Observational Study

Medicine®

OPEN

Mechanism underlying the effect of SO_2-induced oxidation on human skin keratinocytes

Junqin Liang, MD[a], Lina Liu, MS[b], Xiaojing Kang, MD, PhD[a,*], Fengxia Hu, MS[a], Lidan Mao, BS[a]

Abstract

This study aimed to study the effect and mechanism of action of SO_2-induced oxidation on human skin keratinocytes.

Different concentrations of SO_2 derivatives (0, 25, 50, 100, 200, 400, and 800 μM) were used for treating HaCaT keratinocytes for 24 hours. MTT was used to evaluate the effect of each concentration on cell proliferation. HaCaT cells were randomly divided into control and SO_2 groups. The control group received no treatment, whereas the SO_2 group was treated with SO_2 derivatives of selected concentrations for 24 hours. The levels of reactive oxygen species (ROS), malondialdehyde (MDA), and superoxide dismutase (SOD), tumor necrosis factor TNF-α (TNF-α), and interleukin-1 (IL-1-β) in cell supernatants were detected using enzyme-linked immunosorbent assay. Real-time polymerase chain reaction was used to detect the expression of nuclear transcription factor (Nrf2) and heme oxygenase (HO)-1 mRNA. The Western blot analysis was used to test the expression levels of Nrf2, HO-1, activated caspase-3, Bcl-2, Bax, IκB, NF-κB p65 (p65), ERK1/2, p38, phospho-NF-κB p65 (p-p65), p-ERK1/2, and p-p38.

SO_2 derivatives (100, 200, 400, and 800 μM) could inhibit cell proliferation. SO_2 derivatives increased the level of ROS, MDA, TNF-α, IL-1β, Nrf2, HO-1, and p-p65/p65 and decreased the levels of SOD, IκB, p-ERK1/2/ERK1/2, and p-p38/p38 compared with the control group, but they had no effect on the levels of caspase-3, Bcl-2, and Bax.

SO_2 could inhibit the proliferation of human skin keratinocytes and induce oxidative stress and inflammation via the activation of the NF-κB pathway to inhibit the ERK1/2 and p38 pathways.

Abbreviations: AMPK = Adenosine 5'-monophosphate (AMP)-activated protein kinase, Bax = BCL2-Associated X, Bcl-2 = B-cell lymphoma-2, Caspase-3 = cysteinyl aspartate-specific proteinase-3, ERK-1/2 = extracellular regulated protein kinases-1/2, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, HO-1 = heme oxygenase-1, HRP = Horseradish peroxidase, IκB = inhibitor of nuclear factor kappa-B, IL-1β = interleukin-1β, MDA = malondialdehyde, NF-κB = nuclear factor kappa-B, Nrf2 = nuclear transcription factor, PM = particulate matter, ROS = reactive oxygen species, SOD = superoxide dismutase, TNF-α = tumor necrosis factor TNF-α.

Keywords: human skin keratinocytes, inflammatory injury, mechanism, oxidative damage, SO_2 derivative

1. Introduction

Air pollution has become the most serious environmental issue in China, especially the haze that is the primary pollutant. The composition of haze is quite complex and can be divided into 2 categories (gaseous state and aerosol) according to its existing state. The gaseous pollutants mainly include SO_2, NO_x, CO, and O_3, while aerosol pollutants comprise atmospheric particulates (mainly including PM2.5 and PM10). Besides endangering traffic safety, haze can also cause harm to human respiratory and cardiovascular systems.[1,2] Recent studies have found that some haze components can also cause skin damage. Magnani et al[3] used concentrated atmospheric particulates to treat the reconstructed human epidermal tissue model and found that atmospheric particulates penetrated into the skin tissue and increased the production of reactive oxygen species (ROS), leading to lipid peroxidation. Also, nuclear transcription factor-κB (NF-κB) increased the expression of cyclooxygenase-2 and cytochrome P450, induced inflammatory reaction, and caused apoptosis of skin epidermal cells after exposure to atmospheric particulates.[3] O_3 could damage skin cells by facilitating the production of ROS, free radicals or free radical–dependent toxin products, and nonradical molecules such as aldehydes.[4] Short-term exposure to NO_2 in patients with ectopic dermatitis led to changes in the skin surface and damaged skin barrier function.[5] Exposure to NO_2 in the environment was also associated with an increase in the number of freckles on the face.[6] SO_2 is an important part of the haze. Recent studies have shown that SO_2 may also be associated with skin diseases. Foreign studies showed that a high concentration of SO_2 positively correlated with the occurrence of pruritus and rash.[7] Kathuria et al[8] found that childhood eczema was also associated with higher annual average SO_2 levels. A 2-year

Editor: Arjun Ballal.

JL and LL contributed equally to this work.

This work was supported by the 2018 Chinese Medical Association-L'Oreal Chinese Healthy Skin/Hair Research Project (2018151101).

The authors of this work have no conflicts of interest to disclose.

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

[a] Department of Dermatology, People's Hospital of Xinjiang Uygur Autonomous Region, Urumqi, Xinjiang, [b] Departmental of medical research, Naval Medical Center of PLA, Shanghai, China.

[*] Correspondence: Xiaojing Kang, Department of Dermatology, People's Hospital of Xinjiang Uygur Autonomous Region, No. 91 Tianchi Road, Tianshan District, Urumqi 830001, Xinjiang, China (e-mail: drkangxj666@163.com).

Copyright © 2020 the Author(s). Published by Wolters Kluwer Health, Inc. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial License 4.0 (CCBY-NC), where it is permissible to download, share, remix, transform, and buildup the work provided it is properly cited. The work cannot be used commercially without permission from the journal.

How to cite this article: Liang J, Liu L, Kang X, Hu F, Mao L. Mechanism underlying the effect of SO_2-induced oxidation on human skin keratinocytes. Medicine 2020;99:48(e23152).

Received: 22 July 2019 / Received in final form: 1 October 2020 / Accepted: 10 October 2020

http://dx.doi.org/10.1097/MD.0000000000023152

longitudinal study of 10 junior middle schools in Taiyuan, Shanxi Province, showed that outdoor SO_2 concentrations positively correlated with skin symptoms (skin rash, pruritus, eczema, and so forth).[9] However, a few laboratory studies explored the skin damage caused by SO_2 exposure, and hence the molecular mechanism of the action of SO_2 is still unclear. SO_2 is a highly water-soluble gas, which is converted into sulfite and hydrogen sulfite after inhalation. Keratinocytes are the main components of the epidermis. Therefore, this study explored the effect and mechanism of action of SO_2 on human skin keratinocytes using SO_2 derivatives.

2. Materials and methods

Ethical approval was not needed for this study owing to unnecessary data connected with individual patient information.

2.1. Materials

Human keratinocyte line (HaCaT) was purchased from the typical Chinese Culture Preservation Center of Wuhan University. DMEM high-glucose medium was purchased from Hyclone Inc. Pancreatin and fetal bovine serum was purchased from Sigma (USA). $NaHSO_3$ and Na_2SO_3 were purchased from Tianjin Comeio Reagent Co., Ltd. Enzyme-linked immunosorbent assay (ELISA) kits, detecting reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD), tumor necrosis factor-α (TNF-α), and interleukin (IL)-1β, were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd., China. An MTT test kit and RIPA lysate solution were purchased from Biyuntian Biotechnology Research Institute, Shanghai, China. Amido Black dyeing solution (0.1%) was purchased from Beijing Jimei Biotechnology Co., Ltd., China. A nitrocellulose membrane was purchased from Pall Company (USA). A mouse anti-β-actin antibody was purchased from Bioworld Inc (USA). Mouse anti-caspase-3 was purchased from Cell Signaling Technology Company (USA). Rabbit anti-Bcl-2, rabbit anti-Bax, rabbit anti-Nrf2, rabbit anti-HO-1, rabbit anti-IκB, rabbit anti-NF-κB (p65), rabbit anti-phosphorylated NF-κB (p-p65), rabbit anti-ERK1/2, rabbit anti-phosphorylatedERK1/2 (p-ERK1/2), rabbit anti-p38, and rabbit anti-phosphorylated p38 (p-p38) were purchased from Abcam Company (USA). Horseradish peroxidase (HRP)-goat anti-mouse secondary antibodies and HRP-goat anti-rabbit secondary antibody were purchased from Jakson Company (USA). TRIzol reagent was purchased from Life Technology Company (USA). RevertAid First Strand cDNA Synthesis kits were purchased from Thermo Company (USA). A Fast Start Essential DNA Green Master real-time quantitative kit was purchased from Roche (USA). A real-time polymerase chain reaction (PCR) primer was designed and synthesized by a biologist from Shanghai. The primer sequences were as follows: HO-1 upstream primer: 5“-CCTCCCTGTACCACATCTAT-3”, and downstream primer: 5“-AGCTCTTCTGGGAAGTAGAC-3”; Nrf2 upstream primer: 5“-CAAGTTTGGGAGGAGCTATTAT-3”, and downstream primer: 5“-CAGTTTGGCTTCTGGACTT-3”; glyceraldehyde phosphate dehydrogenase (GAPDH) upstream primer: 5“-TCCAAAATCAAGTGGGGCGA-3”, and downstream primer: 5“-TGATGACCCTTTTGGCTCCC-3”. The configuration of SO_2 derivatives in the present study was $NaHSO_3$:Na_2SO_3 = 1:3 (molar ratio, medium configuration before use).[10] The mother liquor with a concentration of 1 M was first prepared and diluted as required in the study.

2.2. Methods

2.2.1. Cell culture and grouping. HaCaT cells were cultured in DMEM medium containing 10% fetal bovine serum. They were incubated at 37°C in a 5% CO_2 cell incubator. They were randomly divided into 2 groups: control and SO_2 groups. The control group received no treatment. However, different concentrations of SO_2 derivatives were added in the SO_2 group.

2.3. Cell viability test

HaCaT cells in the logarithmic growth phase were taken, digested using trypsin, and inoculated into the 96-well plate at a density of 5×10^3/well. After overnight culture, different concentrations of SO_2 derivatives were added (0, 25, 50, 100, 200, 400, and 800 μ M). After 24-hour treatment, 10 μl of MTT solution was added to each well and incubated at 37°C for 4 hour. Subsequently, the absorbance of each well was measured at 570 nm using an enzyme labeling instrument.

2.4. Enzyme-linked immunosorbent assay

The levels of ROS, MDA, and SOD in the cells and the concentrations of TNF-α and IL-1β in the plasma were measured following the instructions on the ELISA kit.

2.5. Extraction of total RNA and real-time PCR detection

The total RNAs of HaCaT cells were extracted using TRIzol reagent following the manufacturers protocol. The concentration and purity of total RNA were determined using a NanoDrop 2000 spectrophotometer. The high-purity 500 ng total RNA was reverse transcribed into cDNA. Then, the mRNA expression levels of HO-1 and Nrf2 were detected using real-time PCR. The relative expression of target genes was calculated using the $2^{-\triangle\triangle Ct}$ method, with GAPDH as an internal reference.

2.6. Total protein extraction and Western blot detection

RIPA lysate was added to the cultured HaCaT cells. After ice cracking for 1.5 hour and centrifugation at 15,000 rpm for 10 minutes, the total protein was extracted from the supernatant. After the concentration of the extracted protein was determined using the amido black method, 25 μg total protein was boiled and denatured for 5 minutes in each sample, and sodium dodecyl sulfate–polyacrylamide electrophoresis was performed. After electrophoresis, the separated protein was transferred to the nitrocellulose membrane. Then, the protein was sealed for 2 hour using defatted milk powder and incubated overnight with an antibody at 4°C. It was washed with phosphate-buffered saline with 23 to 4 times and then incubated with secondary antibody for 1 hour. After adding the enhanced chemiluminescence solution, the film was photographed and the gray value was analyzed.

2.7. Statistical analysis

All experiments were repeated at least 3 times, and the data were expressed as mean ± standard deviation ($\bar{x} \pm s$). SPSS13.0 software was used for the statistical analysis of data. The single-factor analysis of variance and Bonferroni post-test were used for evaluating the differences among the groups. A P value <.05 indicated a statistically significant difference.

3. Results

3.1. Effects of SO_2 derivatives on the proliferation of HaCaT cells

The MTT assay was used to detect the proliferation of HaCaT cells with different concentrations of SO_2 derivatives (0, 25, 50, 100, 200, 400, and 800 μM), as shown in Figure 1. SO_2 derivatives (100, 200, 400, and 800 μM) could inhibit cell proliferation compared with cells without SO_2 derivatives, and the difference was statistically significant ($P < .05$). The effect of 25 and 50 μM SO_2derivatives on cell proliferation was not significant ($P > .05$). Compared with 100 μM, 200, 400, and 800 μM SO_2 derivatives further inhibited cell proliferation, and the difference was statistically significant ($P < .05$). However, no significant difference was observed in the inhibition of cell proliferation between these 3 concentrations ($P > .05$). Considering that a high concentration of SO_2 derivatives might lead to cell death, 100 and 200 μM SO_2 derivatives were chosen for the following experiments.

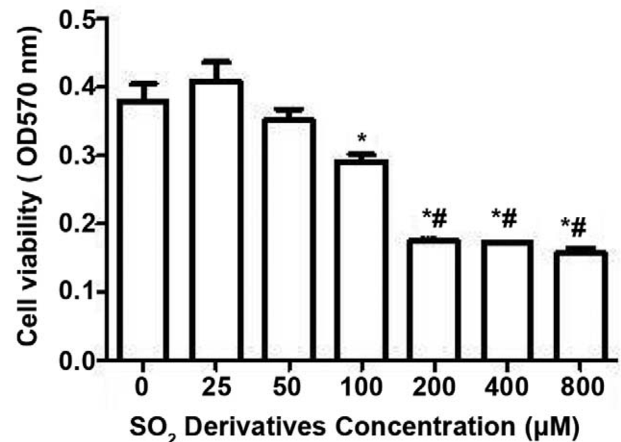

Figure 1. Effects of different concentrations of SO_2 derivatives on the proliferation of HaCaT cells. $^{*}P < .05$, compared with 0 μM. $^{\#}P < .05$, compared with 100 μM SO_2 derivatives.

3.2. Effect of SO_2 derivatives on the apoptosis of HaCaT cells

This study also examined the effects of SO_2 derivatives on cell apoptosis, as shown in Figure 2. SO_2 derivatives (100 and 200 μM) had no significant effect on the expression levels of activated caspase-3, Bax, and Bcl-2 compared with the control group, and the difference was not statistically significant ($P > .05$). The effect

Figure 2. Effect of SO_2 derivatives on the apoptosis of HaCaT cells.

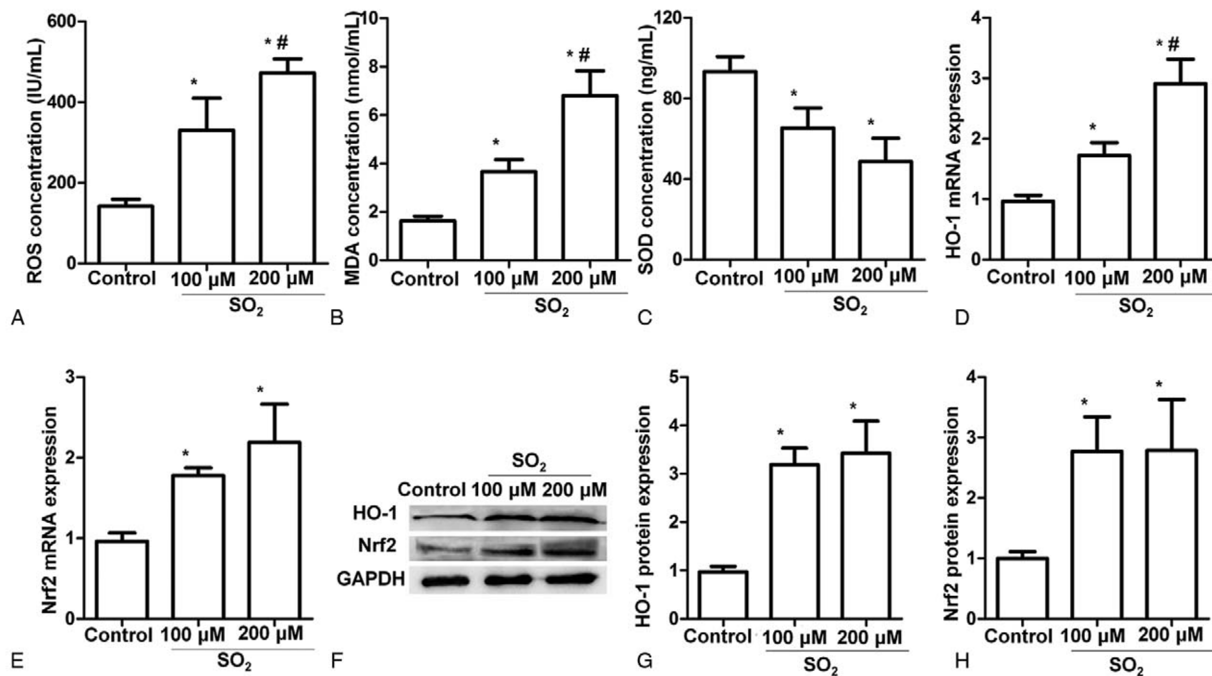

Figure 3. Effects of SO_2 derivatives on oxidative stress in HaCaT cells. ELISA was used to detect the changes in the levels of ROS (A), MDA (B), and SOD (C) in the cells. Real-time PCR was used to detect the changes in the mRNA expression levels of HO-1 (D) and Nrf2 (E). (F–H) Western blot analysis was used to detect the changes in the protein expression levels of HO-1 and Nrf2. $^{*}P < .05$, compared with the control group. $^{\#}P < .05$, compared with 100 μM SO_2 derivatives.

of SO_2 derivatives on oxidative stress in HaCaT cells was evaluated using the levels of ROS, MDA, and SOD in cells, as shown in Figure 3A–C. ROS and MDA concentrations increased, and SOD concentration decreased in the SO_2 group compared with the control group. Besides, the effect of high concentrations of SO_2 derivatives on the changes in the levels of ROS and MDA was more obvious, and the difference was statistically significant ($P < .05$).

The mRNA and protein expression levels of Nrf2 and HO-1 were detected using real-time PCR and Western blot analysis, as shown in Figure 3D–H. The mRNA and protein expression levels of Nrf2 and HO-1 increased in the SO_2 group compared with the control group, and the difference was statistically significant ($P < .05$).

The effect of SO_2 derivatives on the expression of inflammatory factors in HaCaT cells was detected, as shown in Figure 4. The levels of TNF-α and IL-1β were higher in the SO_2 group than in the control group. A high concentration of SO_2 derivatives increased the contents of TNF-α and IL-1β more obviously, and the difference was statistically significant ($P < .05$).

The possible mechanism underlying the damaging effect of SO_2 derivatives on HaCaT cells was explored, as shown in Figure 5. The phosphorylation level of NF-κB was upregulated and the expression level of IκB was downregulated in the SO_2 group

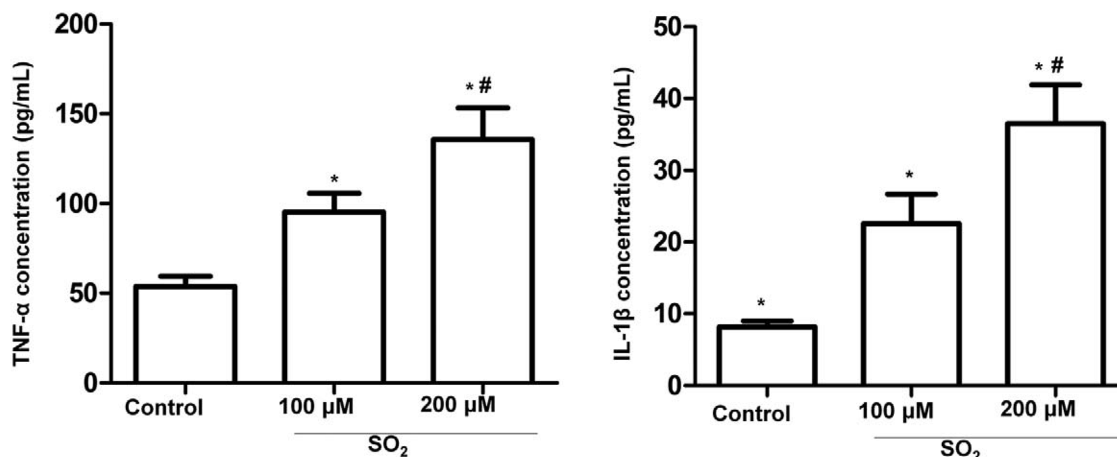

Figure 4. Effect of SO_2 derivatives on the expression of inflammatory factors in HaCaT cells. $^{*}P < .05$, compared with the control group. $^{\#}P < .05$, compared with 100 μM SO_2 derivatives.

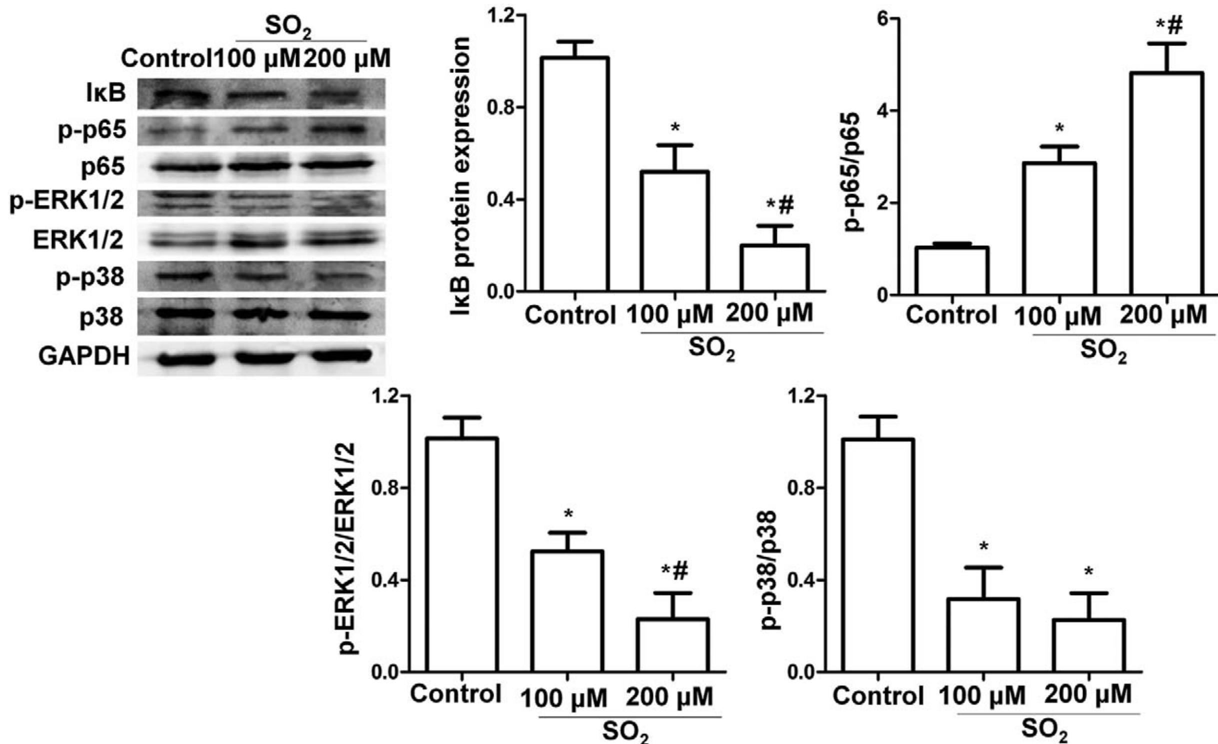

Figure 5. Effects of SO_2 derivatives on NF-κB ERK1/2 and p38 signaling pathway in HaCaT cells. *$P<.05$, compared with the control group. #$P<.05$, compared with 100 μM SO_2 derivatives.

compared with the control group. The effect of high concentrations of SO_2 derivatives on the aforementioned 2 levels was more obvious, and the difference was statistically significant ($P<.05$). The phosphorylation levels of ERK1/2 and p38 were downregulated in the SO_2 group compared with the control group, but the effect of high concentrations of SO_2 derivatives on the phosphorylation level of p38 was more obvious. The difference was statistically significant ($P<.05$).

4. Discussion

The skin is the largest organ of the human body. It is in direct contact with the environment. It is a barrier between the inside and outside environments of the human body and the first line of defense of the body to resist the outside invasion. The skin and its appendages also have roles in secretion, excretion, absorption, and body temperature regulation; they also participate in immune responses. When affected by internal and external environments, the skin gradually appears dry and yellow with wrinkles. Many factors cause skin damage; the most common is ultraviolet radiation.[11] Previous studies explored the effects of some components of haze, such as atmospheric particulates including O_3 and NO_2, on skin cells.[3–6] However, the effect of SO_2, an important component of haze, on skin cells was unclear. In this study, SO_2 derivatives were used to stimulate HaCaT cells so as to explore the effect and mechanism of action of SO_2 on skin cells.

At first, HaCaT cells were treated with different concentrations of SO_2 derivatives. The results showed that the proliferation of HaCaT cells was inhibited at a concentration of 100 μM. The cell proliferation was almost no longer inhibited at 200 μM. Therefore, 100 μM and 200 μM were selected as the concentrations of SO_2 derivatives for subsequent experiments. The activation of caspase-3 is an important biochemical indicator of early and late apoptosis in tissues and cells.[12] The members of the Bcl-2 family form a complex protein–protein interaction network that regulates apoptosis by regulating the permeability of mitochondrial outer membranes. Bax and Bcl-2 are important pro-apoptotic and anti-apoptotic proteins in this family, respectively.[13] Therefore, the present study investigated the effect of SO_2 derivatives on the apoptosis of HaCaT cells by detecting the levels of activated caspase-3, Bax, and Bcl-2. The results showed that SO_2 derivatives had no effect on the expression of caspase-3, Bax, and Bcl-2, indicating that SO_2 derivatives did not affect the apoptosis of HaCaT cells.

The present study also examined the effects of SO_2 derivatives on oxidative stress in HaCaT cells. The results showed that SO_2 derivatives decreased the levels of ROS and MDA and increased the levels of antioxidant SOD compared with the control group. HO-1 and Nrf2 are important receptors for oxidative stress.[14] The activation of the Nrf2 pathway mediates the expression of downstream defense enzymes to resist oxidative stress and damage caused by exogenous toxic substances. HO-1 is an important target gene downstream of the Nrf2 signaling pathway.[14] This study also found that SO_2 derivatives could upregulate the expression of Nrf2 and HO-1, further suggesting that SO_2 could induce oxidative stress injury in HaCaT cells. The results showed that the effect of high concentrations of SO_2 derivatives on the levels of ROS and MDA was more obvious, but the effect on the levels of SOD was not different from that of a low concentration of SO_2 derivatives, which might be related to the regulation of Nrf2. Furthermore, the present study examined the effects of SO_2 derivatives on the inflammatory response of HaCaT cells. The results showed that the levels of TNF-α and IL-1β were higher in

the SO_2 group than in the control group, suggesting that SO_2 could induce inflammation in human skin keratinocytes.

Previous studies found that the activation of NF-κB pathway was related to the O_3-induced inflammation of human keratinocytes.[14] This study found that SO_2 derivatives decreased the expression of NF-κB inhibitor IκB and increased the phosphorylation of NF-κB, suggesting that the SO_2-induced inflammatory response was closely related to the activation of the NF-κB pathway. The MAPK signaling pathway had an important role in cell proliferation, differentiation, apoptosis, and inflammation. P38 ERK and JNK are the 3 most widely studied pathways.[15] Liu et al[10] found that SO_2 inhibited the proliferation of vascular smooth muscle cells by inhibiting the ERK/MAPK pathway. The co-treatment of PM2.5 and SO_2 could induce lung structure damage and inflammation through the TLR4/p38/NF-κB pathway in rats.[16] This study found that SO_2 derivatives reduced the phosphorylation of ERK1/2 and p38, suggesting that SO_2 inhibited the proliferation of human keratinocytes by inhibiting the activation of ERK1/2 and p38 pathways. In conclusion, the present study found that SO_2 could inhibit the proliferation of human keratinocytes and induce oxidative stress and inflammation. These effects might be mediated by activating the NF-κB pathway and inhibiting the ERK1/2 and p38 pathways.

5. Conclusions

In general, SO_2 could inhibit the proliferation of human skin keratinocytes and induce oxidative stress and inflammation via the activation of the NF-κB pathway to inhibit the ERK1/2 and p38 pathways.

Author contributions

All authors agreed to be accountable for all aspects of this work.
Conceptualization: Xiaojing Kang, Junqin Liang.
Data Curation: Fengxia Hu, Lidan Mao.
Formal analysis: Junqin Liang.
Funding acquisition: Junqin Liang.
Investigation: Fengxia Hu, Lidan Mao.
Methodology: Lina Liu.
Project administration: Xiaojing Kang.
Resources: Fengxia Hu.
Software: Lina Liu.
Supervision: Xiaojing Kang, Junqin Liang.
Validation: Xiaojing Kang, Junqin Liang.
Visualization: Lina Liu.
Writing – original draft: Junqin Liang.
Writing – review & editing: Xiaojing Kang, Junqin Liang.

References

[1] Jin LF, Feng FF, Wu WD. Research progress on the harm of fine particulate pollutants to respiratory system. J Xinxiang Med Univers 2015;32:91–5.
[2] Wang R, Wang JF. Research progress on the effects of atmospheric fine particulate matter (PM25) on cardiovascular and cerebrovascular diseases. Chin J Integrative Medicine Cardio-/Cerebrovascular Dis 2015;13:1183–4.
[3] Magnani ND, Muresan XM, Belmonte G, et al. Skin damage mechanisms related to airborne particulate matter exposure. Toxicol Sci 2016;149:227–36.
[4] Valacchi G, Sticozzi C, Pecorelli A, et al. Cutaneous responses to environmental stressors. Ann N Y Acad Sci 2012;1271:75–81.
[5] Eberlein-Konig B, Przybilla B, Kuhnl P, et al. Influence of airborne nitrogen dioxide or formaldehyde on parameters of skin function and cellular activation in patients with atopic eczema and control subjects. J Allergy Clin Immunol 1998;101((1 Pt 1)):141–3.
[6] Huls A, Vierkotter A, Gao W, et al. Traffic-related air pollution contributes to development of facial lentigines: further epidemiological evidence from Caucasians and Asians. J Invest Dermatol 2016;136: 1053–6.
[7] Wiwatanadate P. Acute air pollution-related symptoms among residents in Chiang Mai, Thailand. J Environ Health 2014;76:76–84.
[8] Kathuria P, Silverberg JI. Association of pollution and climate with atopic eczema in US children. Pediatr Allergy Immunol 2016;27:478–85.
[9] Zhang X, Li F, Zhang L, et al. A longitudinal study of sick building syndrome (SBS) among pupils in relation to SO2, NO2, O3 and PM10 in schools in China. PLoS One 2014;9:e112933.
[10] Liu D, Huang Y, Bu D, et al. Sulfur dioxide inhibits vascular smooth muscle cell proliferation via suppressing the Erk/MAP kinase pathway mediated by cAMP/PKA signaling. Cell Death Dis 2014;5:e1251.
[11] Deshmukh J, Pofahl R, Haase I. Epidermal Rac1 regulates the DNA damage response and protects from UV-light-induced keratinocyte apoptosis and skin carcinogenesis. Cell Death Dis 2017;8:e2664.
[12] Choudhary GS, Al-Harbi S, Almasan A. Caspase-3 activation is a critical determinant of genotoxic stress-induced apoptosis. Methods Mol Biol 2015;1219:1–9.
[13] Borkan SC. The role of BCL-2 family members in acute kidney injury. Semin Nephrol 2016;36:237–50.
[14] Valacchi G, Sticozzi C, Belmonte G, et al. Vitamin C compound mixtures prevent ozone-induced oxidative damage in human keratinocytes as initial assessment of pollution protection. PLoS One 2015;10:e0131097.
[15] Kim EK, Choi EJ. Compromised MAPK signaling in human diseases: an update. Arch Toxicol 2015;89:867–82.
[16] Li R, Zhao L, Tong J, et al. Fine particulate matter and sulfur dioxide coexposures induce rat lung pathological injury and inflammatory responses via TLR4/p38/NF-kappaB Pathway. Int J Toxicol 2016; 1091581816682225.