Minocycline protects melanocytes against H₂O₂-induced cell death via JNK and p38 MAPK pathways

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Abstract. Vitiligo is an acquired and progressive disorder manifested by the selective destruction of melanocytes in the skin. An extremely high level of hydrogen peroxide (H_2O_2) in plasma as well as in lesional skin has been reported in vitiligo patients. High H_2O_2 level has been suggested to be responsible for the disappearance of melanocytes in vitiligo. JNK and p38 MAPK are strongly induced by oxidative stress and related to neuron loss in neurodegenerative disorders. Minocycline, an antibiotic possessing antioxidant activity, is capable of attenuating oxidative stress-induced neurotoxicity. To investigate whether minocycline rescues melanocytes from H_2O_2-induced apoptosis, cultured mouse melanocytes (B10BR) were treated with H_2O_2 in the presence or absence of minocycline. Our data showed that H_2O_2 decreases cell viability in a concentration-dependent manner which is attenuated by minocycline. Also, H_2O_2 treatment activates JNK and p38 MAPK, and executive caspase 3 in B10BR cells. Minocycline significantly inhibits H_2O_2-induced activation of JNK, p38 MAPK and caspase 3. Collectively, we concluded that minocycline protects melanocytes against H2O2-induced apoptosis *in vitro*. Its protective effect is associated with the inhibition of JNK and p38 MAPK. Our findings suggest that minocycline, a clinically well-tolerated, safe antibiotic, may be used to prevent melanocyte loss in the early stage of vitiligo.

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Introduction

Vitiligo is an acquired pigmentary disorder characterized by well-circumscribed depigmented patches. Autoimmune, self-destruction, neural, and genetic theories have been proposed for the pathogenesis of vitiligo. Although none of these theories is sufficient to explain the pathogenesis of the disease, reactive oxygen species may play an important role in the pathology of melanocyte damage (1). There are several lines of evidence for the accumulation of hydrogen peroxide (H_2O_2) in the epidermis of acute vitiligo patients, and the concentration is in the 10^{-3} M range (2,3). Moreover, the level of H_2O_2 has been reported to increase in the plasma and urine of vitiligo patients as well as in lesional skin. The increases mainly occur at the onset and progression phase, possibly contributing to the disappearance of melanocytes in vitiligo (4-6). Thus it implies impaired antioxidant defense capacity in vitiligo.

The mechanism that accounts for apoptosis induced by H_2O_2 in melanocytes is still under investigation. There is much evidence that suggests constant accumulation of H_2O_2 leading to a cascade of impaired signals in active vitiligo (7-11). Moreover, the calmodulin-regulated calcium homeostasis is altered by H_2O_2-mediated stress due to the loss of calcium binding on the protein (6). JNK and p38 MAPK activation is reportedly involved in dopamine-induced melanocyte apoptosis (12). However, mitogen-activated protein kinase (MAPK) signal transduction has still not been thoroughly studied in H_2O_2-induced apoptosis in melanocytes. Thus the current study was carried out to investigate whether MAPKs, especially JNK and p38 MAPK, contribute to melanocyte loss after H_2O_2 treatment.

H_2O_2 is a putative mediator of melanocyte death in vitiligo. Removal of H_2O_2 by antioxidant has been proven to be beneficial to patients. Several reports suggested that a topical substitution with a UVB-activated pseudocatalase can successfully remove epidermal H_2O_2 and achieve remarkable repigmentation in vitiligo (13,14). In contrast, other researchers found that topical pseudocatalase was not effective for vitiligo in an open, single-center study (15). In such a case,

resort to other drugs which could alleviate oxidative stress damage would be necessary. Such drugs might be used to treat vitiligo in clinics.

Minocycline possesses antioxidant activity and is capable of inhibiting both free radical production and lipid peroxidation in a concentration-dependent manner (16-18). It has been reported that minocycline attenuates 6-hydroxydopamine-induced neurotoxicity in rat cerebellar granule neurons (19) and exerts protective effects against oxidative stress in the mouse brain (20). However, whether minocycline can protect melanocytes, one kind of epidermal cell differentiated from neural crest cells, against apoptosis induced by H_2O_2, is still unknown.

Given the above, the purpose of the present study was to investigate the underlying molecular mechanism responsible for H_2O_2-induced apoptosis and whether minocycline exerts a protective effect on B10BR mouse melanocytes.

Materials and methods

Cell culture. As described previously (21), spontaneously immortalized mouse melanocyte B10BR cell line (purchased from the Department of Dermatology, Yale University) was maintained in F10 medium (Sigma, St. Louis, MO) supplemented with a 7% horse serum (Hyclone, USA), 50 nM tetradecanoyl phorbol acetate (TPA, Sigma), penicillin/streptomycin (1:100, Sigma) and 4 mM L-glutamine, in a humid atmosphere incubator with 5% CO_2 at 37˚C. For Western blot analysis, cells were reseeded in 6-well plates at a density of $1x10^5$ cells/ml with fresh complete culture medium. Morphological changes were observed under phase contrast microscopy. Unless otherwise indicated, cultures were grown to 70-80% confluence and then serum-starved overnight in F10 medium prior to treatment.

Antibodies and inhibitors. Rabbit anti-phospho-JNK, rabbit anti-phospho-p38 and rabbit anti-caspase 3 were obtained from Cell Signaling Technology (Danvers, MA). Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP antibody were received from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-ß-actin, minocycline (Mino) and H_2O_2 were obtained from Sigma. JNKi and SB 203580 were from Calbiochem (San Diego, CA).

MTT dye assay. Cell viability was assessed using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide dye (MTT) assay (22). The assay was performed according to the instructions of the manufacturer. Briefly, B10BR cells were cultured in 96-well plates, 20 μl of 5 mg/ml MTT labeling reagent was added to each well containing cells in 150 μl of medium, and the plate was incubated for 4 h in a humidified incubator at 37˚C to allow the MTT to be metabolized. The media were removed and cells were re-suspended in formazan (MTT metabolic product) in 200 μl dimethyl sulfoxide (DMSO). The plate was placed on a shaker for 5 min to thoroughly mix the formazan into the solvent. The absorbance of the samples was measured at a wavelength of 490 nm. The extent of MTT conversion in cells exposed to H_2O_2 is expressed as a percentage of the control.

FACS analysis. Twenty-four hours after H_2O_2 and minocycline treatment, both detached and attached cells were harvested and combined. Mild trypsinization was used to collect the attached cells. Cells were centrifuged at 200 x g for 5 min, washed twice with PBS, stained with FITC-conjugated annexin-V (BD Pharmingen) and propidium iodide (PI) for 30 min in the dark at room temperature, and subjected to two-color analysis using FACScalibur (Becton Dickinson, Mountain View, CA).

Western blot analysis. As reported previously (23), cultured mouse melanocytes with and without treatment were washed with cold PBS and harvested by scraping into 150 μl of RIPA buffer. Cell lysates were incubated in 4˚C for 30 min. Proteins (20 μg) were denatured in 5X SDS-PAGE sample buffer for 5 min at 95˚C. The proteins were separated by 10% SDS-PAGE and transferred onto PVDF membrane (Millipore, Bedford, MA). Nonspecific binding was blocked with 10% dry milk in TBST for 1 h at room temperature. After blocking, membranes were incubated with specific antibodies in dilution buffer (2% BSA in TBS) overnight at 4˚C. The blots were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG at appropriate dilutions and room temperature for 1 h. Antibody binding was detected using enhanced chemiluminescence (ECL) detection system (GE Biosciences, NJ) following manufacturer's instructions and visualized by autoradiography with Hyperfilm.

Melanin quantification. As previously reported (24), B10BR cells were seeded in 6-well culture plates and were treated with various concentrations of H_2O_2 and minocycline for 24 h. Then cells were washed twice with PBS, detached with 0.25% Tripsin/EDTA. Cell pellets were disrupted in a lysis buffer (20 mM Tris, pH 7.5, 0.1% Triton X-100) on ice for 30 min. After centrifugation, the pellets were washed twice with 5% trichloroacetic acid, twice with a cold mixture of ethanol and ethyl ether (3:1), and once with cold ethyl ether. The dried pellets were dissolved in 0.85 N NaOH by boiling for 10 min, and the absorbance values were then measured at a wavelength of 405 nm. Amounts of melanin were calculated from the standard curves for synthetic melanin.

Statistical analysis. The values in the figures are expressed as the means ± SE. The figures in this study were representative of 3 different experiments. Statistical analysis of the data between the control and treated groups was performed by a Student's t-test. Values of $p<0.05$ were considered to be statistically significant.

Results

H_2O_2 decreases melanocyte viability in B10BR cells. To test whether H_2O_2 directly induces melanocyte viability, B10BR cells were cultured in 96-well plates, as described in Materials and methods, and treated with H_2O_2 at concentrations of 50, 100 and 250 μM. The data shows that H_2O_2 (50-250 μM) induced a concentration-dependent decrease in cell viability measured by MTT assay (Fig. 1A). To further determine H_2O_2-induced apoptosis in melanocytes, B10BR cells were cultured in 6-well plates and treated with H_2O_2 for 24 h. Cells
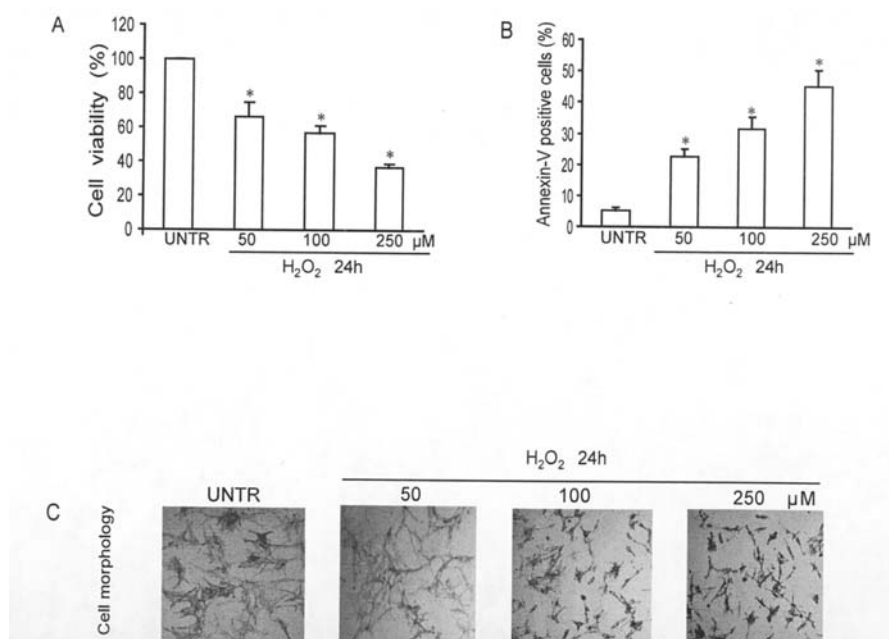
Figure 1. H_2O_2 induced cell death in cultured mouse melanocytes. B10BR cells were treated with different concentrations of H_2O_2 (50, 100 and 250 μM) for 24 h. Cell viability was detected with MTT dye assay (A). Cell apoptosis was measured by flow cytometry with annexin-V-FITC and PI counterstaining (B). Cell morphology was observed using an optical microscope (Olympus IX-50) equipped with a JVC color video camera (TKC1380U), (C). $^*P<0.05$ vs. control group for B10BR cells. Data are presented as the mean ± SEM for three independent experiments.

were then collected and the apoptosis rate was quantified by flow cytometry. The apoptotic cells were positively stained by FITC-labeled annexin-V and showed an increase in the number of positively stained cells as the concentration of H_2O_2 increased (Fig. 1B). We also observed the morphological changes of melanocytes after H_2O_2 treatment. Melanocytes were treated with various concentrations (50-250 μM) of H_2O_2 for 24 h. Many melanocytes were degenerated after H_2O_2 treatment (Fig. 1C) and the cytotoxic effects of H_2O_2 on melanocytes were in a concentration-dependent manner.

H_2O_2 induces p38 MAPK and JNK phosphorylation and caspase 3 activation in B10BR cells. To investigate the mechanism of H_2O_2-induced apoptosis in melanocytes, we next focused on the signal transduction pathways in H_2O_2-induced melanocytes. Mitogen-activated protein kinases (MAPKs) are a large family of protein kinases that phosphorylate and sequentially activate one another in a series of distinct cascades in response to diverse sets of stimuli. JNK and p38 MAPK pathways have been shown to form the core signaling unit of oxidative stress responses in melanocytes. Caspase 3 plays a major role in cell apoptosis induced by oxidative stress. To determine whether p38, JNK and capase 3 are involved in H_2O_2-induced apoptosis in cultured melanocytes, B10BR cells were deprived of serum overnight and treated with H_2O_2. Cell lysates were collected at 5, 10, 20, 40 and 60 min post H_2O_2 treatment to detect p38 and JNK phosphorylation. To detect caspase 3 activation, cell lysates were collected at 6, 12 and 24 h post H_2O_2 treatment. Western blot analysis indicated that 250 μM of H_2O_2 transiently induced p38 and JNK phosphorylation, as shown in Fig. 2A-D. H_2O_2-induced p38 and JNK phosphory-

lation peaked at 20 min post treatment and the activity remained elevated for 1 h. The activation of caspase 3 peaked at 12 h and remained for 24 h, as shown in Fig. 2E and F.

Minocycline inhibits the activation of p38 MAPK, JNK and caspase 3 induced by H_2O_2. The data above clearly demonstrate that p38 and JNK activation is involved in H_2O_2-induced apoptosis. However, whether this activation or other mechanisms are involved in the protective effects of minocycline against H_2O_2-induced apoptosis has not been studied. We therefore pretreated melanocytes with minocycline for 30 min before adding H_2O_2 into medium and collected cells at 20 min after H_2O_2 treatment. As shown in Fig. 3A and C, the phosphorylation of p38 and JNK is significantly inhibited, capase 3 activation is also blocked accordingly, as shown in Fig. 3E. The inhibitory effects of minocycline on the activation of p38, JNK and capase 3 are concentration-dependent. Treatment with SB203580 and JNKi, classic p38 and JNK activation inhibitors, not only blocks H_2O_2-induced p38 and JNK phosphorylation, but also blocks caspase 3 activation significantly, confirming the signal transduction pathways in H_2O_2-induced melanocyte apoptosis, shown in Fig. 3B and D-F. These results demonstrate that inhibition of p38 and JNK activation as well as an inhibitory role in caspase 3 activation is involved, at least in part, in the protective effects of minocycline against H_2O_2-induced apoptosis in melanocytes.

Minocycline protects against H_2O_2 induced apoptosis in B10BR cells. The above data demonstrate that H_2O_2 induced melanocyte apoptosis in a concentration-dependent manner. To determine whether minocycline protects against H_2O_2-induced apoptosis, B10BR cells were pre-treated with different
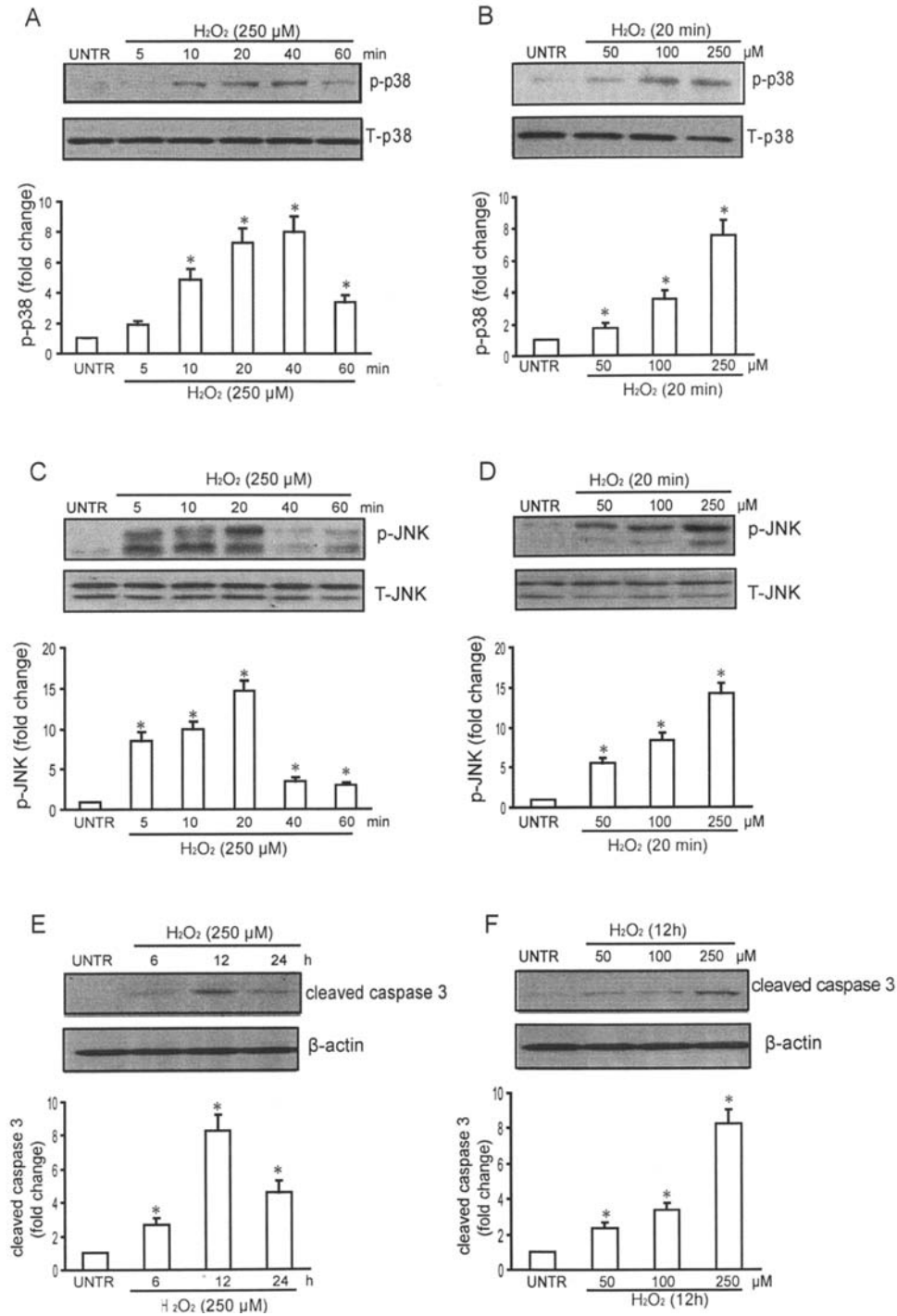
Figure 2. H_2O_2 induced JNK, p38 phosphorylation and caspase 3 activation in cultured mouse melanocytes. B10BR cells were treated with H_2O_2 (250 μM) and harvested at different time points (5, 10, 20, 40 and 60 min): phosphor-p38 (p-p38) was detected by Western blot analysis and quantified in A; phosphor-JNK (p-JNK) was detected by Western blot analysis and quantified in C. B10BR cells were treated with various concentrations of H_2O_2 (50, 100, 250 μM) and harvested at 20 min: p-p38 was detected by Western blot analysis and quantified in B; p-JNK was detected by Western blot analysis and quantified in D. B10BR cells were treated with H_2O_2 (250 μM) for different time points (6, 12 and 24 h, shown in E) or treated with various concentrations of H_2O_2 (50, 100, 250 μM, shown in F) for 12 h. Cleaved-caspase 3 was detected by Western blot anaysis and quantified and normalized to ß-actin. *P<0.05 vs. control group for B10BR cells. Data are presented as the mean ± SEM for three independent experiments.

concentrations of minocycline (10-100 μM) for 30 min before 250 μM H_2O_2 was added. The MTT data showed that minocycline caused concentration-dependent protective effects on cell viability, as shown in Fig. 4A. p38 inhibitor, SB, or JNK inhibitor, JNKi, also showed the protective effects against H_2O_2-induced cell death (Fig. 4B). The apoptosis rate

was further quantified by flow cytometry and the data showed that minocycline protected against H_2O_2-induced cell death in a concentration-dependent manner, as shown in Fig. 4C. In addition, the melanocyte morphological changes induced by H_2O_2 were reversed by pretreatment with minocycline (Fig. 4D). The above data demonstrate that p38 and
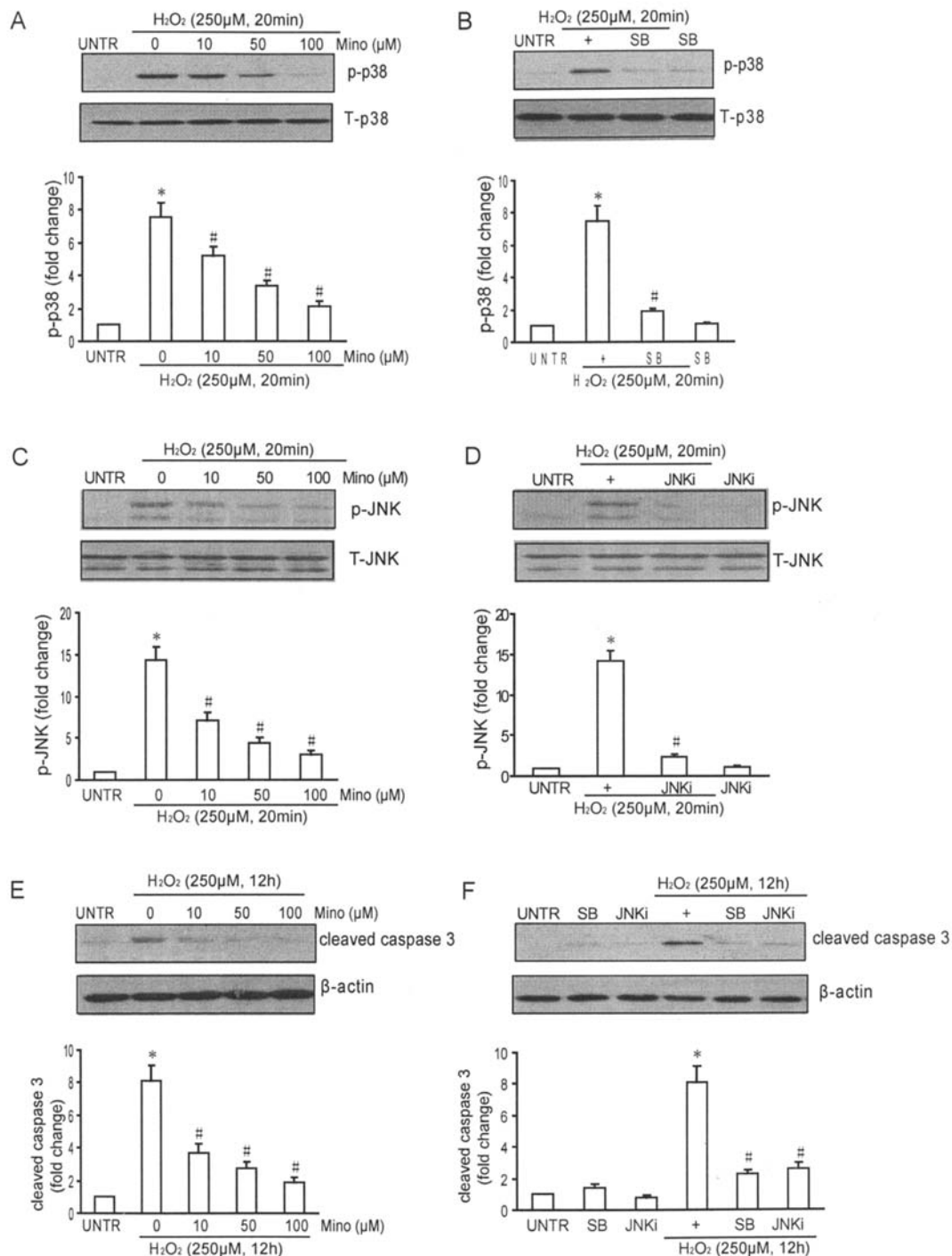
Figure 3. Inhibitory effects of minocycline on JNK, p38 and caspase 3 activation induced by H_2O_2. B10BR cells were pretreated with minocycline (10, 50, 100 μM) for 1 h, followed by H_2O_2 (250 μM) treatment for 20 min: phosphor-p38 (p-p38) was detected by Western blot analysis and quantified in A; phosphor-JNK (p-JNK) was detected by Western blot analysis and quantified in C. B10BR cells were pretreated with p38 inhibitor SB203580 (SB, 10 μM) or JNK inhibitor JNKi (10 μM) for 1 h, followed by H_2O_2 (250 μM) treatment for 20 min: p-p38 was detected by Western blot analysis and quantified in B; p-JNK was detected by Western blot analysis and quantified in D. B10BR cells were pretreated with minocycline (10, 50, 100 μM, shown in E) or pretreated with SB and JNKi (10 μM, shown in F) for 1 h, followed by H_2O_2 (250 μM) treatment for 12 h. Cleaved-caspase 3 was detected by Western blot analysis and quantified and normalized to ß-actin. *$P < 0.05$ vs. control group for B10BR cells. #$P < 0.05$ vs. H_2O_2 (250 μM) group for B10BR cells. Data are presented as the mean ± SEM for three independent experiments.

JNK were involved in the melanocyte apoptosis pathway. To further study whether inhibition of p38 and JNK phophorylation protects melanocytes against apoptosis, B10BR cells were pretreated with p38 inhibitor, SB, or JNK inhibitor, JNKi. Results showed that SB and JNKi recover the morphological changes induced by H_2O_2 (Fig. 4D).

H_2O_2 treatment decreases melanin synthesis which is blocked by minocycline. To further investigate whether melanin synthesis is decreased after H_2O_2 treatment and whether minocycline could attenuate H_2O_2 effect, we directly measured melanin synthesis after treatments. The results showed that melanin synthesis is significantly decreased after H_2O_2
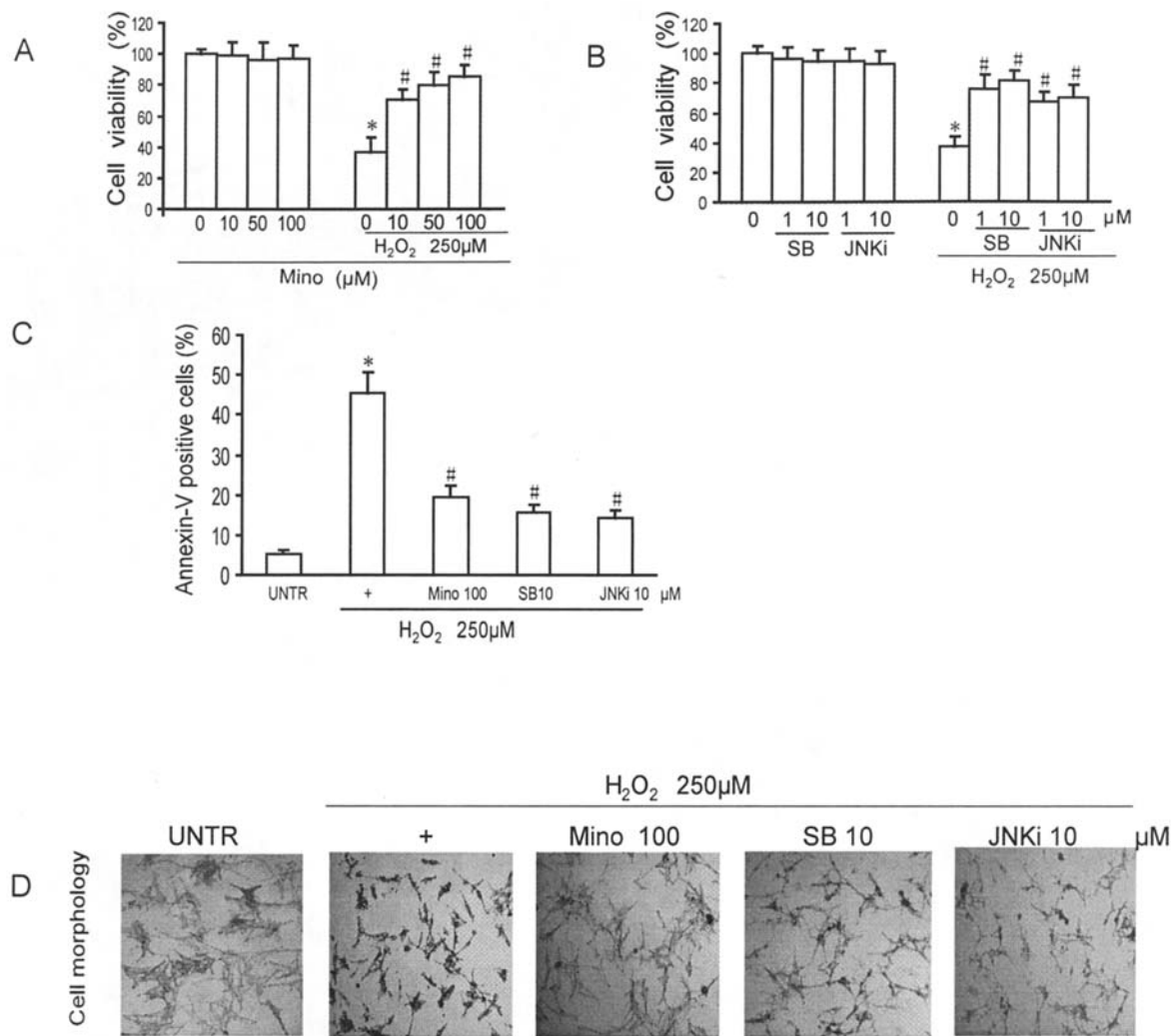
Figure 4. Minocycline protects B10BR cells from apoptosis induced by H_2O_2. B10BR cells were pretreated with minocycline (10, 50, 100 μM) or SB or JNKi (10 μM) for 1 h, followed by H_2O_2 (250 μM) treatment for 24 h. Cell viability was detected with MTT dye assay, shown in A and B. Cell apoptosis was measured by flow cytometry with annexin-V-FITC and PI counterstaining, shown in C. Cell morphology was observed using an optical microscope equipped with a JVC color video camera, shown in D. *$P<0.05$ vs. control group for B10BR cells. #$P<0.05$ vs. H_2O_2 (250 μM) group for B10BR cells. Data are presented as the mean ± SEM for three independent experiments.
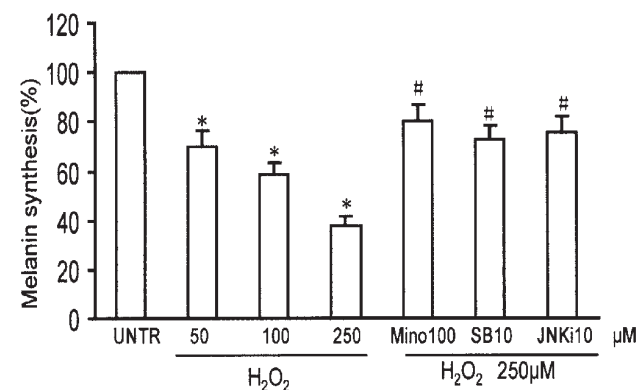

Figure 5. Minocycline reversed melanin synthesis of B10BR cells decreased by H_2O_2. B10BR cells were treated with different concentrations of H_2O_2 (50, 100 and 250 μM) for 24 h; melanin synthesis was detected. B10BR cells were pretreated with minocycline (10, 50, 100 μM), SB (10 μM) and JNKi (10 μM) for 1 h, followed by H_2O_2 (250 μM) treatment for 24 h; melanin synthesis was detected. *$P<0.05$ vs. control group for B10BR cells. #$P<0.05$ vs. H_2O_2 (250 μM) group for B10BR cells. Data are presented as the mean ± SEM for three independent experiments.

treatment and the decrease is in an H_2O_2 concentration-dependent manner. Pretreatment with minocycline reverses H_2O_2 effect. Similar results were seen in melanocytes pretreated with SB and JNKi (Fig. 5).

Discussion

Previous studies have shown that H_2O_2-mediated oxidative stress plays a key role in the onset as well as in the progression of vitiligo (3). Both the epidermis and the vascular system in patients with active vitiligo are characterized by constant accumulation of H_2O_2, leading to impaired redox state and cell signal cascade (8,10,25,26). H_2O_2 is the vital mediator of oxidative stress which leads to cell death. Our results indicate that H_2O_2 treatment decreases cell viability in a concentration-dependent manner (50-250 μM) in B10BR cells. Previous studies have demonstrated that a reduction in epidermal H_2O_2 levels correlates with a cessation of the disease in 95% of affected vitiligo patients (14,27). Thus

elimination of excessive epidermal H_2O_2 is critical in promoting repigmentation and for the eventual development of vitiligo therapy drugs.

Excessive epidermal H_2O_2 is closely related to vitiligo progression. Previous study has shown that H_2O_2 deactivates methionine sulfoxide reductases A and B in the epidermis of patients with vitiligo eventually and decreases antioxidant capability (26). Also H_2O_2 was reported to mediate oxidation of epidermal ACTH, α-MSH, and ß-endorphin in vitiligo, and oxidized endorphin loses its function in the promotion of pigmentation in melanocytes (10). Furthermore, H_2O_2-mediated oxidation affects calcium binding in calmodulin, leading to perturbed calcium homeostasis and perturbed l-phenylalanine-uptake in the epidermis in acute vitiligo (28). However, the signal cascade by which H_2O_2 induces apoptosis in melanocytes has not been well elucidated. While studies on neurodegenerative disorders have indicated that H_2O_2 activates p38 MAPK and JNK which account for neuron loss (29,30) and neurotoxicity (31), the question whether they are involved in H_2O_2-induced melanocyte apoptosis remains unknown. Our results demonstrate that H_2O_2 does activate p38 MAPK and JNK, but not ERK (data not shown) and also activates caspase 3. Using specific kinase inhibitors, our study further supports the notion that p38 and JNK are the most important kinases involved in H_2O_2-induced cell death in melanocytes, suggesting that p38 and JNK inhibitors may be developed to treat vitiligo.

Minocycline exerts protective effects on neurons. Minocycline treatment significantly reduces pro-nerve growth factor production in microglia by inhibiting phosphorylation of p38 and alleviates oligodendrocyte death (32). Also, minocycline inhibits JNK1/2 and ERK1/2 activation and exerts anti-inflammatory properties in the central nervous system (33). Moreover, minocycline inhibits both caspase-dependent and -independent apoptotic pathways and possesses neuroprotective effects against hippocampal damage (34,35). We hypothesized that minocycline can inhibit H_2O_2-induced p38 and JNK activation and exert protective effects on melanocytes. Our results showed that minocycline inhibits p38 and JNK activation and caspase 3 activation. Inhibition of p38 and JNK activation is involved, at least in part, in the protective effect of minocycline in melanocytes, against H_2O_2-induced apoptosis. Our data suggest that minocycline may be useful for vitiligo therapy by preventing melanocyte loss.

In the present study, we showed that minocycline pretreatment inhibited apoptosis induced by H_2O_2 in melanocytes. Our study suggests that minocycline may be a promising drug in preventing the loss of melanocytes and promoting repigmentation in vitiligo patients. Moreover, we found that pretreatment with minocycline reverses melanin synthesis impaired by H_2O_2. We propose that minocycline has no direct effects on melanin synthesis or tyrosinase activity (data not shown), and the up-regulated melanin synthesis by minocycline is most likely a consequence of an increase of melanocyte viability. Collective data from clinical application have revealed that skin pigmentation occurs in some rheumatoid patients with chronic minocycline administration (36,37). Although the underlying mechanism has not yet been elucidated, it has been observed that minocycline-induced pigment deposit in papillary dermis but not in epidermis has been observed (38,39). Given the above, minocycline may be effective in promoting repigmentation in vitiligo patients. Further study will be carried out focusing on the protective effects of minocycline *in vivo* in vitiligo patients.

In conclusion, our data have shown that pretreatment with minocycline inhibited H_2O_2-induced p38 and JNK phosphorylation and attenuated cell death in melanocytes. This study provides insights into the understanding of the molecular mechanism of the beneficial effect of minocycline in treatment of vitiligo patients.

Acknowledgements

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