Mechanism underlying the effect of SO₂-induced oxidation on human skin keratinocytes

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Abstract
This study aimed to study the effect and mechanism of action of SO₂-induced oxidation on human skin keratinocytes. Different concentrations of SO₂ 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 SO₂ groups. The control group received no treatment, whereas the SO₂ group was treated with SO₂ 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-1β) in cell supernatants were detected using enzyme-linked immunosorbent assay. Real-time polymerase chain reaction was used to detect the expressions of nuclear transcription factor (NFκB) and heme oxygenase (HO)-1 mRNA. The Western blot analysis was used to test the expression levels of NFκB, HO-1, activated caspase-3, Bcl-2, Bax, caspase-3, NF-κB p65 (p65), ERK1/2, p38, phospho-NF-κB p65 (p-p65), p-ERK1/2, and p-p38.

SO₂ derivatives (100, 200, 400, and 800 μM) could inhibit cell proliferation. SO₂ derivatives increased the level of ROS, MDA, TNF-α, IL-1β, NFκB, HO-1, and p-p65/p65 and decreased the levels of SOD, IC50, p-ERK1/2/p-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.

SO₂ could inhibit the proliferation of human skin keratinocytes and induce oxidative stress and inflammation via the activation of the NFκB 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, IC50 = inhibitor of nuclear factor kappa-B, IL-1β = Interleukin-1β, MDA = malondialdehyde, NF-κB = nuclear factor kappa-B, IC50 = nuclear transcription factor, PM = particulate matter, ROS = reactive oxygen species, SOD = superoxide dismutase, TNF-α = tumor necrosis factor

Keywords: human skin keratinocytes, inflammatory injury, mechanism, oxidative damage, SO₂ 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 SO₂, NOₓ, CO, and O₃, 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. 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 lipoperoxidation. Also, nuclear transcription factor-kβ (NF-kβ) increased the expression of cylooxygenase-2 and cytochrome P450, induced inflammatory reaction, and caused apoptosis of skin epidermal cells after exposure to atmospheric particulates.[3] O₃ could damage skin cells by facilitating the production of ROS, free radicals or free radical-dependent toxin products, and nonradical molecules such as aldehydes. Short-term exposure to NO₂ in patients with eczematous dermatitis led to changes in the skin surface and damaged skin barrier function.[6] Exposure to NO₂ in the environment was also associated with an increase in the number of freckles on the face.[6] SO₂ is an important part of the haze. Recent studies have shown that SO₂ may also be associated with skin diseases. Foreign studies showed that a high concentration of SO₂ 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 SO₂ levels. A 2-year...
longitudinal study of 10 junior middle schools in Taiyuan, Shanxi Province, showed that outdoor SO2 concentrations positively correlated with skin symptoms (skin rash, pruritus, eczema, and so forth). However, a few laboratory studies explored the skin damage caused by SO2 exposure, and hence the molecular mechanism of the action of SO2 is still unclear. SO2 is a highly water-soluble gas, which is converted into sulfate and hydrogen sulfite after inhalation. Keratinocytes are the main components of the epidermis. Therefore, this study explored the effect and mechanism of action of SO2 on human skin keratinocytes using SO2 derivatives.

2. Materials and methods

Ethical approval was not needed for this study owing to unnessary 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. Pancreat and fetal bovine serum was purchased from Sigma (USA). NaHSO3 and Na2SO3 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-beta-actin antibody was purchased from Biorwold 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-κB, rabbit anti-ERK1/2, and downstream primer: 5'-AGCTCTTCTGGGAAGTAGAC-3', and downstream primer: 5'-TCCAAAATCAAGTGGGGCGA-3', were used for evaluating the differences among the groups. All experiments were repeated at least 3 times, and the data were expressed as mean ± standard deviation (X ± 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 SO₂ derivatives on the proliferation of HaCaT cells

The MTT assay was used to detect the proliferation of HaCaT cells with different concentrations of SO₂ derivatives (0, 25, 50, 100, 200, 400, and 800 μM), as shown in Figure 1. SO₂ derivatives (100, 200, 400, and 800 μM) could inhibit cell proliferation compared with cells without SO₂ derivatives, and the difference was statistically significant (P < .05). The effect of 25 and 50 μM SO₂ derivatives on cell proliferation was not significant (P > .05). Compared with 100 μM, 200, 400, and 800 μM SO₂ 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 SO₂ derivatives might lead to cell death, 100 and 200 μM SO₂ derivatives were chosen for the following experiments.

3.2. Effect of SO₂ derivatives on the apoptosis of HaCaT cells

This study also examined the effects of SO₂ derivatives on cell apoptosis, as shown in Figure 2. SO₂ 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
of SO₂ 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 SO₂ group compared with the control group. Besides, the effect of high concentrations of SO₂ 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 SO₂ group compared with the control group, and the difference was statistically significant (P < .05).

The effect of SO₂ 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 SO₂ group than in the control group. A high concentration of SO₂ 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 SO₂ 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 SO₂ group.
compared with the control group. The effect of high concentrations of SO2 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 SO2 group compared with the control group, but the effect of high concentrations of SO2 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.\(^{[13-6]}\) However, the effect of \(SO_2\), an important component of haze, on skin cells was unclear.

In this study, SO2 derivatives were used to stimulate HaCaT cells so as to explore the effect and mechanism of action of SO2 on skin cells. At first, HaCaT cells were treated with different concentrations of SO2 derivatives. The results showed that the proliferation of HaCaT cells was inhibited at a concentration of 100 \(\mu M\). The cell proliferation was almost no longer inhibited at 200 \(\mu M\). Therefore, 100 \(\mu M\) and 200 \(\mu M\) were selected as the concentrations of SO2 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 SO2 derivatives on the apoptosis of HaCaT cells by detecting the levels of activated caspase-3, Bax, and Bcl-2. The results showed that SO2 derivatives had no effect on the expression of caspase-3, Bax, and Bcl-2, indicating that SO2 derivatives did not affect the apoptosis of HaCaT cells.

The present study also examined the effects of SO2 derivatives on oxidative stress in HaCaT cells. The results showed that SO2 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 SO2 derivatives could upregulate the expression of Nrf2 and HO-1, further suggesting that SO2 could induce oxidative stress injury in HaCaT cells. The results showed that the effect of high concentrations of SO2 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 SO2 derivatives, which might be related to the regulation of Nrf2. Furthermore, the present study examined the effects of SO2 derivatives on the inflammatory response of HaCaT cells. The results showed that the levels of TNF-\(\alpha\) and IL-1\(\beta\) were higher in
the SO2 group than in the control group, suggesting that SO2 could induce inflammation in human skin keratinocytes.

Previous studies found that the activation of NF-κB pathway was related to the O3-induced inflammation of human keratinocytes.\(^{[14]}\) This study found that SO2 derivatives decreased the expression of NF-κB inhibitor IκB and increased the phosphorylation of NF-κB, suggesting that the SO2-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 SO2 inhibited the proliferation of vascular smooth muscle cells by inhibiting the ERK/MAPK pathway. The MAPK signaling pathway had an important role in cell proliferation, differentiation, apoptosis, 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, SO2 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.

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