Redox Biology 14 (2018) 354–360

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research paper

A novel *S*-sulfhydrated human serum albumin preparation suppresses melanin synthesis

Mayumi Ikeda[a,1], Yu Ishima[a,*,1], Ryo Kinoshita[b], Victor T.G. Chuang[c], Nanami Tasaka[a], Nana Matsuo[a], Hiroshi Watanabe[b], Taro Shimizu[a], Tatsuhiro Ishida[a], Masaki Otagiri[d], Toru Maruyama[b,*]

[a] Department of Pharmacokinetics and Biopharmaceutics, Institute of Biomedical Sciences, Tokushima University, 1-78-1, Sho-machi, Tokushima 770-8505, Japan
[b] Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan
[c] School of Pharmacy, Faculty of Health Sciences, Curtin University, GPO Box U1987, Perth 6845, Western Australia
[d] Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan

ARTICLE INFO

Keywords:
Ultraviolet irradiation
Human serum albumin
Reactive sulfur species
Whitening agent
Oxidative stress

ABSTRACT

Products of ultraviolet (UV) irradiation such as reactive oxygen species (ROS) and nitric oxide (NO) stimulate melanin synthesis. Reactive sulfur species (RSS) have been shown to have strong ROS and NO scavenging effects. However, the instability and low retention of RSS limit their use as inhibitors of melanin synthesis. The free thiol at Cys34 on human serum albumin (HSA) is highly stable, has a long retention and possess a high reactivity for RSS. We report herein on the development of an HSA based RSS delivery system. Sulfane sulfur derivatives released from sodium polysulfides (Na_2S_n) react readily with HSA. An assay for estimating the elimination of sulfide from polysulfide showed that almost all of the sulfur released from Na_2S_n bound to HSA. The Na_2S_n-treated HSA was found to efficiently scavenge ROS and NO produced from chemical reagents. The Na_2S_n-treated HSA was also found to inhibit melanin synthesis in B16 melanoma cells and this inhibition was independent of the number of added sulfur atoms. In B16 melanoma cells, the Na_2S_n-treated HSA also inhibited the levels of ROS and NO induced by UV radiation. Finally, the Na_2S_n-treated HSA inhibited melanin synthesis from L-DOPA and mushroom tyrosinase and suppressed the extent of aggregation of melanin pigments. These data suggest that Na_2S_n-treated HSA inhibits tyrosinase activity for melanin synthesis via two pathways; by directly inhibiting ROS signaling and by scavenging NO. These findings indicate that Na_2S_n-treated HSA has potential to be an attractive and effective candidate for use as a skin whitening agent.

1. Introduction

Ultraviolet (UV) irradiation produces reactive oxygen species (ROS) that ultimately cause cell death [1]. To protect the skin from UV damage, melanin, a dark colored pigment, is produced by melanocytes [2]. While melanin is essential for skin health, a demand for melanin scavenging preparations exists. Chloasma (melasma) is a condition in which the skin develops discolored areas that are caused by overproduction of melanin and are sometimes regarded as a metaphor of aging. In addition, inhibitors of melanin synthesis are popular cosmetics for brightening the skin, especially in Asian countries [3].

Tyrosinase catalyzes the production of melanin from tyrosine via DOPA and dopaquinone in melanocytes [4]. Its activity is regulated by a variety of factors such as ERK1/2 and Akt signaling [5]. ROS such as hydrogen peroxide produced by UV irradiation, activates tyrosinase and promotes melanin synthesis in melanocytes [2]. UV also causes the production of nitric oxide (NO) and stimulates tyrosinase activity via cGMP [6], a second messenger of NO.

On the other hand, thiol compounds with anti-oxidant effect has been widely used as supplements, radioprotection agents and perm agents [7]. Thiol-containing compounds undergo self-oxidation to form sulfonic acid, sulfenic acid and sulfonic acid [8]. Thiol also scavenges NO via *S*-nitrosation [8]. Because of these effects, thiol-containing compounds are often used in treating chloasma [7,9]. However, the skin whitening effect of thiols is very weak, a demand for more effective ROS and NO scavenging agents exists.

Reactive sulfur species (RSS) including cysteine persulfide have recently been reported to have stronger anti-oxidant effects than thiols.

* Corresponding authors.
E-mail addresses: ishima.yuu@tokushima-u.ac.jp (Y. Ishima), tomaru@gpo.kumamoto-u.ac.jp (T. Maruyama).
[1] Contributed equally to the results of this work.

http://dx.doi.org/10.1016/j.redox.2017.10.007
Received 20 September 2017; Received in revised form 6 October 2017; Accepted 10 October 2017
Available online 11 October 2017

RSS contain a reactive thiol group [10] and the p*K*a of most RSS are much lower than that for thiols [11]. Therefore, RSS can react effectively with both ROS and NO and predicted to reduce the extent of melanin production. Sodium polysulfides (Na_2S_n), diallyltrisulfide (DATS) and dimethyltrisulfides (DMTS) are commonly used as RSS donors [12]. However, Na_2S_n has a low retention at neutral pH and has an offensive smell. In addition, DATS and DMTS, which are produced by garlic and onions are also odorous and their potential for such treatments is limited [13]. Furthermore, the half-life of Na_2S_n is very short in serum and, based on in vivo models, multiple injections are needed for them to be effective. Thus, the development of novel RSS-delivery-systems would be highly desirable.

Human serum albumin (HSA) is the most abundant protein in serum and is widely used as a drug carrier because of its biocompatibility and long plasma retention properties [14,15]. HSA contains a total of 35 Cys residues and one of them, Cys^{34}, is present in the form of a free thiol group [16]. Cys^{34} is sometimes a target for a drug binding site, because of its reactive thiol group [17,18]. For example, in the presence of nitric oxide (NO) the Cys^{34} thiol group is *S*-nitrosated. We previously demonstrated that *S*-nitrosated HSA (SNO-HSA) allows NO to be retained for long periods in serum [19]. SNO-HSA has various biological functions, including a liver protective effect against ischemia/reperfusion [20] and tumor suppressing effects [21].

Consequently, we hypothesized that HSA could be used as a RSS carrier (such as SNO-HSA) via the *S*-sulfhydration of Cys^{34}-SH. As a source of polysulfur, DATS and DMTS are limited because of their lipophilicity and volatility. Hence, commercially available Na_2S_n (Na_2S, Na_2S_2, Na_2S_3 and Na_2S_4) was used in this study. Ogasawara et al. previously prepared sulfur-bound serum albumin reacted with sodium sulfide (NaHS) by a simple mixing of the reagents [22]. The sulfur from NaHS was added to Cys^{34} and the resulting preparation protected liver damage caused by lipid peroxide. We adopted this method for preparing RSS-added-HSA using Na_2S_n for RSS delivery.

In this work, we reported the preparation of Na_2S_n-treated HSA and its use as a novel delivery system of RSS. The added sulfur was analyzed by means of a sulfane sulfur probe [23] and the elimination of sulfide from polysulfide [24]. To evaluate the effect of Na_2S_n-treated HSA on skin whitening, the effect of the Na_2S_n-treated HSA on melanin synthesis was studied using a B16 melanoma cell line.

2. Material and methods

2.1. Materials

Human serum albumin (HSA) was purchased from KAKETSUKEN (Kumamoto, Japan) and all HSA samples were defatted by a charcoal treatment. Sodium sulfide and sodium tetrasulfide were purchased from DOJINDO Laboratory (Kumamoto, Japan). Sulfane sulfur probe 4 (SSP4) was prepared as previously described [23]. L-DOPA, glutathione, (DTNB), ascorbic acid and sodium satiric Griess reagent (sulfanilamide, naphthylethylenediamine-HCl) were purchased from Nakarai Chemicals (Kyoto, Japan). Sephadex G-25 desalting column (φ 1.6 × 2.5 cm) was purchased from GE Healthcare (Kyoto, Japan). Dulbecco's modified Eagle medium (DMEM) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Wako Pure Chemical (Osaka, Japan). Mushroom tyrosinase was purchased from Sigma-Aldrich. All other chemicals were of the best grade that was commercially available, and all solutions were prepared in deionized and distilled water.

2.2. BCA protein assay

Protein concentrations were measured using a BCA protein assay. 10 μL aliquots of samples and bovine serum albumin (BSA) standards were incubated in 100 μL of reaction buffer at 25 °C for 30 min. After the reaction, micro-plate reader was used to measure the absorbance of 540 nm. BSA was used to construct a standard curve.

2.3. Synthesis of Na_2S_n treated-HSA

HSA (300 μM) was incubated with 1 mM of sodium polysulfides (Na_2S_n) in PBS (pH 7.4) for 1 h at 37 °C. After the reaction, excess sodium polysulfides were removed by gel filtration with a Sephadex G-25 column.

2.4. Determination of sulfur binding rate by elimination method for sulfide from polysulfide (EMSP)

EMSP was prepared as previously described (3 × EMSP by addition of 792 mg of L-ascorbic acid to 5 mL of 3 N of NaOH) [24]. Samples (7.5 μM, 133 μL) were incubated with 66.7 μL of 3 × EMSP for 3 h at 37 °C. A 1% zinc acetate solution (600 μL) was then added to the reaction solution, followed by vortexing immediately. The samples were centrifuged at 8,000 × *g* for 5 min and washed with phosphate buffered saline (PBS) twice. After removing the supernatants, deionized and distilled water (200 μL) was added to the precipitates. After adding 1% zinc acetate (300 μL), 50 μL of 20 mM *N,N*-dimethyl-*p*-phenylenediamine and 20 mM $FeCl_3$ in 7.2 N HCl, the solution was incubated for 30 min at 25 °C. Samples were centrifuged at 8000 × *g* for 1 min and transferred into 96-well plates and the OD at 665 nm measured. Na_2S was used to construct a standard curve.

2.5. Detection of sulfane sulfur with SSP4

Each sample (20 μM) was incubated with 5 μM of SSP4 in 1 mM Cetyltrimethylammonium Bromide / PBS (pH 7.4) for 10 min at 25 °C. After incubation, the fluorescence measured by a spectrophotometer (JASCO Corporation) with excitation at 457 nm, emission at 490–535 nm.

2.6. DPPH radical tests

DPPH (250 μM) in ethanol was mixed with the same amount of MES buffer (50 mM, pH 7.4). Na_2S_n-treated HSA (40 μM) was the added to this DPPH solution, which was then incubated for 30 min at 25 °C and the absorbance of the DPPH radicals was measured at 540 nm. Scavenged radical rates were converted using the following formula;

$$\text{Scavenged radical (\%)} = (Abs_{sample}\text{-}Abs_{pbs})/\ Abs_{pbs} \times 100$$

2.7. NO and SNO analysis

Na_2S_n-treated HSA (50 μM) was incubated with an NO donor, NOC7 (200 μM), for 30 min at 25 °C. After the reaction, the concentration of NO and SNO were measured by a Griess assay with minor modifications [25]. The Griess reagent solution was prepared by mixing 0.1% N-1-Naphtylethylene-diamide dihydrochloride and 1% sulfanilamide in 2% phosphoric acid. The reaction buffer was composed of 0.1 M NaCl, 0.5 mM DTPA and 10 mM AcONa·AcOH (pH 5.5). Samples (20 μM) were reacted with the Griess reagent solution (60 μL) in reaction buffer (110 μL) with 3 mM $HgCl_2$ in 10 mM Na Acetate (pH 5.5). After a 15 min incubation, the absorbance of 540 nm was measured by means of a microplate reader. The remaining NO/SNO ratio (%) was calculated and compared to PBS values for the samples.

2.8. Cell culture

B16 melanoma cells were provided by the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan), and were cultured in DMEM containing 10% fetal bovine serum and an antibiotics solution. Cells were grown with maintained at 37 °C in humidified air containing 5% CO_2 in incubator (passage number 10–20).