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Minocycline Modulation of Alpha-MSH Production by Keratinocytes *In vitro*

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The anti-inflammatory mechanisms of minocycline, an antibiotic used in the treatment of the inflammatory component of acne, are only partially understood. In addition to inflammation due to cytokines (IL-1, IL-6, TNF-α, etc.), recent studies have shown that neuropeptide-mediated neurogenic inflammation may play an important role in cutaneous inflammation. The purpose of this study was to investigate minocycline-induced modulation of cutaneous production of alpha-melanocyte-stimulating hormone (α-MSH), a neuropeptide with known anti-inflammatory activity. Two different skin models were used: explants of inflammatory skin and reconstituted skin, both incubated with minocycline at different concentrations and for different time periods. Epidermal production of α-MSH, as evaluated by immunofluorescence and immunoperoxidase techniques, showed increased expression in both models. This neuropeptide, which has an anti-inflammatory activity (notably through production of IL-10, antagonism of IL-1 and inhibition of the chemotaxis of polymorphonuclear leukocytes), thus plays a role in the anti-inflammatory action of minocycline. ***Key words: inflammation; α-MSH; cyclines.***

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Minocycline is an antibiotic used in the treatment of acne, particularly of its inflammatory component. In addition to its bacteriostatic effect on *Propionibacterium acnes* (1), minocycline acts through its own anti-inflammatory properties by inhibiting the bacterial production of lipases and TNF-α (2, 3), the chemotaxis of polymorphonuclear leukocytes (4) and the activity of superoxide dismutase (5). However, the mechanism of minocycline action is only partially understood and could involve the modulation of other mediators, such as neuropeptides. These protein compounds released by nerve fibres in the skin or synthesized by some skin cells contribute to inflammatory events through pharmacological properties, such as modulation of mastocyte degranulation, lymphocytic activity or chemotaxis of polymorphonuclear leukocytes (6). Among the 20 or so neuropeptides present in the skin, alpha-melanocyte-stimulating hormone (α-MSH) seems of particular interest since it displays a clearly established anti-inflammatory activity (7) notably by modulating interleukine production.Thus, the anti-inflammatory activity of minocycline could be related not only to a modulation of cytokines, but also to α-MSH. Moreover, α-MSH contributes to the melanogenesis, is synthesized by keratinocytes (8) and so may possibly be related to the pigmentation induced by minocycline. Thus, the purpose of our study was to investigate minocyline modulation *in vitro* of cutaneous production of α-MSH.

Two different skin models were used: explants of inflammatory skin and reconstituted skin, both incubated with minocycline at different concentrations and for different time periods. The epidermal production of α-MSH was evaluated by immunofluorescence and immunoperoxidase techniques.

MATERIALS AND METHODS

Explant technique in inflammatory skin

Culture. Punches (4 mm in diameter) from biopsies of psoriatic skin, considered as an inflammatory model, were incubated at 37°C in a moist atmosphere in the presence of 5% CO_2 for 3, 6, 8 or 24 h in KSFM medium (Gibro BRL, France) supplemented with penicillin 200 IU/ml-streptomycin 200 μg/ml (Boehringer-Mannheim, Germany), Fungizone 0.25 μg (Bristol-Meyers Squibb, France), epidermal growth factor 0.5 ng/ml (Gibco BRL), and bovine pituitary gland 25 μg/ml (Gibo BRL). The medium contained minocycline at the following concentrations: 1.5, 3 or 6 μg/ml. These concentrations were chosen to match that in serum, which is evaluated between 0.7 and 6.5 μg/ml (9). Medium without minocycline was used as a control. Each minocycline concentration was done in triplicate. After incubation, explants were removed from the culture medium and frozen at –80°C. Sections (6 μm thick) were then cut with a cryostat, fixed in acetone at 4°C for 10 min and frozen.

Immunofluorescence. α-MSH was detected by an immunofluorescence amplification technique. The sections were incubated for 30 min at room temperature with a polyclonal rabbit anti-α-MSH IgG (1:200) (Amersham, France) and then, after washing, with a $F(ab)'_2$ fragment of biotinylated goat anti-rabbit antibody (1:50). Labelling was done using fluorescein-conjugated streptavidin (1:100). Counterstaining was performed with propidium iodide. Two types of negative control were provided: use of an irrelevant monoclonal antibody, CD2 of the same isotype as that of the first antibody, and omission of the first antibody. Moreover the control with pre-immune serum was negative. Slides were interpreted blindly by 2 observers using a Leitz fluorescence microscope. A mean was determined for 4 different fields (×40 magnification) per slide. When keratinocyte staining was detected, the semi-quantitative estimation of fluorescence intensity in the epidermis was scored as follows: 0 = negative; 1 = very low intensity; 2 = low intensity; 3 = moderate intensity; and 4 = high intensity.

Reconstituted skin technique

Culture (Prunieras' technique as modified by Basset-Seguin et al. (10)). One-centimetre squares were cut out of defatted mammaplasties and de-epidermized by heating at 56°C for 15 min. The dermis was then killed by 10 successive freezing/thawing steps for 10 min in liquid nitrogen (–180°C). Two-millimeter punches from biopsies of defatted normal skin were then placed in the centre of killed dermis. After 10 days of culture at 37°C in a moist atmosphere and in the pre-

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Table I. *Modulation by minocycline of the expression of α-MSH by keratinocytes on reconstituted skin after 3 and 6 h of incubation with different concentrations of minocycline.*

The results represent the mean of 3 experiments. Intensity of labelling: + very low intensity; ++ low intensity; +++ moderate intensity

Minocycline (μg/ml)	3 h	6 h
0	+	+
1.5	+	+/++
3	+/++	+/++
6	+/++	++
10	+/++	++/+++
100	++/+++	+++
1000	++/+++	+++

sence of CO_2, the reconstituted skin was incubated for 3 or 6 h with 7 ml Eagle's minimum essential medium (MEM) ×1 (Gibco BRL) supplemented with 10% foetal calf serum (Gibco BRL), penicillin 200 IU/ml-streptomycin 200 μg/ml (Boehringer-Mannheim), epidermal growth factor 20 ng/ml (Gibco BRL), cholera toxin 10-10 (Sigma, France) and hydrocortisone 0.4 μg/ml (Roussel, France). MEM contained minocycline at the various concentrations (Table I), all of which were done in triplicate. Medium without minocycline was used as a control. After incubation, the reconstituted skin was removed from the culture medium and frozen at −80°C. Sections were then cut with a cryostat, fixed in acetone at 4°C for 10 min and frozen.

Immunoperoxidase. α-MSH was detected with an immunoperoxidase technique (DAKO kit), using phosphate-buffered saline for dilution and TBS for washes. At each step, sections were incubated for 30 min at room temperature, first with a rabbit anti-α-MSH IgG (1:200) (Amersham), then, after washing, with a second antibody, i.e. an $F(ab)'_2$ fragment of biotinylated goat anti-rabbit antibody diluted in sodium azide buffer, and finally with peroxidase-conjugated streptavidin. Revelation was performed with hydrogen peroxide and 3-amino-9-ethylcarbazole (5 s). Counterstaining was done with haemalum. Two types of negative controls were provided: use of an irrelevant monoclonal antibody of the same isotype as that of the first antibody, and omission of the first antibody. Slides were interpreted blindly by 2 observers using a Leitz white light microscope. A mean was determined for 4 different fields (×40 magnification) per slide. Fluorescence intensity was scored semi-quantitatively: 0 = negative; 1 = very low intensity; 2 = low intensity; 3 = moderate intensity; and 4 = high intensity.

Statistical analysis

Statistical opinion was sought (Dr N'Guyen) and it was concluded that no statistical test could be adequately used in this system.

RESULTS

Explants of inflammatory skin

The results are summarized in Table II.

In the presence of minocycline, an increase in epidermal staining of α-MSH over baseline values was noted for all time periods studied (3, 6, 8 and 24 h), with a maximum for a concentration of 6 μg/ml at 24 h.

Table II. *Modulation by minocycline of the expression of α-MSH on epidermis by using explants of inflammatory skin.*

This study is performed at 4 different durations of incubation with minocycline and 4 different concentrations of minocycline. The results represent the mean of 3 different experiments. Intensity of labelling: + very low intensity; ++ low intensity; +++ moderate intensity

Minocycline (μg/ml)	3 h	6 h	8 h	24 h
0	+/−	+/−	+/++	+
1.5	+	+/++	++	+/++
3	+	+/++	+/++	+
6	++	++	++	++/+++

Reconstituted skin

Results are summarized in Table I. In the presence of minocycline, an increase in keratinocyte staining of α-MSH over baseline values was noted for all time periods studied, from 3 μg/ml at 3 h and from 1.5 μg/ml at 6 h. Intensity was maximal for minocycline concentrations of 100 and 1,000 μg/ml at 6 h.

DISCUSSION

This *in vitro* study shows that α-MSH production increased in both inflammatory and reconstituted skin beginning with the lowest minocycline concentrations tested (3 μg/ml). α-MSH is a 13 amino acid neuropeptide derived from pro-opionmelanocortin (POMC) (11). In skin, POMC-derived peptides are detected in C fibres (12) as well as in various types of cells: peripheral blood mononucleate cells, immune cells and skin cells (keratinocytes, melanocytes, sebocytes, Merkel cells) (13). Weak epidermal staining was found at basal state in inflammatory and reconstituted skin, which is concordant with the results of previous studies showing a lack of α-MSH keratinocytic production in the absence of stimulation (14). Epidermal staining was increased in the presence of minocycline in both inflammatory and reconstituted skin at all incubation times studied. The skin explant model allowed us to evaluate global α-MSH production in skin, whereas the reconstituted skin model provided more specific assessment of keratinocytic production. In this study, skin biopsies of psoriasis were used as inflammatory skin model, however the results obtained on the modulation of α-MSH production by minocycline may be relevant in acne where minocycline is often used. In both models, the dose-dependent increase was maximal at the highest minocycline concentrations tested, respectively 1000 μg/ml for explant model and 6 μg/ml for reconstituted skin model. These doses are higher than the mean concentrations of minocycline noted in serum (0.7–6.5 μg/ml) (9) for explant skin model, but are similar for reconstituted skin. Increased α-MSH production in the presence of minocycline could affect the different activities of the antibiotic. First, it could play a role in the anti-inflammatory activity of minocycline, since α-MSH has both a central and peripheral anti-inflammatory effect. Its central effect is apparently dependent on a modulation of the release of substance P, a neuropeptide with pro-inflammatory activity (15), and its peripheral effect on inhibition of the release of certain pro-inflammatory cytokines (IL-1, IL-6, TNF-α) (16), production of interferon-gamma (17) and

chemotaxis of polymorphonuclear leukocytes (18). Moreover, it stimulates the production of IL-10, an anti-inflammatory cytokine (19). Thus, α-MSH could contribute to the anti-inflammatory effects of minocycline by a mechanism of release from the nerve fibres containing it and subsequent synthesis by keratinocytes. In this way, it could limit the cytokine cascade, which sustains inflammatory reaction. Moreover, the increase in α-MSH production could affect the pigmentation phenomena relative to minocycline, which are rather common with this antibiotic since α-MSH stimulates melanogenesis (20). It may be concluded from this study that minocycline induces keratinocytic production of α-MSH in *in vitro* models, which is an additional demonstration of the anti-inflammatory properties of this antibiotic.

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