaling Technology, 9624S, 1 : 1000) and anti-GAPDH (GeneTex, GTX100118, 1 : 5000). On the following day, the membranes were incubated with HRP-linked secondary antibodies (Cell Signaling Technology, 7074P2, 1 : 1000) at room temperature for 1 h and visualized using a WesternBright™ ECL (advansta, K-12045-D50).

3.5. Resiniferatoxin-Induced Hair Greying Mice Model. All the animal experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University (approval no. SYSU-YXYSZ-20210332). C57BL/6 female mice of 7 weeks old were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). After depilation, the mice were injected with RTX (AdipoGen Life Sciences, AG-CN2-0534-MC05, 20 μg/kg) in the flank for 7 days, and RTX was prepared in 2% DMSO with 0.15% Tween 80 in PBS. An hour before (0 hour) and 11 hours after (12 hours) the RTX injection, mice were coated with a transdermal preparation of propranolol of 1 mg/ml (RTX + propranolol group)/or isoliensinine of 1 mg/ml (RTX + isoliensinine Group), respectively. At day 30, the mice were photographed, and the ratio of grey hair was calculated. Use ImageJ to measure the overall back area and the grey area of the photographs, and calculate the proportion of grey hair.

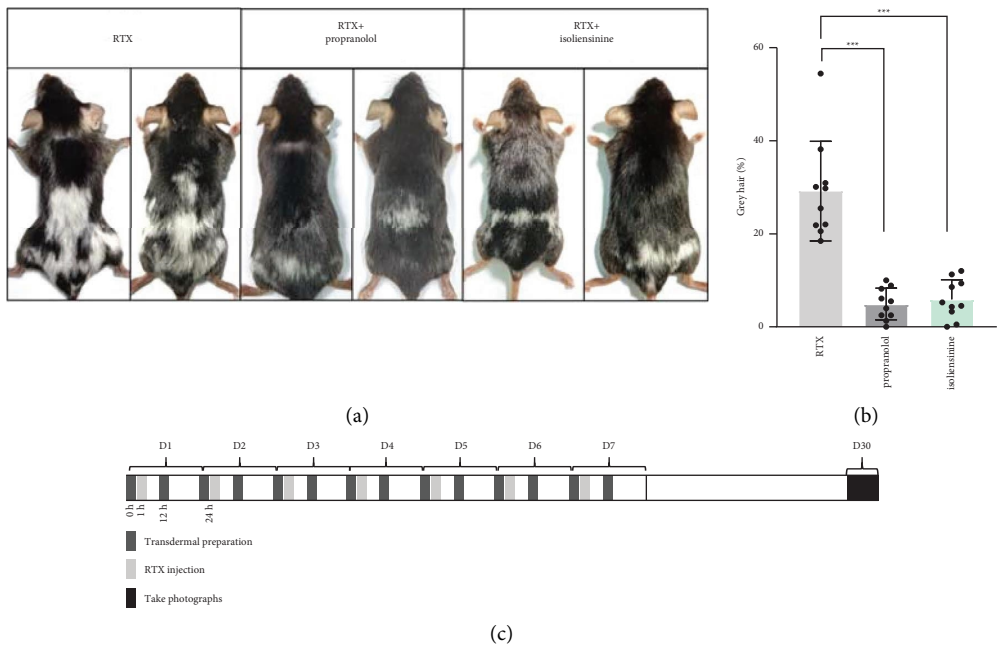


Figure 3: Isoliensinine prevented stress-induced hair greying: (a) RTX induces hair greying of C57/BL6 mice; applying the transdermal preparation of propranolol or isoliensinine with the injection of RTX reduces the proportion of grey hair ($n = 10$ mice for each condition). (b) Quantification of the body area covered by grey hairs ($n = 10$ mice for each condition; one-way ANOVA with Tukey's multiple comparisons). (c) Time of application of transdermal preparation and injection of RTX.

4. Discussion

More people are experiencing stress-induced hair greying, but there is no medication for hair greying prevention or treatment. Due to long term of the clinical trial for the hair greying prevention drugs, natural compounds provide an opportunity to rapidly develop a ready-to-use antihair greying product. Natural products have been used by people all over the world since ancient times. There have been many reports that some natural products can prevent and treat hair greying, but there is no solid basis or evidence [2]. The mechanism by which stress causes grey hair has been explored recently, and computer-aided drug design could be helpful to screen out natural compounds with good structure and reliable safety from the compound library. Isoliensinine is a natural compound that has been included in the catalog of Used Cosmetics Raw Materials in 2021 and has been proven to be effective in preventing stress-induced hair whitening in this study. Thus, the data of this study prove isoliensinine is an efficient natural $\beta 2$ blocker and with great potential to develop a natural antihair greying product.

Data Availability

All data included in this study are available from with the corresponding authors upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Xusheng Wang, Xuejuan Xu, Xiaojing Zhao, and Yuanqiang Ling conceptualized the study; Lingchen Yan, Miaomiao Li, and Meidi Zhu investigated the study; Ying Gao analyzed the data; Weiwei Chu and Ying Gao wrote the manuscript; Xusheng Wang and Weiwei Chu performed funding acquisition. All authors have read and agreed to the published version of the manuscript. Lingchen Yan, Miaomiao Li, and Meidi Zhu contributed to this work equally.

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