

Effects of Oxymatrine on the NF-kappa B expression of HaCaT cells

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Abstract

Objective: To study the effect of oxymatrine on the expression of nuclear factor-kappa B (NF- κ B) of Human benign epidermal keratinocytes line (HaCaT cells). **Methods:** HaCaT cells were cultured with different concentration of Oxymatrine (10 μ g/ml, 50 μ g/ml and 100 μ g/ml) for 24 h, then 10^{-5} mol/L Substance P was added to the cells. After 30 min, NF- κ B expression in the cells was observed by immunocytochemistry, NF- κ B P65 protein expression was evaluated by Western blot, and the mRNA expression of NF- κ B P65 was evaluated by reverse transcription polymerase chain reaction (RT-PCR). The 10^{-5} mol/L Substance P and culture medium were added to the Substance P group and control group, respectively. **Results:** In control group, expression rate of positive cells, the expressions of protein and mRNA of NF- κ B were all low. In Substance P group, when 10^{-5} mol/L Substance P was added, the expressions were all increased ($P < 0.05$). But in Oxymatrine groups, the expression rate of positive cells, the expressions of protein and mRNA were all descended in a concentration-dependent manner ($P < 0.05$ or $P < 0.01$). **Conclusion:** Oxymatrine can down-regulate the expression of NF- κ B of the HaCaT cells and may play an important role in regulating anti-inflammation and immunity.

Key words: Oxymatrine; NF- κ B; HaCaT cells; Substance P

INTRODUCTION

Many skin diseases are related to the Substance P and the activation of NF- κ B, such as psoriasis, urticaria, and so on. NF- κ B is a kind of cellular factor and participates in the expression and regulation of many kinds of inflammation mediators^[1]. Substance P is a neuropeptide with many biological activities, one of which has been proved to participate in the regulatory process of skin immune and inflammation. The inter-regulation of the cytokine and Substance P has also been found to exist^[2]. Oxymatrine is an extract from *Sophora flavescens Ait* (a kind of traditional Chinese medicine), which has the effects of anti-inflammatory, anti-allergy, anti-virus and anti-tumor. Oxymatrine has been used in the treatment of skin diseases such as eczema, alopecia and psoriasis. In order to explore the effect of oxymatrine on the NF- κ B in HaCaT cells, the present study was designed.

MATERIALS AND METHODS

Reagents, cell culture and grouping

HaCaT cells and Substance P were purchased from ATCC (Manassas, VA), NF- κ B P65 rabbit anti-human polyclonal antibody (1:500) and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). DAB immunohistochemical kit, Western blotting kit and PCR kit were all purchased from Shanghai Shenggong Bioengineering Technology Service Co. Ltd. Oxymatrine was purchased from China Institute for Drug Control.

HaCaT cells were cultured with DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (PC/SM) at 37°C in a humidified atmosphere of 5% CO₂. When cells were cultured at confluent state, cells between passages 2 and 3 were used in the test. Cells were divided as follows: control group (only culture medium), Substance P group (10^{-5} mol/L Substance P) and Oxymatrine treated groups (10 μ g/ml Oxymatrine, 50 μ g/ml Oxymatrine and 100 μ g/ml Oxymatrine).

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Immunocytochemistry

Confluent cells in 6-well plates were pre-incubated with different concentration of Oxymatrine or culture medium, respectively. After 24 h, the 10^{-5} mol/L Substance P was added to the cells. Thirty minutes later, the cells were washed with ice-cold PBS, and fixed with 4% formaldehyde on ice for 15 min. The fixed cells were permeated by treatment with PBS containing 0.1% Triton X-100 and blocked with 5.5% goat serum at room temperature for 1 h. A primary antibody to detect NF- κ B p65 was incubated at 48°C overnight. Subsequently, addition of secondary antibody and staining(DAB substrate kit) were carried out according to the manufacturer's instructions. NF- κ B expressions in the cells were observed by immunocytochemistry, when the cells nuclei were stained brown or buffy, they were determined positive. Twenty fields of vision from each slide were randomly chosen under microscope. The positive cells expression rate=positive cells amount/total cells amount \times 100%. Then, the average value and standard error were calculated. This experiment was repeated three times.

Western blotting

HaCaT cells were regularly cultured in the 50 ml culture bottle. After the cells were cycled for the logarithm growth period, the drug groups were treated by different concentrations of Oxymatrine for 24 h, respectively. Then 10^{-5} mol/L Substance P was added to the cells for 30 min. At the end, 1×10^6 cells were collected. Equivalent amounts of protein in each sample were separated by 10% SDS-PAGE and blotted onto a PVDF membrane, and then incubated with NF- κ B P65 rabbit anti-human polyclonal antibody for target protein. The sample protein was placed at 4°C overnight and incubated with the secondary antibody. At last, DAB coloration, picturing and scanning were performed. The results were analyzed by Gel doc1000 Automatic Image software to determine the band density value with β -actin as the internal control. The relative expression value of NF- κ B P65 protein was expressed as the NF- κ B P65 density value / β -actin density value. This experiment was repeated three times.

Semi-quantification of RT-PCR

Confluent cells were treated with Oxymatrine of 10 μ g/ml, 50 μ g/ml and 100 μ g/ml for 24 h, respectively, and then stimulated with Substance P of 10^{-5} mol/L for 30 min. After the incubation, the cells were treated with ice-cold PBS. Total RNA was isolated by one-step method of Trizol. Those RNAs were identified for their integrity by agarose gel electrophoresis. RT procedure was carried out by reverse transcription kit, the 2 μ l cDNA were used as templates

for PCR with upstream primer 5' -GACCAACAACAACCCCTTC-3' and downstream primer 5' -ACCTCAATGTCCTCTTTCTGC-3', product size was 273 bp. And β -actin as the internal control with upstream primer 5' -ATCATGTTTG-AGACCTCA-3', downstream primer 5' -CATCTC-TTGCTCGAAGC-3', the product size was 249 bp. The conditions for PCR were as follows: one cycle of 5 min at 94°C, 94°C 30 s, 51°C 30 s, 72°C 1 min, 30 cycles, the final cycle 10 min at 72°C. The products(10 μ l) were separated by 1% agarose gel electrophoresis and analyzed by image analysis system, mRNA expression was calculated as the ratio of the density of the corresponding band to the β -actin band by densitometry, respectively. This experiment was repeated three times.

Statistical analysis

All analyses were performed at least in triplicate. Statistics tests were performed by SPSS 12.0 software. All values are expressed as mean \pm SD($\bar{x} \pm s$) and performed by one ANOVA test. The *P* value of less than 0.05 was considered statistically significant.

RESULTS

Immunocytochemistry

Cells in the control group grew in a confluent state, when part of cytoplasm and a few nuclei were stained brown(and the expression rate of positive cells was low), the mitoses could be seen(Fig 1a). In Substance P group, many cell nuclei were stained brown or buffy(Fig 1b), the expression rate of positive cells increasing($P < 0.05$). The nuclear condensation and deep stain could be seen, and the cells didn't grow well. But in Oxymatrine groups. The phenomena were different, the expression rates of positive cells were all descended($P < 0.05$ or $P < 0.01$), compared with Substance P group, especially in the group of Oxymatrine of 100 μ g/ml.(Fig 1c-e and Tab 1)

Effects of Oxymatrine on the expressions of NF- κ B by western bolting

Compared with the control group, the band density of protein and the ratio of NF- κ B P65/ β -actin in Substance P group all increased($P < 0.05$) by $(38.89 \pm 1.05)\%$, suggesting that Substance P could increase the protein expression of NF- κ B. But both the density and the ratio of NF- κ B P65/ β -actin in Oxymatrine groups decreased significantly ($P < 0.01$), which decreased by $(28.74 \pm 2.36)\%$, $(43.79 \pm 6.23)\%$ and $(98.68 \pm 9.87)\%$ respectively, compared with the Substance P group, indicating that Oxymatrine can inhibit the protein expression of NF- κ B, especially in the group of 100 μ g/ml. In addition, significant difference between the control group and Oxymatrine group of 100 μ g/ml also existed in the density and the ratio($P < 0.05$). (Fig 2, Tab 2)

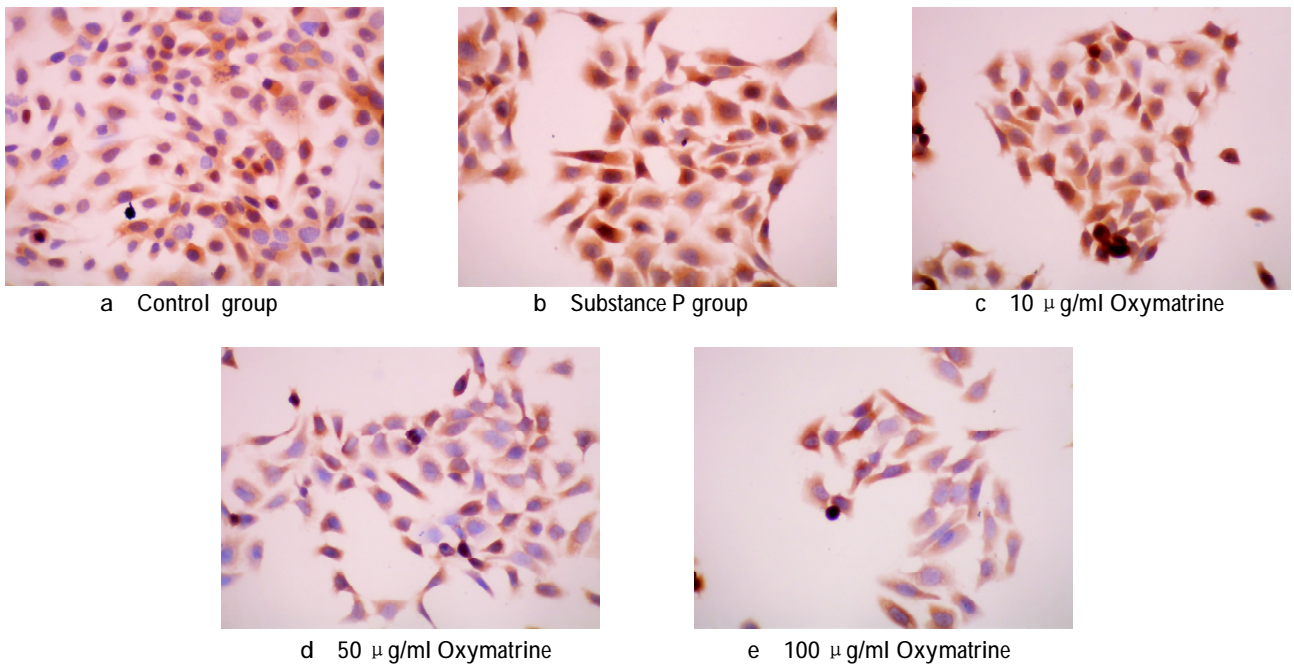
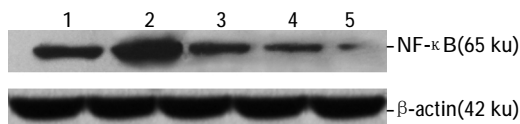


Fig 1 The NF-κ B expression of HaCat cells in each group(IHE, × 200)

Tab 1 The expression rate of NF-κ B positive cells in HaCaT cells (% , $\bar{x} \pm s$)

Control	Substance P	Oxymatrine(μg/ml)		
		10	50	100
13.14 ± 1.55	31.45 ± 2.59 [△]	22.29 ± 1.94 ^{*△}	17.84 ± 2.17 [*]	12.59 ± 4.46 ^{**}

Compared with the control group, [△]*P* < 0.05; compared with the substance P group, ^{*}*P* < 0.05, ^{**}*P* < 0.01.



1:control; 2:Substance P; 3:10 μg/ml Oxymatrine; 4:50 μg/ml Oxymatrine; 5:100 μg/ml Oxymatrine.

Fig 2 Protein expression of NF-κB in different groups

Effects of oxymatrine on the mRNA expressions of NF-κ B

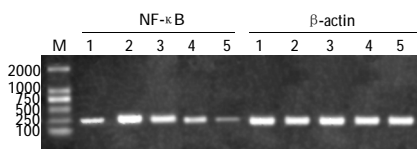
In contrast with the control group, the mean relative density of mRNA and the ratio of NF-κ B P65/ β-actin

all increased in Substance P group(*P* < 0.05), which increased by(38.89 ± 3.56)%, indicating that Substance P could enhance the mRNA expression of NF-κ B. But in the Oxymatrine groups with different concentration, the densities and the ratios all decreased by(57.51 ± 2.32)%,(64.61 ± 6.87)% and(82.84 ± 9.42)%, respectively, in contrast with Substance P group(*P* < 0.01 or *P* < 0.05), suggesting that Oxymatrine can inhibit the mRNA expression of NF-κ B. In addition, the mRNA expression of NF-κ B was also much lower in Oxymatrine group than that in the control group (*P* < 0.05). (Fig 3, Tab 3)

Tab 2 Protein expression of NF-κ B P65 in different groups (n=3, $\bar{x} \pm s$)

Item	Control	Substance P	Oxymatrine(μg/ml)		
			10	50	100
Protein density	265.18 ± 86.32	355.46 ± 59.65 [△]	253.29 ± 76.35 [*]	199.79 ± 38.45 [*]	4.66 ± 32.25 ^{△*}
NF-κ B P65/β-actin	0.78 ± 0.01	1.06 ± 0.03 [△]	0.68 ± 0.34 [*]	0.65 ± 0.34 [*]	0.17 ± 0.02 ^{△*}

Compared with the control group, [△]*P* < 0.05; compared with the substance P group, ^{*}*P* < 0.01.



M: marker; 1: control; 2: SubstanceP; 3: 10 μg/ml Oxymatrine; 4: 50 μg/ml Oxymatrine; 5: 100 μg/ml Oxymatrine.

Fig 3 mRNA expression of NF-κ B in different groups

DISCUSSION

Skin is one of the most important mammalian organs and is the largest organ of the body. It responds to frequent environmental stimuli while maintaining a balance between cellular proliferation and cell loss due to desquamation. Human skin is composed of four different layers: basal, squamous, granular and horny layers. Keratinocytes are

Tab 3 mRNA expression of NF- κ B P65 in different groups (n=3, $\bar{x} \pm s$)

Item	Control	Substance P	Oxymatrine(μ g/ml)		
			10	50	100
mRNA density	18.78 \pm 12.28	27.75 \pm 6.66 $^{\Delta}$	16.82 \pm 1.52 *	8.02 \pm 0.94 **	4.76 \pm 1.05 $^{\Delta**}$
NF- κ B P65/ β -actin	0.76 \pm 0.09	1.52 \pm 0.01 $^{\Delta}$	0.63 \pm 0.03 *	0.41 \pm 0.02 **	0.24 \pm 0.11 $^{\Delta**}$

Compared with the control group, $^{\Delta} P < 0.05$; compared with the Substance P group, $^* P < 0.05$, $^{**} P < 0.01$.

derived from epithelial stem cells that have the ability to proliferate and differentiate into all of the cell types. So keratinocytes are not only the most important skin structure cells, but also the function and target cells of the skin.

Psychological factors and the activation of NF- κ B are associated with many skin diseases in the process of their onset, development and the curative effect. In the process, the cutaneous nervous system and neuroendocrine system play very important roles. The cutaneous nervous system modulates physiological and pathophysiological effects, including cell growth and differentiation, immunity and inflammation as well as tissue repair^[3]. The neuroendocrine system plays a role in the modulation of inflammatory and immune responses^[4]. Substance P is not only a kind of immune regulator, but also a member of tachykinin peptide family which is widely distributed in the central and peripheral nervous system and is an important messenger in neuron-immune system^[2]. Substance P has been proved to be involved in the pathogenesis of inflammatory skin disorders such as atopic dermatitis and psoriasis^[5-7]. On the other hand, Substance P is known to activate a variety of cutaneous cells through high affinity neuropeptide receptors or by direct activation of intracellular G-protein signaling cascade^[3,8]. Via the modulation of transcription factor activation(NF- κ B, AP-1, STAT-3), Substance P regulates the expression of adhesion molecules and proinflammatory cytokines in different cells as modulators of immune and inflammatory reactions^[3].

NF- κ B, which is located in cytoplasm in an inactivated form due to association with its inhibitors, I κ B proteins, plays pivotal roles in inflammatory processes^[9]. NF- κ B exists as a dimer composed of various combinations of structurally related proteins, in which P65 is the transcriptional component of the most common form of the NF- κ B heterodimer^[9-10]. The NF- κ B family of eukaryotic transcription factors plays multiple roles in the regulation of diverse biological processes, including development, immune/inflammatory responses, cell growth/death, stress responses and carcinogenesis^[11-13]. The activation of the transcription factor NF- κ B is central to the control of the cellular response triggered by many stimuli^[14], such as TNF- α , LPS and ultraviolet. In addition, there has been a study to show that neu-

ropeptide Substance P can activate the NF- κ B^[15].

Because many skin diseases are associated with the activation of NF- κ B, such as psoriasis, urticaria and skin tumors, we often use drugs to inhibit the activation of NF- κ B for treating and controlling inflammatory skin diseases. However, these drugs such as glucocorticoids, cyclosporin A, usually have many side-effects. For example, if these drugs are used too long and/or the NF- κ B activity is over-inhibited or in long-term inactivation, dramatic side-effects might occur, such as the depression of immunity, leading to an increased sensitivity to bacterial infection^[15]. It is widely believed that the identification of safe, selective inhibitors of NF- κ B activation will result in powerful new agents for the treatment of chronic inflammatory disorders.

Oxymatrine is a kind of alkaloid extracted from the traditional Chinese medicine, *Sophora flavescens Ait*, which has been extensively used in China for the treatment of viral hepatitis, cancer, cardiac diseases and skin diseases. Oxymatrine has many pharmacological activities such as antiviral, anti-inflammation, immunoregulation, anti-tumor. Furthermore, it can induce apoptosis of keratinocytes^[17].

Evidence showed that Oxymatrine has hormone-like function but no remarkable side effects^[18], and is used in immune and allergic skin diseases such as psoriasis, urticaria, eczema and SLE. Because there is no study on the effect of Oxymatrine on the NF- κ B expression in HaCaT cells, the present experiment was designed. In control group, because NF- κ B was mainly located in the cytoplasm, a few nuclei were stained brown. The positive rate of cells expressing NF- κ B, protein and mRNA expression of NF- κ B were all low. After Substance P was added, NF- κ B trans-located from cytoplasm to the nuclear, so there were many nuclei stained brown or buffy. And the cells displayed NF- κ B positive expression, both protein and mRNA expression of NF- κ B increased compared with control group. These phenomena suggested that Substance P could activate the NF- κ B of HaCaT cells. But in Oxymatrine groups pretreated by Oxymatrine, the expressions were all markedly decreased. The results indicate that Oxymatrine may down-regulate the NF- κ B expression leading to anti-inflammation and antiviral effects, and also regulate the immunity.

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