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ORIGINAL ARTICLE

Metformin Promotes HaCaT Cell Apoptosis through Generation of Reactive Oxygen Species *via* Raf-1-ERK1/2-Nrf2 Inactivation

Xiaoyan Wang,[1,2] Ronghua Li,[1] Xintong Zhao,[1] Xiaojing Yu,[1,3] and Qing Sun[1,3]

Abstract— Although metformin (MET) may be useful for the treatment of psoriasis, the mechanisms underlying its method of action have yet to be fully elucidated. Here, the relationship between MET function and reactive oxygen species (ROS) levels and the underlying mechanism were explored in human immortalized keratinocyte cell line (HaCaT). HaCaT cells were incubated with MET at 0, 10, 20, 40, and 60 mM for 24 h. The cell viability was evaluated by the CCK-8 assay. The cell apoptosis rate and intracellular ROS levels were examined using flow cytometry. The protein expression and the phosphorylation levels of nuclear factor erythroid-derived 2 related factor 2 (Nrf2), Raf-1, and ERK1/2 were assessed by Western blot. The specific ROS scavenger *N*-acetyl-cysteine (NAC) and the specific Nrf2 agonist Oltipraz (OPZ) were used to analyze the effect of MET. MET decreased HaCaT cell proliferation and induced HaCaT cell apoptosis in a dose-dependent manner. MET was found to elevate intracellular ROS levels in a dose-dependent manner, while pretreatment with NAC attenuated these effects. MET inhibits the protein expression and the phosphorylation levels of Nrf2. The combination of OPZ and MET can significantly increase the cell viability, decrease the rate of apoptosis, and attenuate the intracellular ROS levels relative to MET alone. MET inhibits the protein expression and the phosphorylation levels of Raf-1 and ERK1/2. MET was found to attenuate Raf-1-ERK1/2 signaling in HaCaT cells to suppress the expression and phosphorylation levels of Nrf2, which contributed to the intracellular generation of ROS and the pro-apoptotic effects of MET.

KEY WORDS: psoriasis; metformin; ROS; Raf-1; ERK; Nrf2.

INTRODUCTION

Psoriasis is a common, recurrent, chronic inflammatory skin disease affecting 3% of the general population and leads to significant morbidity. Psoriasis is characterized by the development of erythematous papules and overlying scaly plaques [1]. The etiology of psoriasis is complex and remains unclear. Although many therapeutic strategies have been developed for the treatment of psoriasis, many psoriasis patients still suffer from frequent relapse, adverse drug effects, and other unwanted reactions [2]. It is necessary to identify new therapies.

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[1] Department of Dermatology, Qilu Hospital of Shandong University, Jinan, Shandong 250000, China
[2] Department of Dermatology, Qingdao Municipal Hospital, Qingdao, Shandong 266000, China
[3] To whom correspondence should be addressed at Department of Dermatology, Qilu Hospital of Shandong University, Jinan, Shandong 250000, China. E-mails: yuxiaojing96@163.com; sunqing7226@163.com

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Metformin (1,1-dimethylbiguanide hydrochloride, MET), the oral hypoglycemic agent, is commonly used for the treatment of type 2 diabetes mellitus [3]. Recently, the roles of MET in the treatment of psoriasis have been evaluated. Like other insulin-sensitizing agents, MET can significantly decrease the occurrence of psoriasis in diabetic patients [4, 5]. MET can also significantly down-regulate the severity of psoriasis compared with a placebo [6]. Our previous study showed that MET has anti-proliferative roles in human immortalized keratinocyte cells (HaCaT) *via* inhibition of the mTOR signaling pathway [7]. The studies cited above found that MET is effective in psoriasis patients, but the underlying mechanism has not yet been fully elucidated.

Reactive oxygen species (ROS) are reactive molecules that can regulate cell signaling and homeostasis [8]. Generally, elevated ROS under oxidative stress conditions are involved in the pathogenesis of various diseases, including cancer and inflammation, by damaging cellular components [8, 9]. However, accumulating evidence has also demonstrated that ROS have dual roles in tumors. Lower levels of ROS can promote cell survival and tumorigenesis, while elevated levels of ROS can induce tumor cell apoptosis or senescence and inhibit cancer cell growth [10–13]. The anti-tumor potency of metformin has been linked to the generation of ROS [14–16].

Nuclear factor erythroid-derived 2 related factor 2 (Nrf2) is a transcription factor that can regulate the expression of antioxidant proteins and regulate cellular defenses against oxidative stress [17, 18]. An inducible antioxidant program modulated by Nrf2 and its repressor protein can tightly control ROS levels [11]. The interaction between Nrf2 and the promoters of its target protein can be heightened through activation of the Raf/ERK signal pathway [19]. Previous studies have shown that Nrf2 can regulate the expression of key markers for keratinocyte hyperproliferation [20, 21], so the dysregulation of Nrf2 may contribute to the pathogenesis of psoriasis. MET can reduce the expression of Nrf2 through the inactivation of Raf-ERK signaling in cancer cells [22].

Studies have yet to establish whether MET promotes human keratinocyte apoptosis *via* ROS and whether the Raf-ERK-Nrf2 signaling pathway participates in the process. HaCaT cells with full epidermal differentiation capacity have been widely used as cellular models for the study of psoriasis [7, 23]. The present study explores the effects of MET on the proliferation and apoptosis of HaCaT cells, on the regulation of ROS, and the underlying mechanisms.

MATERIALS AND METHODS

Materials

MET and *N*-acetyl-cysteine (NAC) (Sigma Chemical Co., St. Louis, MO, USA), Cell Counting Kit-8 (CCK-8), and Annexin V–PI cell Apoptosis Detection Kit (BestBio, Shanghai, China); ROS detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); BCA protein concentration detection kit (Beyotime Institute of Biotechnology, Haimen, China); antibodies against Raf-1 (EP 4969), p-Raf-1 [S259 EPR3433(2)], ERK1/2, p-ERK1/2, p-Nrf2 (EP 1809Y), and Nrf2 (EP 1808Y) (Abcam, Cambridge, MA, USA); antibodies against GAPDH and secondary antibody (horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG) (Cell Signaling Technologies, Beverly, MA, USA); U0126 and Oltipraz (OPZ) (Selleck Chemicals, Houston, TX, USA); quantitative automatic microplate reader (Anthos Labtec Co., Ltd., Uckfield, Sussex, UK). All other chemicals and reagents were of analytical grade. The study was approved by the ethics committee of Qilu Hospital, affiliated with Shandong University (Jinan, China).

Cell Culture and Treatments

The HaCaT cell line was obtained from the Chinese Academy of Sciences Shanghai Institute for Biological Sciences–Cell Resource Center (Shanghai, China). HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sangon Biotech Co., Ltd., Shanghai, China), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2 to 70–80% confluency for use in the assays. MET and NAC were directly dissolved in DMEM. U0126 and OPZ were dissolved in dimethyl sulfoxide (DMSO) and diluted to the desired concentration with serum-free culture medium. The final concentration of DMSO did not exceed 0.1% (*v*/*v*) and did not affect cell viability. The control group was treated with DMEM alone. HaCaT cells were collected during the logarithmic growth phase and plated in

Table 1. Primer Sequences for Quantitative Real-Time PCR

Gene name	Primer sequences (5′ to 3′)
Nrf2	F TCAGCGACGGAAAGAGTATGA
	R CCACTGGTTTCTGACTGGATGT
GAPDH	F GCACCGTCAAGGCTGAGAAC
	R TGGTGAAGACGCCAGTGGA

triplicate in 96-well (2×10^4 cells/well) and 6-well plates (4×10^5 cells/well). MET groups with different concentrations (10, 20, 40, and 60 mM) and control (without MET) group were established.

Determination of Cell Viability by the CCK-8 Assay

The proliferation of different groups was measured by the CCK-8 assay according to the manufacturer's protocol. In brief, HaCaT cells during the logarithmic growth phase were collected and plated into 96-well plates at an initial

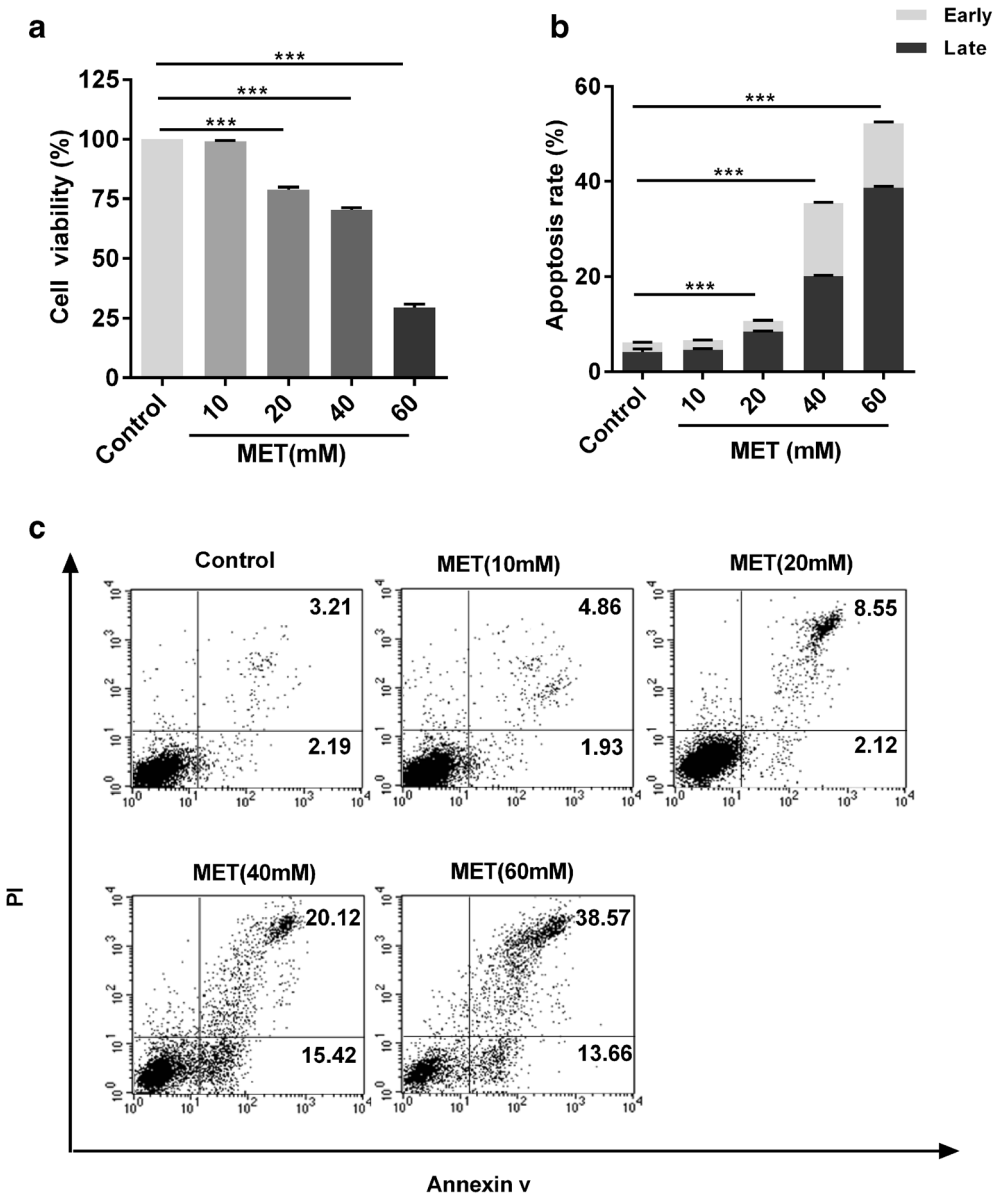
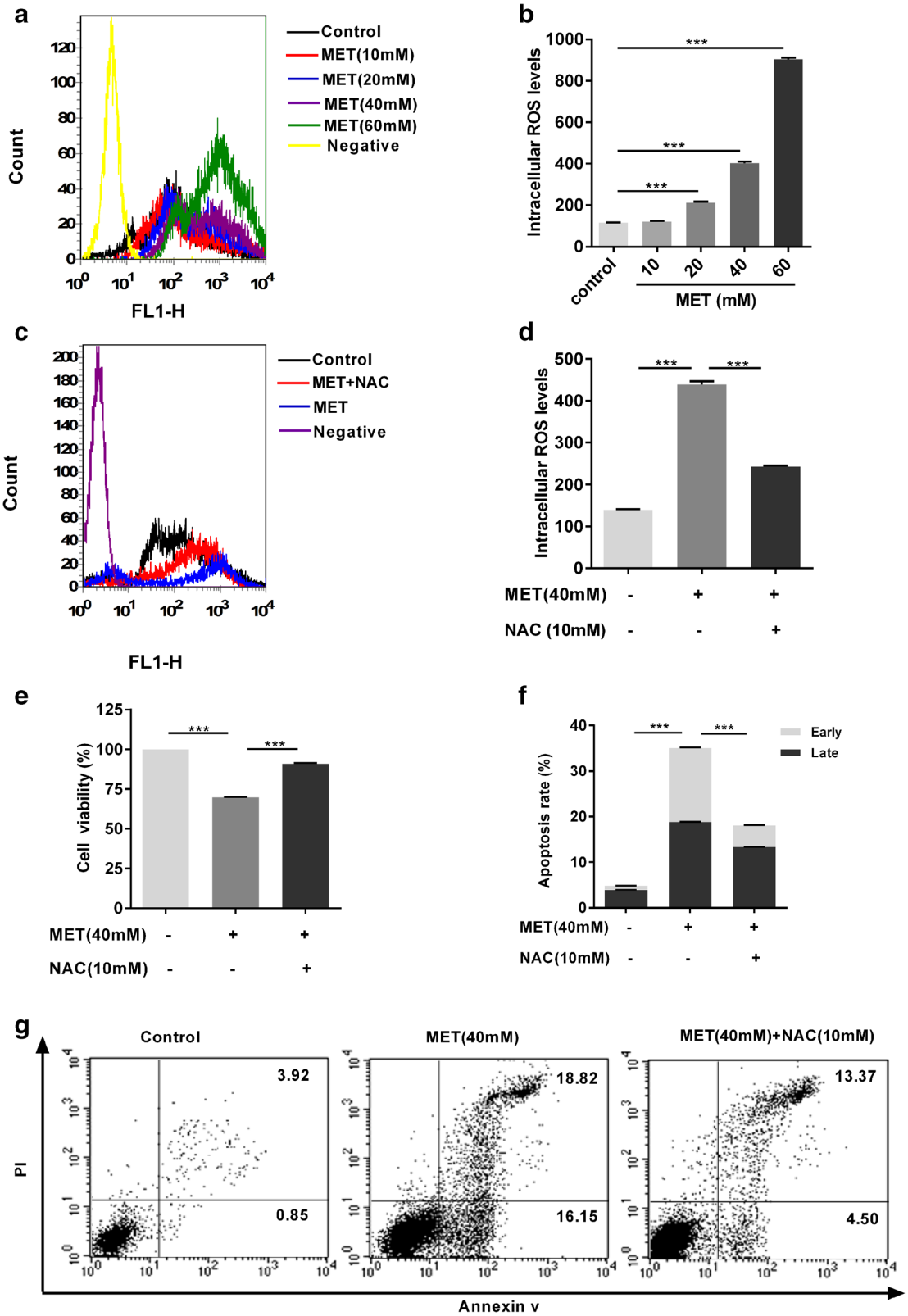


Fig. 1. Effects of MET on HaCaT cells' viability and apoptosis. HaCaT cells were exposed to various concentrations of metformin (MET) (10, 20, 40, and 60 mM) for 24 h. **a** Cell viability was determined by the CCK-8 assay. **b** Cellular apoptosis was assayed by Annexin V-FITC and PI counterstaining and analyzed with flow cytometry. This composite diagram demonstrates early and late apoptotic cell rate. The light gray bar indicates rate of cells undergoing early apoptosis and the dark bar for late apoptotic cell rate. The apoptosis rate (%) = (the number of early and late apoptotic cells/the number of total cells observed) × 100%. **c** The original representative flow cytometry figures of (**b**). Data are here presented as the mean ± SEM of three independent experiments. ***$P < 0.001$.


a
Count
FL1-H
Control
MET(10mM)
MET(20mM)
MET(40mM)
MET(60mM)
Negative
b
Intracellular ROS levels
control
10
20
40
60
MET (mM)
***
c
Count
FL1-H
Control
MET+NAC
MET
Negative
d
Intracellular ROS levels
MET(40mM)
NAC (10mM)
e
Cell viability (%)
MET(40mM)
NAC(10mM)
f
Apoptosis rate (%)
Early
Late
MET(40mM)
NAC(10mM)
g
Control
MET(40mM)
MET(40mM)+NAC(10mM)
PI
Annexin v
3.92
0.85
18.82
16.15
13.37
4.50

density of 2×10^4 cells/well ($n = 3$ per group). After 24 h of inoculation, the culture medium in MET groups was replaced with DMEM containing different concentrations of MET. A group of untreated control wells (cells treated with medium) and a group of blank control wells (without cells) were established at the same time. After another 24 h of incubation with the indicated drugs, 10 μl of kit reagent was added to 100 μl cell solution and incubated for a further 60 min at 37 °C. The optical densities were measured at 450 nm using a quantitative automatic microplate reader. The cell viability rate (%) was calculated using the following formula:

$$\left(OC_{\text{experimental group}} - OD_{\text{blank control}}\right) / \left(OD_{\text{control group}} - OD_{\text{blank control}}\right) \times 100.$$

Apoptosis as Measured Using an Annexin V–PI Cell Apoptosis Detection Kit and Flow Cytometric Analysis

HaCaT cells were cultured in six-well plates (4×10^5 cells/well) and incubated with appropriate drugs for 24 h. The cells were harvested, washed twice with PBS, and stained according to the manufacturer's instructions. The rate of apoptosis was immediately determined using a FACSCalibur flow cytometer (BD Biosciences, CA, USA).

ROS Assay

The changes in the intracellular ROS levels were quantified using a DCFH-DA probe according to the manufacturer's instructions. In brief, the HaCaT cells were grown in six-well plates, rinsed once with PBS, and then treated with corresponding drugs. After 24 h of incubation, the culture medium was removed and incubated with DCFH-DA (diluted with PBS to a concentration of 1:5000) for 30 min at 37 °C, and shaken up and down once every 10 min. The cells were then washed three times with FBS-free medium, resuspended in 500 μl PBS, and analyzed using a flow cytometer.

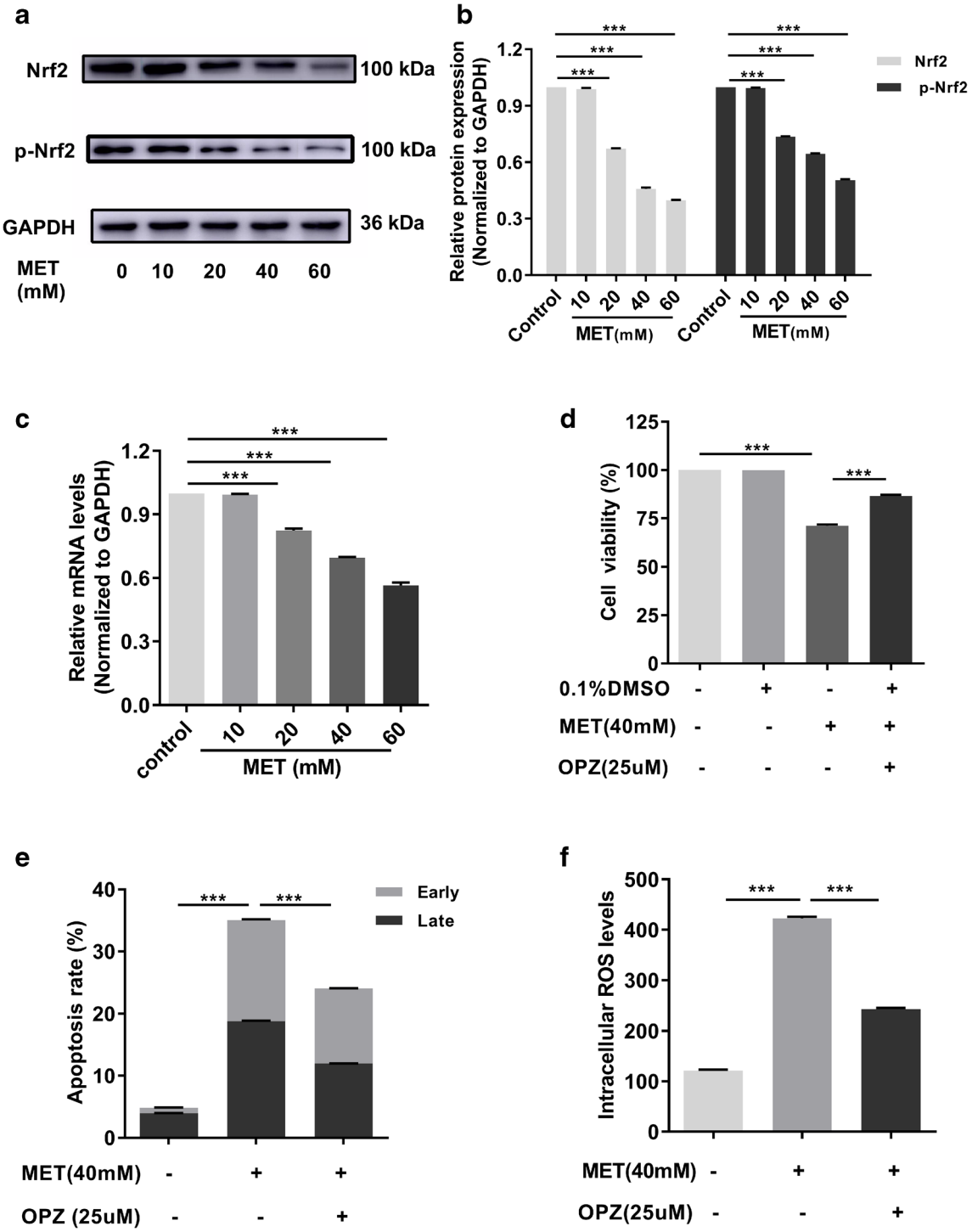
◀ **Fig. 2.** The HaCaT cells were exposed to 10 mM NAC for 1 h prior to 40 mM MET treatment for 24 h. The effect of MET on intracellular ROS levels and effect of the antioxidant NAC on MET-induced ROS generation in HaCaT cells. **a** Representative images of ROS production after MET treatment. **b** The fluorescence intensity of the cells after MET treatment. **c** Representative images of ROS production after MET-plus-NAC treatment. **d** The fluorescence intensity of the cells after MET-plus-NAC treatment. **e** The effect of the antioxidant NAC on MET-induced cytotoxicity after 24 h was analyzed with CCK8. **f** The effect of NAC on the necroptotic death induced by MET was assessed by Annexin–PI assay and flow cytometry. This composite diagram demonstrates early and late apoptotic cell rate. The light gray bar indicates rate of cells undergoing early apoptosis and the dark bar for late apoptotic cell rate. The apoptosis rate (%) = (the number of early and late apoptotic cells/the number of total cells observed) × 100%. **g** The original representative flow cytometry figures of (**f**). The data shown in (**b**), (**d**), (**e**), and (**f**) are here presented as the mean ± SEM of three independent experiments. ***$P < 0.001$.

Western Blot Analysis

Cells were harvested and total proteins were extracted from each sample. Proteins were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and then transferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in TBST buffer (Tris 2.42 g, NaCl 8.8 g, Tween 1 ml, then diluted to a constant volume to 1 l with double-distilled water) for 1 h at room temperature. After incubation with the appropriate primary antibodies overnight at 4 °C, the membranes were washed three times with TBST and followed by a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membranes were washed thoroughly and detected by chemiluminescence with ECL-Plus (EMD Millipore, Darmstadt, Germany) and imaged by the imaging systems (Bio-Rad, Richmond, CA, USA). To investigate multiple protein targets under the same treatment condition, the blot was stripped and reused. Equal loading of samples was confirmed by GAPDH levels in the whole-cell lysates. The integrated optical density for the protein bands was then analyzed using ImageJ software (version 1.46r; NIH, USA), and the values were normalized to GAPDH. The relative absorbance ratios of target protein to GAPDH were defined as the respective relative values of target protein.

Quantitative Real-Time-PCR (qRT-PCR)

HaCaT cells were treated with MET (0, 10, 20, 40, and 60 mM) for 24 h. Total RNA was extracted from the treated cells using the Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) and the general complementary DNA was synthesized using the general complementary DNA (cDNA) synthesis kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. The qRT-PCR experiment was performed using a Bio-Rad CFX Manager quantitative PCR instrument (Bio-Rad, Richmond, CA, USA). The cycling conditions were as follows: predegeneration at 95 °C for 30 s (one cycle), followed by 40 cycles with denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. Accumulated PCR


a
Nrf2
100 kDa
p-Nrf2
100 kDa
GAPDH
36 kDa
MET (mM)
0 10 20 40 60
b
Relative protein expression (Normalized to GAPDH)
Nrf2
p-Nrf2
Control
MET(mM)
c
Relative mRNA levels (Normalized to GAPDH)
control
MET (mM)
d
Cell viability (%)
0.1%DMSO
MET(40mM)
OPZ(25uM)
e
Apoptosis rate (%)
Early
Late
MET(40mM)
OPZ (25uM)
f
Intracellular ROS levels
MET(40mM)
OPZ(25uM)

products were directly detected by inspecting the increase in the SYBR® reporter dye. The primer sequences are shown in Table 1. The relative expression levels of Nrf2 in the MET-treated cells were compared to those in control cells using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) with GAPDH as the reference gene.

Statistical Analysis

All the data in our study were obtained from at least three independent experiments and are presented here as the mean ± SEM, with $n = 3$. A two-tailed unpaired Student's t test was used to analyze data between two groups. One-way ANOVA followed by Bonferroni's multiple comparison test comparisons were performed within three or more groups. These analyses were performed using GraphPad Prism (GraphPad Software 3.0; San Diego, CA, USA). Differences between groups were considered significant at $P < 0.05$.

RESULTS

MET Decreased HaCaT Cell Proliferation and Induced Apoptosis in HaCaT Cells in a Dose-Dependent Manner

The effects of MET on the proliferation and viability of HaCaT cell were analyzed using the CCK-8 assay. MET decreased cell proliferation in a dose-dependent manner. Compared to the control group, the proliferation of HaCaT cells was significantly inhibited by MET at concentrations over 10 mM after 24 h of treatment ($P < 0.001$, respectively) (Fig. 1a). The apoptosis rates of HaCaT cells treated with different concentrations of MET for 24 h were explored. As shown in Fig. 1b, MET also induced apoptosis in HaCaT cells in a dose-dependent manner. HaCaT cells in the groups treated with concentrations of MET over 10 mM for 24 h showed higher rates of apoptosis than in the control group ($P < 0.001$, respectively).

◀ **Fig. 3.** Nrf2 inhibited by MET contributed to the ROS generation and the pro-apoptosis effects of MET in HaCaT cells. **a** Nrf2 and p-Nrf2 protein levels were measured by Western blot after treatment with MET (0, 10, 20, 40, and 60 mM) for 24 h. **b** The intensity of each band was quantified by densitometry analysis. All protein expression levels were normalized to the internal control GAPDH. **c** Nrf2 mRNA level was analyzed using qRT-PCR. **d**–**f** HaCaT cells were treated with Oltipraz (OPZ, 25 μM, a specific agonist of Nrf2) and MET (40 mM) for 24 h. The addition of OPZ was found to significantly inhibit the necroptosis rate and elevate the cell viability rate. What is more, increased intracellular ROS levels induced by MET was also decreased by OPZ. The data shown in (**b**), (**c**), (**d**), (**e**), and (**f**) are here presented as the mean ± SEM of three independent experiments. ***$P < 0.001$.

MET Induced HaCaT Cell Apoptosis through Oxidative Stress

A previous study demonstrated that MET can promote the accumulation of ROS to cause the apoptosis in tumor cells [15]. In the present study, intracellular ROS levels of HaCaT cells after MET treatment were investigated. The DCFH-DA assay revealed that the intracellular ROS levels of HaCaT cells treated with concentrations of MET over 10 mM were all significantly higher than those of the control cells ($P < 0.001$, respectively). MET could elevate the intracellular ROS levels of HaCaT cells in a dose-dependent manner (Fig. 2a, b). NAC is a specific ROS scavenger [24], and it was used here to assess the effects of ROS on MET-induced apoptosis. Considering the cell viability and the apoptosis rate, MET at the concentration of 40 mM was selected to continue the study. The HaCaT cells were exposed to 10 mM NAC for 1 h prior to 40 mM MET treatment. After 24 h, the HaCaT cells were harvested to measure the intracellular ROS levels, cell viability, and the rate of apoptosis. As shown in Fig. 2c and d, the DCFH-DA assay indicated that the intracellular ROS levels in HaCaT cells induced by MET can be significantly attenuated by pretreatment with NAC ($P < 0.001$). Meanwhile, the cell viability was significantly higher (Fig. 2e) and the apoptosis rate (Fig. 2f, g) was significantly lower in HaCaT cells pretreated with NAC than in HaCaT cells treated with MET alone ($P < 0.001$). These results indicated that ROS plays a central role in MET-triggered apoptosis of HaCaT cells.

Nrf2 Inhibited by MET Contributed to the Generation of ROS and the Pro-Apoptotic Effects of MET in HaCaT Cells

The oxidative stress sensor Nrf2 can play an important role in the regulation of ROS levels [11]. MET can also suppress the expression and activity of Nrf2 in cancer cells [22, 25]. According to the studies cited above, Western blot and qRT-PCR were first used to analyze the protein expression, the phosphorylation level, and mRNA level of Nrf2 in HaCaT cells treated with different doses of MET for 24 h. Results showed that MET at concentrations over 10 mM could significantly inhibit the expression of Nrf2 and p-Nrf2 at the protein level (Fig. 3a, b) and that of Nrf2 at the mRNA level (Fig. 3c) in HaCaT cells relative to control cells ($P < 0.001$). This suppressive role of MET is dose dependent.

We then determined whether Nrf2 inhibition mediated the pro-apoptosis roles of MET in HaCaT cells and the underlying mechanism. HaCaT cells were treated with a combination of OPZ (25 μM, a specific agonist of Nrf2) and MET (40 mM) or MET alone (40 mM) for 24 h. Then the cells were harvested to assess cell viability, the rates of apoptosis, and intracellular ROS levels. The combination of OPZ and MET was associated with significantly greater cell viability than MET alone (Fig. 3d), a lower rate of apoptosis (Fig. 3e and Fig. S1), and attenuation of intracellular ROS levels (Fig. 3f and Fig. S2) in HaCaT cells ($P < 0.001$, respectively). The results suggested that Nrf2 inhibited by MET contributed to ROS generation and the pro-apoptotic effects of MET in HaCaT cells.

MET Inhibits Nrf2 Expression in HaCaT Cells *via* Raf-ERK Inactivation

The Raf/ERK signal pathway is essential to the activation of Nrf2 [19]. Raf-1/ERK1–2 proteins and their phosphorylation levels in HaCaT cells treated with MET were evaluated by Western blot. Results showed that MET at concentrations from 20 mM strongly suppressed the total protein levels and the phosphorylated levels of Raf-1 and ERK1/2 in a dose-dependent manner ($P < 0.001$) (Fig. 4a, b). In addition, to confirm whether the inhibition of Raf-ERK signaling pathway resulted in the suppression of Nrf2, HaCaT cells were treated with 10 μM U0126 (MEK1/2 inhibitor) for 1 h, and Nrf2 and p-Nrf2 protein levels were assessed by Western blot. Surprisingly, we found that the administration of U0126 can render the protein expression levels of ERK 1/2, p-ERK 1/2, Nrf2, and p-Nrf2 in HaCaT cells significantly lower than in control cells ($P < 0.001$) (Fig. 4c, d). The results demonstrated that MET may inhibit the expression and activity of Nrf2 in HaCaT cells *via* Raf-ERK inactivation.

DISCUSSION

Psoriasis is a multifactorial skin disease that inconveniences many patients. Because hyperproliferation and abnormal differentiation of epidermal keratinocytes are essential to the pathophysiology of psoriasis [26], inhibition of these cellular events is the principal method of treating psoriasis [27]. Considering the side effects and drug resistance associated with current therapies, it is necessary to identify new therapies.

MET, an insulin sensitizer, may be useful in the treatment of psoriasis according to the results of clinical investigations [4–6]. Our previous study showed that MET can inhibit HaCaT cell proliferation by suppressing the mTOR signaling pathway [7]. In the present study, we found that MET decreased both HaCaT cell proliferation and induced HaCaT cell apoptosis in a dose-dependent manner (Fig. 1). Our results were consistent with the effects of MET in psoriasis.

Increased ROS production and decreased antioxidant system functions have been demonstrated to be associated with the pathogenesis of psoriasis [28–30]. However, like the dual function in cancer, ROS can also act as a regulator or a suppressor of immune-mediated diseases and inflammation [31]. ROS deficiency could exacerbate mannan-induced psoriasis and arthritis, whereas the specific restoration of ROS production could ameliorate these two diseases [32]. In addition, ROS at high levels could promote the function of Treg and so prevent the psoriatic dermatitis induced by imiquimod [33]. These studies indicate the protective role of ROS in psoriasis. Methotrexate, an efficient agent in the treatment of psoriasis, can play anti-inflammatory and immunosuppressive roles through the production of ROS [34]. MET can also induce apoptosis in cancer cells *via* ROS generation [14–16]. However, so far, whether ROS mediate the roles of MET in psoriasis remains unknown. We found that MET could elevate intracellular ROS levels in a dose-dependent manner (Fig. 2a, b), while pretreatment with NAC before administration of MET significantly attenuated the intracellular ROS levels, elevated cell viability, and decreased the apoptosis rate of HaCaT cells (Fig. 2c–g). Our data implied that ROS plays a central role in the MET-triggered apoptosis of HaCaT cells. In addition, the detailed mechanisms of how ROS protects against psoriasis requires investigation in further detail.

Beyond cytoprotection from electrophiles and oxidants, the oxidative stress sensor Nrf2 may play an important role in the pathogenesis of psoriasis [20, 21]. The deletion of Nrf2 can lead to ROS accumulation in Nrf2-KO cells [35]. MET can also suppress the expression and activity of Nrf2 in cancer cells [22, 25]. The roles of Nrf2 in the generation of ROS induced by MET were further explored in the present study. Results showed that MET can reduce the protein expression, phosphorylation, and mRNA levels of Nrf2 in a dose-dependent manner (Fig. 3a–c). The application of Nrf2 agonists OPZ in HaCaT cells can significantly reverse the roles of MET in intracellular ROS production, cell viability, and cell apoptosis (Fig. 3d–f and Fig. S1–S2). Our data demonstrated that Nrf2 was involved in the effects of MET on HaCaT

cells mainly by mediating ROS generation. The role of Nrf2 in the pathogenesis of psoriasis and its relationship with ROS need to be further explored.

ERK activation is associated with cell survival, proliferation, and differentiation in response to mitogens and cell survival factors [36, 37]. Activation of the Raf/ERK signaling cascade in human cancer cells has been demonstrated to be required for Nrf2 activation, which promotes Nrf2 nuclear translocation and binding to specific DNA sequences [19, 38]. Moreover, the expression of Nrf2 can be reduced by MET by inactivating Raf-ERK signaling in cancer cells [22]. The manner by which MET regulates the protein expression and phosphorylation levels of Nrf2 was analyzed here. The results showed that MET can suppress the total protein levels and the phosphorylation levels of Raf-1 and ERK1/2 in a dose-dependent manner (Fig. 4a, b). The administration of the MEK inhibitor U0126 could inhibit the total protein level and the phosphorylation level of ERK 1/2. Meanwhile, Nrf2 and p-Nrf2 expression levels were also decreased (Fig. 4c, d). Our data suggest that the

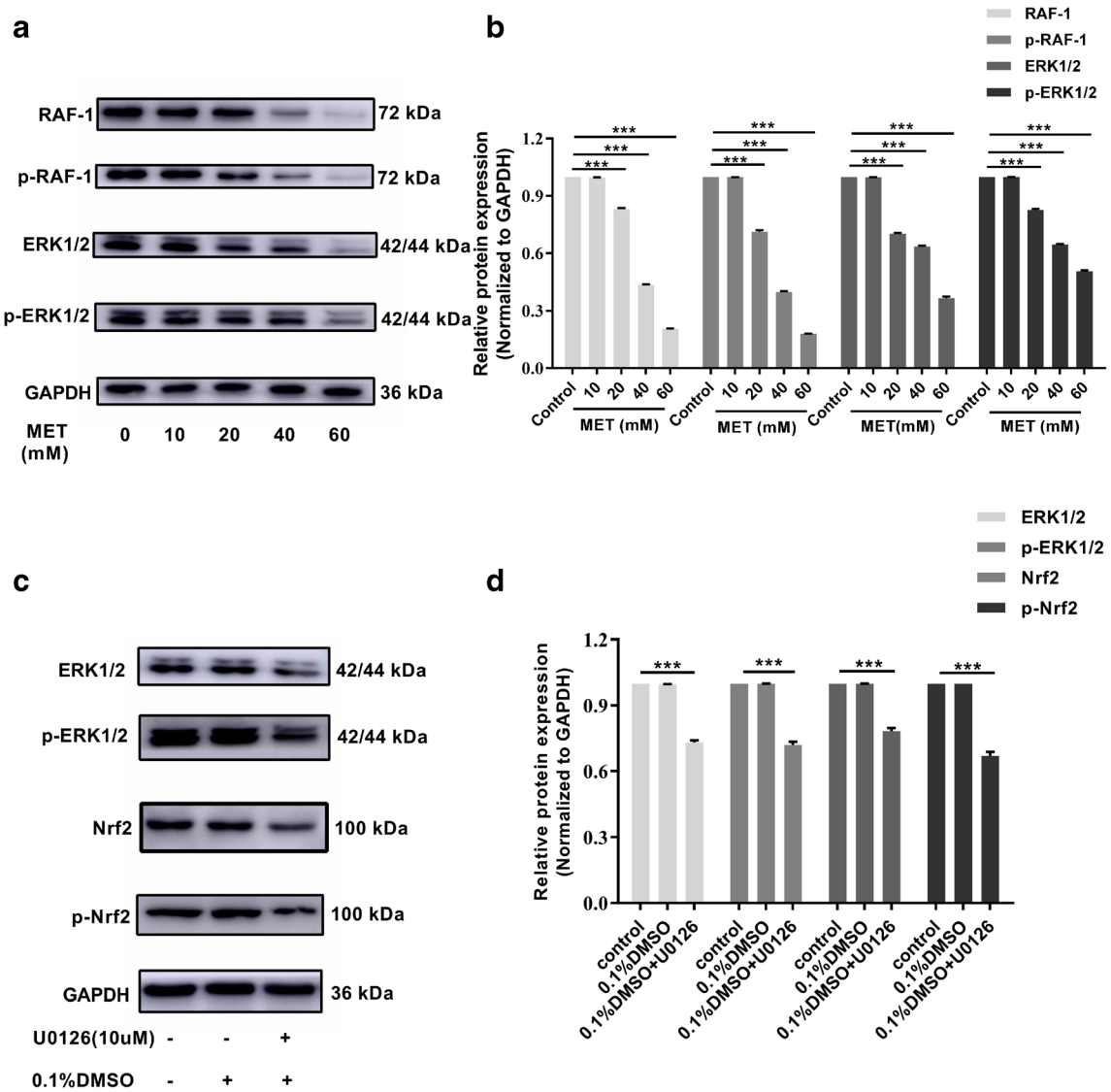


Fig. 4. MET strongly inhibited expression of Raf-1, p-Raf-1, ERK 1/2, and p-ERK 1/2 proteins in a dose-dependent manner. **a** Raf-1, p-Raf-1, ERK 1/2, and p-ERK 1/2 protein levels were measured by Western blot after treatment with MET (0, 10, 20, 40, and 60 mM) for 24 h. **b** The intensity of each band was quantified by densitometry analysis. **c** ERK 1/2, p-ERK 1/2, Nrf2, and p-Nrf2 protein levels were measured by Western blot after treatment with U0126 (10 μM). **d** The intensity of each band was quantified by densitometry analysis. All protein expression levels were normalized to the internal control GAPDH. The data are here presented as the mean ± SEM of three independent experiments. ***$P < 0.001$.

Raf-ERK signaling pathway participates in the suppression of Nrf2 by MET in HaCaT cells.

In summary, MET could attenuate Raf-1-ERK1/2 signaling in HaCaT cells to suppress the expression and phosphorylation levels of Nrf2, which contributed to intracellular ROS generation and the pro-apoptotic effects of MET in the present study. MET may be a good candidate for further development as an anti-psoriasis drug.

However, there are still some questions that remain, such as whether MET regulates the nuclear translocation of Nrf2 and whether other proteins or factors are involved in the functions of MET. These questions will need to be addressed in subsequent experiments.

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COMPLIANCE WITH ETHICAL STANDARDS

This research was approved by the Research Ethics Committee of Qilu Hospital of Shandong University. All participants signed the informed consents.

Conflict of Interest. The authors declare that there is no conflict of interests.

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