Aspirin induces Nrf2-mediated transcriptional activation of haem oxygenase-1 in protection of human melanocytes from H_{2}O_{2}-induced oxidative stress

Zhe Jian #, Lingzhen Tang #, Xiuli Yi, Bangmin Liu, Qian Zhang, Guannan Zhu, Gang Wang, Tianwen Gao *, Chunying Li *

Department of Dermatology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China

Received: October 13, 2015; Accepted: January 15, 2016

Abstract

The removal of hydrogen peroxide (H_{2}O_{2}) by antioxidants has been proven to be beneficial to patients with vitiligo. Aspirin (acetylsalicylic acid, ASA) has antioxidant activity and has great preventive and therapeutical effect in many oxidative stress-relevant diseases. Whether ASA can protect human melanocytes against oxidative stress needs to be further studied. Here, we investigated the potential protective effect and mechanisms of ASA against H_{2}O_{2}-induced oxidative injury in human melanocytes. Human melanocytes were pre-treated with different concentrations of ASA, followed by exposure to 1.0 mM H_{2}O_{2}. Cell apoptosis, intracellular reactive oxygen species (ROS) levels were evaluated by flow cytometry, and cell viability was determined by an Cell Counting Kit-8 assay. Total and phosphorylated NRF2 expression, NRF2 nuclear translocation and antioxidant response element (ARE) transcriptional activity were assayed with or without Nrf2-siRNA transfection to investigate the possible molecular mechanisms. Concomitant with an increase in viability, pre-treatment of 10-90 \mu mol/l ASA resulted in decreased rate of apoptotic cells, lactate dehydrogenase release and intracellular ROS levels in primary human melanocytes. Furthermore, we found ASA dramatically induced NRF2 nuclear translocation, enhanced ARE-luciferase activity, increased both p- NRF2 and total NRF2 levels, and induced the expression of haem oxygenase-1 (HO-1) in human melanocytes. In addition, knockdown of Nrf2 expression or pharmacological inhibition of HO-1 abrogated the protective action of ASA on melanocytes against H_{2}O_{2}-induced cytotoxicity and apoptosis. These results suggest that ASA protects human melanocytes against H_{2}O_{2}-induced oxidative stress via Nrf2-driven transcriptional activation of HO-1.

Keywords: aspirin • melanocyte • nuclear factor E2-related factor 2 • haem oxygenase-1 • hydrogen peroxide • oxidative stress • vitiligo

Introduction

Vitiligo is an acquired depigmenting disorder of great cosmetic importance characterized by loss of melanocytes in the localized epidermis. It affects approximately 0.5–1% of the world population without any racial, sexual predilection in prevalence [1]. Despite continuous progress toward an elucidation of the biochemical, genetic and immunopathological pathways in vitiligo over the past decades, the precise pathogenesis remains elusive [2]. Recently, epidermal oxidative stress has been documented in vitiligo patients [3]. There were several lines of evidence showing impairment in the antioxidant system and reactive oxygen species (ROS)-mediated damage in melanocytes as well as in the whole body of vitiligo patients, supporting a free-radical-mediated damage as an important pathogenic event in melanocyte degeneration [4–9]. Thus, inhibition of oxidative damage may represent prime targets for development of novel therapeutic agents for vitiligo.

Aspirin (acetylsalicylic acid, ASA) is the most common type of nonsteroidal anti-inflammatory drugs (NSAIDs) and is widely used to treat inflammation and pain. Although many of the pharmacological properties of ASA and other NSAIDs are related to their inhibition of prostaglandin biosynthesis, some of their beneficial therapeutic effects are not completely understood. Recent studies have indicated that ASA has free radical scavenging property and is capable of directly protecting different cells from the deleterious effects of oxidative stress [10–13]. The animal and clinical research also showed that

#The authors were equally contributed to this work.
*Correspondence to: Tianwen GAO, M.D., Ph.D. and Chunying LI, M.D., Ph.D.
E-mails: gaotw75401@hotmail.com and lichying@fmmu.edu.cn

© 2016 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

doi: 10.1111/jcmm.12812
untreated control cells, F: forward; R: reverse.

ASA could protect melanocytes from vitiligo patients against oxidative stress and increase their proliferative capacities [19]. Furthermore, a preliminary clinical study has found that the disease activity was suspended in all ASA-treated vitiligo patients [20]. Although Zailaie's studies are inspiring, the research has not been replicated to determine whether ASA can protect melanocytes against oxidative stress.

Although extensive studies have been carried out, the mechanism underlying ASA's antioxidative action remains unclear. Reports have claimed ASA could increase the expression of antioxidative protein haem oxygenase-1 (HO-1), and enhance the nitric oxide production [21, 22]. Nitric oxide are well-known stimulant of nuclear factor E2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway [23]. Haem oxygenase-1 is the downstream molecule of Nrf2-ARE pathway, and degrades haem to CO, iron and biliverdin [21, 23, 24]. Therefore, the antioxidative action of ASA is probably by activating of Nrf2-ARE pathway.

Nrf2-ARE pathway is a central part of molecular mechanisms governing the protective function of phase II detoxification and antioxidative enzymes against oxidative stress [25]. Under normal circumstances, Nrf2 is sequestered in the cytoplasm by a cytosolic repressor Kelch-like ECH-associated protein 1 (Keap1). When inducers disrupt its complex with Keap1, NRF2 translocates to the nucleus, binds to ARE and initiates the transcription of genes coding for detoxifying enzymes and cytoprotective proteins. Therefore, any compound which possesses the capacity to alter the interaction of Nrf2–Keap1 could cause the translocation of NRF2 into nuclear to exert some cytoprotective function. Our previously studies have confirmed that Nrf2-ARE pathway plays an important role in human melanocytes against hydrogen peroxide (H$_2$O$_2$)-induced oxidative injury, and its main effector is HO-1 [26]. Further research demonstrated that the activation of Nrf2-ARE pathway was defective in melanocytes from vitiligo patients, endowing the melanocytes more vulnerable to oxidative stress [27]. Thus, regulation of Nrf2-ARE pathway may be a promising target for vitiligo treatment.

Given the above, the purpose of this study was to investigate whether ASA exerts a protective effect on human melanocytes against H$_2$O$_2$-induced oxidative damage and the underlying molecular mechanism involved. Here, we show that ASA protected human melanocytes against H$_2$O$_2$-induced oxidative stress through activation of Nrf2-ARE pathway and induction of HO-1 expression. These findings corroborate with previous studies that implicate ASA research as a novel therapeutic strategy against vitiligo.

ASA has great preventive and therapeutical effect in many oxidative stress-relevant diseases [12–18]. Zailaie first showed that low-dose ASA could protect melanocytes from vitiligo patients against oxidative stress and increase their proliferative capacities [19]. Furthermore, a preliminary clinical study has found that the disease activity was suspended in all ASA-treated vitiligo patients [20]. Although Zailaie's studies are inspiring, the research has not been replicated to determine whether ASA can protect melanocytes against oxidative stress.

Although extensive studies have been carried out, the mechanism underlying ASA's antioxidative action remains unclear. Reports have claimed ASA could increase the expression of antioxidative protein haem oxygenase-1 (HO-1), and enhance the nitric oxide production [21, 22]. Nitric oxide are well-known stimulant of nuclear factor E2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway [23]. Haem oxygenase-1 is the downstream molecule of Nrf2-ARE pathway, and degrades haem to CO, iron and biliverdin [21, 23, 24]. Therefore, the antioxidative action of ASA is probably by activating of Nrf2-ARE pathway.

Nrf2-ARE pathway is a central part of molecular mechanisms governing the protective function of phase II detoxification and antioxidative enzymes against oxidative stress [25]. Under normal circumstances, Nrf2 is sequestered in the cytoplasm by a cytosolic repressor Kelch-like ECH-associated protein 1 (Keap1). When inducers disrupt its complex with Keap1, NRF2 translocates to the nucleus, binds to ARE and initiates the transcription of genes coding for detoxifying enzymes and cytoprotective proteins. Therefore, any compound which possesses the capacity to alter the interaction of Nrf2–Keap1 could cause the translocation of NRF2 into nuclear to exert some cytoprotective function. Our previously studies have confirmed that Nrf2-ARE pathway plays an important role in human melanocytes against hydrogen peroxide (H$_2$O$_2$)-induced oxidative injury, and its main effector is HO-1 [26]. Further research demonstrated that the activation of Nrf2-ARE pathway was defective in melanocytes from vitiligo patients, endowing the melanocytes more vulnerable to oxidative stress [27]. Thus, regulation of Nrf2-ARE pathway may be a promising target for vitiligo treatment.

Given the above, the purpose of this study was to investigate whether ASA exerts a protective effect on human melanocytes against H$_2$O$_2$-induced oxidative damage and the underlying molecular mechanism involved. Here, we show that ASA protected human melanocytes against H$_2$O$_2$-induced oxidative stress through activation of Nrf2-ARE pathway and induction of HO-1 expression. These findings corroborate with previous studies that implicate ASA research as a novel therapeutic strategy against vitiligo.

### Materials and methods

Please see details of the following in the Supplementary Materials online.
was performed in triplicate and repeated at least three times.

The amount of formazan produced is directly proportional to the number of living cells. Briefly, primary melanocytes or PIG1 cells were seeded into 96-well plates at an initial density of 2 × 10^4 cells/well with 1.0 mM H_2O_2 in the presence or absence of ASA (10, 30 and 90 μM). To further prove the protective action of ASA against oxidative damage, we determined LDH release rates and the level of intracellular ROS after treatment with 1.0 mM H_2O_2 for 24 hrs in primary melanocytes. After exposure to H_2O_2, LDH release was significantly higher in the H_2O_2-treated cells than in the control cells, indicating that H_2O_2 was toxic to primary human melanocytes. In accordance with CCK-8 assay, H_2O_2 treatment markedly increased the LDH release rate of melanocytes and in contrast, the LDH release rate was decreased by pre-treatment with ASA in a dose-dependent manner (Fig. 1D).

Aspirin reduced H_2O_2-induced leakage of LDH and the level of intracellular ROS in human melanocytes

In this study, we first evaluated the effect of ASA on cell proliferation, cell viability, melanin content and tyrosinase activity of primary human melanocytes. As it can be observed in Figure 1A, melanocytes pre-treated with ASA (10–270 μM) induced cell proliferation in a time-dependent manner, whereas melanocytes pre-treated with 810 μM ASA significantly inhibited cell growth compared to untreated group. However, ASA (10–810 μM) did not affect melanin content and tyrosinase activity (Fig. S1). The morphologic changes of melanocytes showed that treatment of ASA alone for 24 hrs had no significant effect on cell morphology at the concentrations ranging from 10 to 90 μM. However, 810 μM ASA resulted in cytotoxicity, including cellular dendrites shortening and partial cell death (data not shown). Although the result obtained from CCK-8 assay demonstrated that 10–270 μM ASA alone had no significant effect on cell viability (Fig. 1C), given the results of proliferation curves and morphologic changes in melanocytes, we decided to use 10–90 μM ASA for the subsequent experiments.

Our previous work has demonstrated that treatment of melanocytes with 1.0 mM H_2O_2 for 24 hrs is the most appropriate way to induce consistent and high degree of oxidative damage [4, 26]. Here, we investigate whether ASA protects melanocytes from H_2O_2-induced cell death. Primary human melanocytes were treated with 1.0 mM H_2O_2 in the presence or absence of ASA (10, 30 and 90 μM), and the cell viability was assessed by cell morphology and CCK-8 assays. After treatment of 1.0 mM H_2O_2 for 24 hrs, the dendrites of melanocytes shortened or disappeared (Fig. 1B, panels b) and cell viability was decreased to about 41% of the control cells (Fig. 1D). However, pre-treatment with 10–90 μM ASA significantly attenuated H_2O_2-induced oxidative damage in a dose-dependent manner, as represented by a decreased number of injured cellular dendrites (Fig. 1B, panels e–f) and an increased cell viability of 62% greater than the control cells (Fig. 1D).

Results

Aspirin attenuated H_2O_2-induced cytotoxicity in human melanocytes

In this study, we first evaluated the effect of ASA on cell proliferation, cell viability, melanin content and tyrosinase activity of primary human melanocytes. As it can be observed in Figure 1A, melanocytes pre-treated with ASA (10–270 μM) induced cell proliferation in a time-dependent manner, whereas melanocytes pre-treated with 810 μM ASA significantly inhibited cell growth compared to untreated group. However, ASA (10–810 μM) did not affect melanin content and tyrosinase activity (Fig. S1). The morphologic changes of melanocytes showed that treatment of ASA alone for 24 hrs had no significant effect on cell morphology at the concentrations ranging from 10 to 90 μM. However, 810 μM ASA resulted in cytotoxicity, including cellular dendrites shortening and partial cell death (data not shown). Although the result obtained from CCK-8 assay demonstrated that 10–270 μM ASA alone had no significant effect on cell viability (Fig. 1C), given the results of proliferation curves and morphologic changes in melanocytes, we decided to use 10–90 μM ASA for the subsequent experiments.

Our previous work has demonstrated that treatment of melanocytes with 1.0 mM H_2O_2 for 24 hrs is the most appropriate way to induce consistent and high degree of oxidative damage [4, 26]. Here, we investigate whether ASA protects melanocytes from H_2O_2-induced cell death. Primary human melanocytes were treated with 1.0 mM H_2O_2 in the presence or absence of ASA (10, 30 and 90 μM), and the cell viability was assessed by cell morphology and CCK-8 assays. After treatment of 1.0 mM H_2O_2 for 24 hrs, the dendrites of melanocytes shortened or disappeared (Fig. 1B, panels b) and cell viability was decreased to about 41% of the control cells (Fig. 1D). However, pre-treatment with 10–90 μM ASA significantly attenuated H_2O_2-induced oxidative damage in a dose-dependent manner, as represented by a decreased number of injured cellular dendrites (Fig. 1B, panels e–f) and an increased cell viability of 62% greater than the control cells (Fig. 1D).

Aspirin reduced H_2O_2-induced leakage of LDH and the level of intracellular ROS in human melanocytes

To further prove the protective action of ASA against oxidative damage, we determined LDH release rates and the level of intracellular ROS after treatment with 1.0 mM H_2O_2 for 24 hrs in primary human melanocytes. After exposure to H_2O_2, LDH release was significantly higher in the H_2O_2-treated cells than in the control cells, indicating that H_2O_2 was toxic to primary human melanocytes. In accordance with CCK-8 assay, H_2O_2 treatment markedly increased the LDH release rate of melanocytes and in contrast, the LDH release rate was decreased by pre-treatment with ASA in a dose-dependent manner (Fig. 2A).
To determine whether ASA modulates the level of ROS generated in human melanocytes in response to H$_2$O$_2$ treatment, we measured the intracellular level of ROS by using fluorescent probe DCFH-DA. As shown in Figure 2B and C, treatment with H$_2$O$_2$ increased the intracellular level of ROS, as indicated by a higher fluorescence intensity in treated cells compared to untreated control cells. The results shown in A and C are presented as the mean ± S.D. of three independent experiments. *P < 0.01 versus H$_2$O$_2$-treated cells, #P < 0.05 and ##P < 0.01 versus untreated control cells.

![Figure 2](image2.png)

Fig. 2 Effects of aspirin on LDH release and intracellular ROS levels in primary human melanocytes following H$_2$O$_2$ challenge. (A) LDH leakage of human melanocytes was determined by an LDH release assay. (B) Representative results for ROS production after pre-treatment. (C) The fluorescence intensity of the cells was calculated relative to that of untreated control cells.

To determine whether ASA modulates the level of ROS generated in human melanocytes in response to H$_2$O$_2$ treatment, we measured the intracellular level of ROS by using fluorescent probe DCFH-DA. As shown in Figure 2B and C, treatment with H$_2$O$_2$ increased the intracellular level of ROS, as indicated by a higher fluorescence intensity in treated cells compared to untreated control cells. The results shown in A and C are presented as the mean ± S.D. of three independent experiments. *P < 0.01 versus H$_2$O$_2$-treated cells, #P < 0.05 and ##P < 0.01 versus untreated control cells.

![Figure 3](image3.png)

Fig. 3 Inhibitory effect of aspirin on H$_2$O$_2$-induced apoptosis in primary human melanocytes. Cells were pre-treated with or without aspirin at the indicated concentrations for 24 hrs and then incubated in the presence or absence of 1.0 mM H$_2$O$_2$ for a further 24 hrs. Cellular apoptosis was assayed by annexin V-FITC and PI counterstaining and analyzed with flow cytometry. (A) The original representative flow cytometry figures. (B) The apoptosis rates of human melanocytes. The data are presented as the mean ± S.D. of three independent experiments. *P < 0.01 versus H$_2$O$_2$-treated cells and #P < 0.01 versus untreated control cells.
H$_2$O$_2$ induced a robust increase in DCF fluorescence level (1.33-fold compared to the control group). Pre-treatment with 10–90 μM ASA for 24 hrs significantly reduced the H$_2$O$_2$-induced ROS accumulation in primary human melanocytes.

Aspirin protects human melanocytes from H$_2$O$_2$-induced apoptosis

To investigate whether ASA protects against H$_2$O$_2$-induced apoptosis, primary human melanocytes were pre-treated with different concentrations of ASA (10, 30 and 90 μM) for 24 hrs before 1.0 mM H$_2$O$_2$ induction. To quantify the rate of cell apoptosis, flow cytometry analysis was performed with both Annexin-V-FITC and PI staining, the average percentages of apoptotic cells were determined as the percentage of Annexin-V-FITC stained cells in both Annexin-V-FITC and PI treated cells, the results are represented in Figure 3. Typical examples are shown in Figure 3A. After H$_2$O$_2$ treatment, the percentage of apoptotic cells increased to 20% from a baseline of 5.2% in the control group, whereas pre-treatment of 10–90 μM ASA markedly inhibited H$_2$O$_2$-induced apoptotic death and reduced the percentages of Annexin-V-stained cells up to 7.8%.

Aspirin dramatically induces Nrf2-mediated transcriptional activation of ARE and HO-1, and increased Nrf2 nuclear translocation, Nrf2 and p-Nrf2 expression in human melanocytes

To rule out the roles of Nrf2-driven transcriptional activation, the mRNA expression profiles of genes regulated by Nrf2 were examined by Real-time PCR amplification from cells treated with or without ASA. In Figure 4A, treatment with ASA induced an early transcriptional activation of various Nrf2-driven mRNAs, including HO-1, NQO1, GCLC and GCLM. Interestingly, pre-treatment with ASA predominantly induced much earlier and augmented expression of HO-1 mRNA only among other Nrf2-driven transcripts. However, transcriptional activation of other Nrf2-driven transcripts, including as NQO1, GCLC and GCLM were not changed. To further determine whether up-regulation of HO-1 by aspirin treatment is caused by Nrf2 pathway, we transfected PIG1 cells with Nrf2 siRNA for 24 hrs, followed by treatment with 90 μM ASA for additional 24 hrs. As shown in Figure 4A, transfection with Nrf2 siRNA significantly reduced ASA-mediated HO-1 mRNA expressions compared to control.

To test our hypothesis that ASA may down-regulate intracellular ROS and protect melanocytes from H$_2$O$_2$-induced oxidative injury by activating Nrf2-ARE pathway, we investigated the effects of ASA on ARE transcriptional activity, Nrf2 nuclear translocation, p-Nrf2 and Nrf2 accumulation. As shown in Figure 4B, ASA significantly increased ARE-luciferase activity in a dose-dependent manner. Immunofluorescent analysis revealed that treatment of cells with 90 μM ASA for 24 hrs induced Nrf2 nuclear translocation and accumulation (Fig. 4C). To further confirm whether ASA could activate Nrf2 in human melanocytes, PIG1 cells were transfected with Nrf2 siRNA for 48 hrs, followed by treatment with 90 μM ASA for additional 24 hrs, and the levels of Nrf2 and p-Nrf2 proteins were determined by Western blot analysis. We found that ASA treatment significantly increased the protein levels of both p-NRF2 and Nrf2, whereas transfection with Nrf2 siRNA reduced ASA-mediated up-regulation of p-Nrf2 and Nrf2 protein expressions (Fig. 4D and E). However, cells treated with NC-siRNA had no effect (Fig. 4D and E). These results demonstrated that ASA could induce HO-1 expression by activating Nrf2-ARE pathway.

Knockdown of Nrf2 expression abrogated the protection action of Aspirin on PIG1 cells against H$_2$O$_2$-induced cytotoxicity and oxidative stress

Next, we investigated whether Nrf2-driven transcriptional activation was directly involved in eliciting the protective effect of ASA against...
Fig. 5 Effect of Nrf2 siRNA and aspirin on cell viability, apoptosis and intracellular ROS level induced by H$_2$O$_2$ in PIG1 cells. (A) Viability of PIG1 cells pre-treated with Nrf2 siRNA, NC-siRNA and/or aspirin (ASA; 90 µM) was determined with CCK-8 assay 24 hrs after exposure to 1.0 mM H$_2$O$_2$ ($n = 3$). (B) The apoptosis rates of PIG1 cells. (C) The effect of Nrf2 siRNA, NC-siRNA and aspirin on H$_2$O$_2$-induced morphological changes in PIG1 cells. (D) The original representative flow cytometry figures. (E) Representative results for ROS production after pre-treatment. (F) The fluorescence intensity of the cells was calculated relative to that of untreated control cells. The data are presented as the mean ± S.D. of three independent experiments. ** $P < 0.01$ and $P > 0.05$ versus H$_2$O$_2$-treated cells; $P < 0.01$ versus untreated control cells. $P > 0.05$ versus H$_2$O$_2$+ ASA treated cells.
Fig. 6 Effect of Znpp and aspirin on cell viability, apoptosis and intracellular ROS level induced by H$_2$O$_2$ in PIG1 cells. (A) Viability of PIG1 cells pre-treated with Znpp and/or aspirin (ASA; 90 μM) was determined with CCK-8 assay 24 hrs after exposure to 1.0 mM H$_2$O$_2$ (n = 3). (B) The apoptosis rates of PIG1 cells. (C) The original representative flow cytometry figures. (D) Representative results for ROS production after pre-treatment. (E) The fluorescence intensity of the cells was calculated relative to that of untreated control cells. The data are presented as the mean ± S.D. of three independent experiments. **P < 0.01 and #P > 0.05 versus H$_2$O$_2$-treated cells; ##P < 0.01 versus untreated control cells.
48 hrs and followed by addition of 1.0 mM H2O2 in the presence of 1.0 mM H2O2 for another 24 hrs. The data demonstrated that Znpp 1316 critical role in the pathogenesis of vitiligo. An extremely high level of oxidative stress plays a role in the pathogenesis of vitiligo.

Discussion

Accumulating evidence indicates that oxidative stress plays a critical role in the pathogenesis of vitiligo. An extremely high level of H2O2 in lesional skin has been reported in vitiligo patients and suggested to be responsible for the disappearance of melanocytes [3]. Anti-oxidative-stress-based remedies are promising strategies for vitiligo; however, the drug development is being hampered by limited understanding of the pharmaceutically relevant molecular targets and structures in most oxidative stress-relevant diseases.

Acetylsalicylic acid, the most widely used NSAID, had been reported to have free radical scavenging property [10, 11] and to be a potent antioxidant in many oxidative stress-related diseases [12–18]. Recently, Zailaie reported that ASA was potently beneficial to vitiligo and other oxidative-relevant diseases.

Nrf2-mediated HO-1 activation is required in protective effect of Aspirin against H2O2-induced cytotoxicity and oxidative stress

To make sure that the increased expressions of HO-1 was needed in the antioxidative property of ASA in human melanocytes against H2O2-derived oxidative injury, we used Znpp (10 μM), the specific inhibitor of HO-1, to pre-treat primary human melanocytes for 24 hrs. The cells were then treated with 90 μM ASA for 24 hrs followed by 1.0 mM H2O2 for another 24 hrs. The data demonstrated that Znpp alleviated cellular protection afforded by ASA against H2O2-mediated toxicity (Fig. 6A). We also verified that pre-treatment with ASA resulted in a marked decrease in apoptotic cells, which was markedly suppressed by addition of Znpp (Fig. 6B and C). In addition, reduction in ROS level by pre-treatment with ASA was also abrogated by the addition of Znpp (Fig. 6D and E).

In conclusion, we have demonstrated that ASA protected melanocytes from oxidative injury by activation of Nrf2-ARE pathway and induction of HO-1 expression. Thus, ASA might represent a promising new therapeutic agent for vitiligo. More experimental and clinical research is needed to further confirm the exact effect and mechanisms of ASA in the treatment of vitiligo patients.
Acknowledgements

This study was supported by the National Natural Science Foundation of China (Nos. 81402599, 81130032 and 81373844). The authors would like to thank Dr. Caroline Le Poole for providing the immortalized human epidermal melanocyte cell line PIG1. The authors also thank Dr. Ariel Hernandez for proofreading the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

References

1. Halder RM, Chappell JL. Vitiligo update. Semin Cutan Med Surg. 2009; 28: 86–92.
2. Schallreuter KU, Bahadoran P, Ricardo M, et al. Vitiligo pathogenesis: autoimmune disease, genetic defect, excessive reactive oxygen species, calcium imbalance, or what else? Exp Dermatol. 2008; 17: 139–40, 141–60.
3. Ricardo M. Bastonini. A new view of vitiligo: looking at normal appearing skin. J Invest Dermatol. 2015; 135: 1713–4.
4. Zhou Z, Li CY, Li K, et al. Decreased methionine sulphoxide reductase A expression renders melanocytes more sensitive to oxidative stress: a possible cause for melanocyte loss in vitiligo. Br J Dermatol. 2009; 161: 504–9.
5. Guan CP, Wei XD, Chen HY, et al. Abnormal nuclear translocation of nuclear factor-E2 related factor 2 in the lesion of vitiligo. Zhongguo Yi Xue Za Zhi. 2008; 88: 2403–6.
6. Schallreuter KU, Elwary SM, Gibbons NC, et al. Activation/deactivation of acetylcholinesterase by H2O2: more evidence for oxidative stress in vitiligo. Biochem Biophys Res Commun. 2004; 315: 502–8.
7. Schallreuter KU, Gibbons NC, Zothner C, et al. Hydrogen peroxide-mediated oxidative stress disrupts calcium binding on calmodulin: more evidence for oxidative stress in vitiligo. Biochem Biophys Res Commun. 2007; 360: 70–5.
8. Schallreuter KU, Gibbons NC, Zothner C, et al. Butyrylcholinesterase is present in the human epidermis and is regulated by H2O2: more evidence for oxidative stress in vitiligo. Biochem Biophys Res Commun. 2006; 349: 931–8.
9. Hasse S, Gibbons NC, Rokos H, et al. Perturbed 6-tetrahydrobiopterin recycling via decreased dihydrosperidine reductase in vitiligo: more evidence for H2O2 stress. J Invest Dermatol. 2004; 122: 307–13.
10. Maniqlia FP, Costa JA. Effects of Acetylsalicylic Acid Usage on inflammatory and oxidative stress markers in hemodialysis patients. Inflammation. 2015; doi: 10.1007/s10753-015-0244-8.
11. Mei L, Daud MK, Ullah N, et al. Pretreatment with salicylic acid and ascorbic acid significantly mitigate oxidative stress induced by copper in cotton genotypes. Environ Sci Pollut Res Int. 2015; 22: 9922–31.
12. Berg K, Langaas M, Ericsson M, et al. Acetylsalicylic acid treatment until surgery reduces oxidative stress and inflammation in patients undergoing coronary artery bypass grafting. Eur J Cardiothorac Surg. 2013; 43: 1154–63.
13. Wu R, Lamontagne D, de Champlain J. Antioxidative properties of acetylsalicylic Acid on vascular tissues from normotensive and spontaneously hypertensive rats. Circulation. 2002; 105: 387–92.
14. El MA, Wu R, de Champlain J. Prevention of hypertension, hyperglycemia and vascular oxidative stress by aspirin treatment in chronically glucose-fed rats. J Hypertens. 2002; 20: 1407–12.
15. Wu R, Laplante MA, De Champlain J. Prevention of angiotensin II-induced hypertension, cardiovascular hypertrophy and oxidative stress by acetylsalicylic acid in rats. J Hypertens. 2004; 22: 793–801.
16. Tauseef M, Sharma KK, Fahim M. Aspirin restores normal baroreflex function in hypercholesterolemic rats by its antioxidative action. Eur J Pharmacol. 2007; 556: 136–43.
17. Tauseef M, Shahid M, Sharma KK, et al. Antioxidative action of aspirin on endothelial function in hypercholesterolaemic rats. Basic Clin Pharmacol Toxicol. 2008; 103: 314–21.
18. Steer KA, Wallace TM, Bolton CH, et al. Aspirin protects low density lipoprotein from oxidative modification. Heart. 1997; 77: 333–7.
19. Zailaie MZ. Short- and long-term effects of acetylsalicylic acid treatment on the proliferation and lipid peroxidation of skin cultured melanocytes of active vitiligo. Saudi Med J. 2004; 25: 1656–63.
20. Zailaie MZ. Decreased proinflammatory cytokine production by peripheral blood mononuclear cells from vitiligo patients following aspirin treatment. Saudi Med J. 2005; 26: 799–805.
21. Grosser N, Abate A, Oberle S, et al. Heme oxygenase-1 induction may explain the antioxidant profile of aspirin. Biochem Biophys Res Commun. 2003; 308: 956–60.
22. Grosser N, Schroder H. Aspirin protects endothelial cells from oxidant damage via the nitric oxide-cGMP pathway. Arterioscler Thromb Vasc Biol. 2003; 23: 1345–51.
23. Mann GE, Rowlands DJ, Li FY, et al. Activation of endothelial nitric oxide synthase by dietary isoflavones: role of NO in Nrf2-mediated antioxidant gene expression. Cardiovasc Res. 2007; 75: 261–74.
24. Polte T, Abate A, Denney PA, et al. Heme oxygenase-1 is a cGMP-inducible endothelial protein and mediates the cytoprotective action of nitric oxide. Arterioscler Thromb Vasc Biol. 2000; 20: 1209–15.
25. Lee JS, Surh YJ. Nrf2 as a novel molecular target for chemoprevention. Cancer Lett. 2005; 234: 171–84.
26. Jian Z, Li K, Liu L, et al. Heme oxygenase-1 protects human melanocytes from H2O2-induced oxidative stress via the Nrf2-ARE

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effect of ASA on Melanogenesis in primary human melanocytes.

Figure S2 The interference efficiency of three different Nrf2-siRNAs.

Data S1 Supplementary materials and methods.
27. Jian Z, Li K, Song P, et al. Impaired activation of the Nrf2-ARE signaling pathway undermines H2O2-induced oxidative stress response: a possible mechanism for melanocyte degeneration in vitiligo. J Invest Dermatol. 2011; 131: 1420–7.

28. Le PI, van den Berg FM, van den Wijngaard RM, et al. Generation of a human melanocyte cell line by introduction of HPV16 E6 and E7 genes. In Vitro Cell Dev Biol Anim. 1997; 33: 42–9.

29. Morita Y, Naka T, Kawazoe Y, et al. Signals transducers and activators of transcription (STAT)-induced STAT inhibitor-1 (SSI-1)/suppressor of cytokine signaling-1 (SOCS-1) suppresses tumor necrosis factor alpha-induced cell death in fibroblasts. Proc Natl Acad Sci USA. 2000; 97: 5405–10.

30. Patrono C. Aspirin and human platelets: from clinical trials to acetylation of cyclooxygenase and back. Trends Pharmacol Sci. 1989; 10: 453–8.

31. Clarke RJ, Mayo G, Price P, et al. Suppression of thromboxane A2 but not of systemic prostacyclin by controlled-release aspirin. N Engl J Med. 1991; 325: 1137–41.

32. Ha CM, Park S, Choi YK, et al. Activation of Nrf2 by dimethyl fumarate improves vascular calcification. Vascul Pharmacol. 2014; 63: 29–36.

33. Mine N, Yamamoto S, Kufe DW, et al. Activation of Nrf2 pathways correlates with resistance of NSCLC cell lines to CBP501 in vitro. Mol Cancer Ther. 2014; 13: 2215–25.

34. Wang W, Wu Y, Zhang G, et al. Activation of the Nrf2-ARE signaling pathway protects the brain from damage induced by epileptic seizure. Brain Res. 2014; 1544: 54–61.

35. Wang C, Li C, Peng H, et al. Activation of the Nrf2-ARE pathway attenuates hyperglycemia-mediated injuries in mouse podocytes. Cell Physiol Biochem. 2014; 34: 891–902.

36. Kolamunne RT, Dias IH, Vernallis AB, et al. Nrf2 activation supports cell survival during hypoxia and hypoxia/reoxygenation in cardiomyoblasts; the roles of reactive oxygen and nitrogen species. Redox Biol. 2013; 1: 418–26.

37. Wink DA, Hanbauer I, Krishna MC, et al. Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. Proc Natl Acad Sci USA. 1993; 90: 9813–7.

38. Chang J, Rao NV, Markowitz BA, et al. Nitric oxide donor prevents hydrogen peroxide-mediated endothelial cell injury. Am J Physiol. 1996; 270: L931–40.

39. Dragomir E, Manduteanu I, Voinea M, et al. Aspirin rectifies calcium homeostasis, decreases reactive oxygen species, and increases NO production in high glucose-exposed human endothelial cells. J Diabetes Complications. 2004; 18: 289–99.