Synergy of H_2O_2 and Methionine Affects Tyrosinase Activity, Causing Repigmentation of Gray Hair

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Abstract: Hair colour demonstrates people's health condition. The most prevalent explanation for hair graying is that peroxides oxidize tyrosinase, preventing this enzyme from activating melanocytes. From Schallreuter's paper in 2004, hydrogen peroxide was found to activate acetylcholinesterase, the enzyme crucial in treatment of vitiligo. Vitiligo is supposed to be caused by deactivation of pigment cells, like the possible cause of hair graying. Meanwhile, methionine has long been considered to eliminate peroxides. Therefore, it's attractive to see that if the combination of the two materials, hydrogen peroxide and methionine, will activate tyrosinase more largely than any one of them do. This research uses the rate of L-Dopachrome formation to determine tyrosinase activity. There are two possible results. The ideal result is the synergy of methionine and hydrogen peroxide activates tyrosinase. However, since hydrogen peroxide can deactivate tyrosinase, the two activations may cancel out so there is no significant change in tyrosinase activity. If synergy of hydrogen peroxide and methioine do activate tyrosinase better, then the problem of hair graying can be better solved.

1 INTRODUCTION

Methionine (Met) plays a critical role in the metabolism of the body. It is an antioxidant that slows down the aging process. Meanwhile, with a high level of Met in the body, the level of the other amino acids will also be enhanced, which strengthens the immune system. Additionally, Met has been confirmed able to detoxify toxic substances like heavy metals and prevent liver damage. Therefore, Met has been used in many health products and is widely accepted (University of Rochester Medical Center). According to Peggy Sextius' research, Met extracted from polygonum multiflorum radix could slow down or prevent hair graying (Sextius, 2017).

Several explanations are proposed for the gradual loss of hair pigmentation. Studies in DNA repair have shown the critical roles of DNA repair, telomerase, Bcl-2, and stem cell factor for hair pigmentation [REFs]. One prevalent theory, the free radical theory of graying, proposes the association between melanogenesis and tyrosinase activity. J. M. Wood and others' study further supports the free radical theory, in which he suggested that H_2O_2 oxidizes Met to Met-S=O and inactivates the enzyme catalase breaking H_2O_2 down to water and oxygen; and he also observed that tyrosinase containing Met is inhibited (Wood, 2009). Thus, it is proved that H_2O_2-induced oxidation of Met directly affects tyrosinase and hinders follicular melanogenesis.

The study by Ikeda et al. revealed Met is able to induce tyrosinase production in two different Streptomyces. The total cellular tyrosinase activity is induced 16.9-folder higher in the presence of Met than in the negative control (Ikeda, 1996).

In the study of Karin U. Schallreuter, there's a return of systemic enzyme activities to normal after application of a pseudocatalase PC-KUS. The experiment is in vitro, conducted on skin biopsies from patients with vitiligo, whose H_2O_2 concentration in vivo is identified to be in the 10-3 M range. Schallreuter et al. observed that the complete removal of H_2O_2 by PC-KUS led to increased epidermal catalase compared to the high concentration control (10-3 M H_2O_2); however, the removal of H_2O_2 led to decreased reaction rates compared to the low concentration control of H_2O_2 (10-6 M H_2O_2) (Schallreuter, 2004).

While high concentrations of H_2O_2 exhibit significant inhibition on the expression of acetylcholinesterase (AchE), low concentrations of H_2O_2 are discovered to activate AchE. The

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understanding of H_2O_2's two roles in vitiligo-the depigmentation disorder can be applied to the hair graying process as well. With the help of Met to prevent H_2O_2-induced inhibition of tyrosinase, I predict that increasing amounts of L-Methionine combined with low amounts H_2O_2 may maximize the tyrosinase activity. The negative control is adding no Met or PC-KUS, one positive control is adding PC-KUS and the other positive control is adding Met.

2 MATERIALS AND METHODS

All research involving human subjects is approved by local ethics committees and adhered to the Helsinki declaration (Wood, 2009).

Hair follicle cells are divided into four groups, three control groups, and one experimental group, from face-lift surgery. As a positive control group (activation), met and grey hair follicle melanocytes (HFMs) are used (group 1). The H_2O_2 concentration in grey and completely white HHFs is determined to be 10-3 M. Another positive-control group consisted of grey HFMs supplemented with PC-KUS (activation) (group 2). The negative control consisted entirely of grey HFMs with no additives (deactivation) (group 3). The experimental group consisted of Met, PC-KUS, and grey HFMs (group 4).

2.1 Cell Culture of HFMs and Preparation of HHF

Hair follicle melanocytes (HFMs) and human hair follicles (HHFs) are harvested from normal human scalp skin obtained from face-lift surgery with gray or completely white hair (n=2, female; n = 20, 10 female, 10 male respectively). By using a microdissection microscope, Anagen VI, HHFs are isolated. Briefly, scalp skin is divided into small pieces, about 1 cm2 each. From the subcutis, the dermis is isolated.

2.2 Preparation of HHF and HHF Cell Extracts

To prevent denaturation, cell extracts are attained from intact anagen HHFs and cell cultures with the use of a mini pestle, a mortar (- 80°C), and fine sand. After 5-minute centrifugation at 7000 g, cell pellets and HHFs are ground in Tris buffer. The collected supernatant is aliquoted and stored at - 80°C and is analyzed by the Dc-protein assay (Wood, 2009).

2.3 In Situ Immunofluorescence Protein Location in the Separated HHFs

Isolated HHFs are kept in OCTTM compound (Sakura, Eastbourne, UK) at -80°C before conducting experiments with the cryostat as described in previous studies [REF]. To briefly describe it, 20 frozen slides were divided into 4 different groups: 5 frozen slides with 5-μm cryosections of gray HHFs(n=3) and shafts are added narrowband UVB-activated pseudocatalase PC-KUS. 5 frozen slides are added extra Methionine (10×10-3 M). 5 frozen slides are added both PC-KUS and extra Methionine. 5 frozen slides are added nothing. The 20 frozen slides are air-dried at room temperature, settled in ice-cold methanol, and blocked-in normal donkey serum, finally washed in phosphate-buffered saline (PBS). Methionine sulfoxide reductase A (MSRA) is detected through a polyclonal rabbit anti-human antibody and then incubated at room temperature. Methionine sulfoxide reductase B (MSRB) detection utilizes a monoclonal mouse anti-human antibody (Autogen Bioclear) and then incubated overnight at 4°C. Following the above, the slides are washed with PBS, air-dried, and then incubated at room temperature with a fluorescent secondary antibody (conjugated donkey anti-rabbit or anti-mouse) with a wash with PBS for 3 times, air-dried, and mounted in Vectashield Mounting Medium containing DAPI (4',6-diamidino-2-phenylindole) in order to identify specific nuclear. Slides are viewed under a Leica DRMIB/E fluorescence microscope, and images are captured with a digital camera, and the imaging software facilitates the observation of imported images on the computer. (Wood, 2009)

2.4 Western Blot

According to the description above, gray and completely white HHF extracts are obtained. Before loading onto a polyacrylamide gel for protein separation, sample buffer (10% SDS, mercaptoethanol, glycerol, and 0.5 M Tris/HCl) is added to the supernatants. The polyacrylamide gel is electroblotted onto a PVDF membrane before any nonspecific binding sites are blocked by immersion at room temperature of the membrane in a gelatin/ TBS-Tween buffer blocking solution. After this step, the primary antibodies are incubated overnight at room temperature in buffer. The antibodies, rabbit antiMSRA, rabbit anti-MSRB, mouse anti-catalase, and goat anti-actin are used. After being washed for 40 min in buffer, the blot is next incubated for 1 h at

room temperature with an antibody listed above. Using moderated enhanced chemiluminescence (ECL) fixed on a film sheet, visualization of the specific protein bands is performed (Wood, 2009).

2.5 Determination of Tyrosinase Activity

The rate of L-Dopachrome formation, measured by a spectrophotometer with optical density of 475 nm, with L-tyrosine as substrate, is utilized to determine tyrosinase action. The rate of reaction is determined in a linear period of 2 min. Since 1×10-3 M L-tyrosine produces 1×10-3 M H_2O_2, 3×10-3 M L-tyrosine is added. Thus, H_2O_2 is produced with a one-to-one ratio, that is, 3×10-3 M. The experiments are performed in the presence of different L-Methionine concentrations (6×10-3 M) and the amount of PC-KUS added (6×10-3 M) in groups 1, 2, and 4. All experiments are conducted twice. Since Met-tyrosinase is activated by L-Dopa, L-Dopachrome formation is followed and recorded from L-Dopa at 570 nm. With a microplate reader, L-Dopachrome will be determined every 2 min over 15 min (Wood, 2009).

2.6 Determination of MSRA and MSRB Enzyme Activities

Consistent with the explanation above, from isolated HHFs for HFMs and other HHFs cells, complete HHFs extracts are acquired. In brief, the reaction mixture contains cell extract and [14C] Methionine sulfoxide, with dithiothreitol (DTT) functioning as an electron donor. Reactions are incubated for 1h at room temperature. Then reaction product is applied to a TLC silica gel plate and graphed by a chromatographer in “isopropanol: formic acid: water” (20:1:10). Ninhydrin is used to detect L-Methionine and Met-S=O. Radiolabeled and scraped from the TLC plates, [14C] L-Methionine spots are added to scintillation fluid and recorded on the 14C channel in a Packard Tricarb Liquid Scintillation Counter. The formation of L-Methionine is standardized to mmol·mg-1 ·(0.5h)-1 of protein (Wood, 2009).

2.7 In Vitro and in Vivo FT-Ramen Spectroscopy for Detection of H2O2, Met-S=O in H2O2-Oxidized Tyrosinase and Gray Hair

Human white hair shafts from the scalp hair are cut into pieces and a FT-Raman spectroscopy is used to analyze it. A Bruker RFS spectrometer together with a liquid-nitrogen-cooled germanium detector help acquire FT-Raman spectra. Near-infrared excitation is produced by a Nd33+: YAG laser. Each spectrum is accumulated over with scans and a resolution of 4 cm-1. Detection of H_2O_2 is by perceiving the O=O stretch. Met-S=O is visualized too. Tyrosinase (10×10-3 M) are lyophilized and measured as solids.

3 RESULTS

There is no H_2O_2 detectable in group 1(HFMs, Met) while there is a presence of 10-3 M H_2O_2 concentrations in group 3(HFMs). The presence of Met-S=O is higher in group 3 than in groups 1 and 2(HFMs, PC-KUS). Results by immunofluorescence and Western blot analysis show that catalase, MSRA and MSRB protein expressions are weaker in group 3 than it is in group 1 and group 2. Tyrosinase in group 1(HFMs, Met) shows activity larger than the negative control group (group 3, nothing added). Tyrosinase in group 2(HFMs, PC-KUS) shows activity larger than the negative control group.

Based on Wood et al's study (2009) (Wood, 2009), H_2O_2 is assigned at 875 cm-1 based on the O=O stretch and Met-S=O is assigned at 1030 cm-1. H_2O_2 oxidized Met to Met-S=O. MSRA and MSRB's function is to repair oxidized Methionine Met-S=O. However, aging decreases the two enzymes' level in the body, MSRA and MSRB are not enough for repairing Met-S=O. Added Met formed Met-S=O with H_2O_2; therefore, added Met reduced or used up H_2O_2. In this way, the inhibition of H_2O_2 could be prevented. As described in possible result 2, Met-S=O isn't all turned back into Met. Thus, gradually melanogenesis is declined. H_2O_2 of low concentration (10-6 M) activated tyrosinase increasing the maximum reaction rates/5min for more than 2 fold over control values in the absence of any H_2O_2. Rates for tyrosinase/5min across the range of H_2O_2 from 0 to 40×10-3 M increased from 49 μmol tyrosinase/5min to 105 μmol tyrosinase/5min.

3.1 Possible Result 1

Experimental group (HFMs, Met, PC-KUS) shows a strong expression of catalase, MSRA, and MSRA. Reaction rate of tyrosinase/5min increases over 4-fold.

Met added scavenged H_2O_2 by forming Met-S=O; and PC-KUS decreases concentration of H_2O_2, leading to a low concentration of H_2O_2 of 50×10-3 M. Tyrosinase is activated.

3.2 Possible Result 2

Experimental group (HFMs, Met, PC-KUS) shows a weaker expression of catalase, MSRA, and MSRA. Reaction rate of tyrosinase/5min increases over 1.5-fold.

Met or PC-KUS is excessive to completely react with H_2O_2. H_2O_2 is absent, making little difference to reaction rate and activity of tyrosinase.

Table 1 Possible Results.

Group (HFMs, 10^{-3} M H_2O_2)	Result 1	Result 2
Group 1(+ Methionine)	++	++
Group 2(+ pseudocatalase)	++	++
Group 3(raw)	-	-
Group 4(+Methionine, pseudocatalase)	+++	+

Note. Table 1 shows the two possible results of the experiment. For result 1, the activation is obvious; for result 2, the effect of combining is not as good as that of separating. "+" represents an increase in tyrosinase activity. "-" represents a decrease in tyrosinase activity. "+-" represents not significantly different from negative control. "?" represents uncertain results.

4 DISCUSSION

This is the first research on the combined functioning of H_2O_2 and Met.

The result of group 1 can be attained from the interpretation that free Methionine alters Methionine on tyrosinase. There's Methionine on tyrosinase. H_2O_2 oxidizes the Methionine and thus inhibits the enzyme. However, if extra Methionine is added, it will be added Methionine which is oxidized instead of Methionine on tyrosinase.

The result of group 2 is obtained on the analogy of PC-KUS's treatment of vitiligo (Schallreuter, 2004). Based on the similarity in the pathology of vitiligo and hair graying, pseudocatalase is supposed to affect tyrosinase similarly as it does to acetylcholinesterase (AchE), whose deactivation is critical for vitiligo. Although it's certain that a low concentration of H_2O_2 (10-6 M) activates AchE, the mechanism is unknown.

In possible result 2, H_2O_2 is excessive, making PC-KUS and Methionine react completely. There may be a slight decrease in H_2O_2 concentration, but the change is not enough for an obvious increase in tyrosinase activity.

5 CONCLUSION

In summary, the research proves the activation of H_2O_2 and Met to tyrosinase, respectively, and it investigates the validity of H_2O_2 and Met to tyrosinase activity in combination. My study's findings will indicate whether the activity of tyrosinase activated by H_2O_2 and Met reaches a higher level than when they are used alone. My research findings may provide a solution to the problem of hair graying. It may be possible to remain "perpetually young and vital" for a longer period.

Even though low concentrations of H_2O_2 can activate enzymes, the mechanism by which it works is unknown. Experiments only demonstrate that increasing the concentration of H_2O_2 within a certain range activates enzymes more and more effectively; and that activation terminates when a certain amount is exceeded. Additional experiments are required to determine the precise ultimate quantity, which may vary between enzymes. Additionally, while pseudocatalase acts similarly to natural catalase in that it degrades H_2O_2 to water and oxygen, the effect of PC-KUS treatment on hair graying requires further investigation.

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