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Effect of glucocorticoids on the synthesis of antimicrobial peptides in amphibian skin

Maurizio Simmaco[a,c], Anita Boman[b], Maria Luisa Mangoni[a], Giuseppina Mignogna[c], Rossella Miele[c], Donatella Barra[c,*], Hans G. Boman[1,b]

[a] *Dipartimento di Scienze Biomediche, Università di Chieti, 66100 Chieti, Italy*
[b] *Department of Microbiology, Stockholm University, S-106 91 Stockholm, Sweden*
[c] *Istituto Pasteur-Fondazione Cenci Bolognetti, Dipartimento di Scienze Biochimiche and CNR Centro di Biologia Molecolare, Università 'La Sapienza', Piazzale Aldo Moro 5, 00185 Rome, Italy*

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Abstract Gene-encoded peptide antibiotics are widespread in insects, plants and vertebrates and confer protection against bacterial and fungal infections. NF-κB is an important transcription factor for many immunity-related mammalian proteins and also for insect immune genes. The activity of NF-κB is regulated by the interaction with an inhibitor, IκB. It was recently demonstrated that glucocorticoids induce the synthesis of IκB in human cell lines. So far, all genes for peptide antibiotics have promoter motifs with NF-κB binding sites, but its actual function in peptide regulation has been studied only in insects. Here we show that glucocorticoid treatment of the frog *Rana esculenta* inhibits the transcription of all genes encoding antibacterial peptides by inducing the synthesis of IκBα. These results suggest that also in vertebrates peptide-mediated innate immunity is controlled by NF-κB-regulated transcription.

Key words: Antimicrobial peptide; Innate immunity; Cortisone; IκB; *Rana esculenta*

1. Introduction

Gene-encoded peptide antibiotics are widespread in nature [1–3]. These molecules confer protection against microorganism infections in the absence of a clonally based immune system. However, both in insects and in vertebrates innate immunity provided by antimicrobial peptides may act as a first line of defence against infections.

It is well known that glucocorticoids (GCs) suppress immunity in mammals, but the precise mechanism was only recently clarified by studies on cultured mammalian cells [4,5]. An important transcription factor, NF-κB, a homo- or heterodimeric, Rel-containing protein, is present in the cytoplasm. The activity of NF-κB is regulated by preventing it from entering into the nucleus when tightly complexed with one member of a family of proteins with ankyrin repeats, termed IκB. The recent finding that GCs induce the constitutive synthesis of IκBα [4,5] explains the immunosuppression exerted by GCs in mammalian cell cultures. Since several genes for antibacterial peptides in mammals have promoter motifs that could bind NF-κB-like factors [6–8], we decided to investigate whether GC treatment of live frogs could influence the synthesis of immunity-related peptides in the skin.

*Corresponding author. Fax: (39) (6) 4440062.
E-mail: barra@axrma.uniroma1.it

[1]Present adress: MTC, Box 280, Karolinska Institut, S-171 77 Stockholm, Sweden.

We selected wild *Rana esculenta* for the present work, since the different antimicrobial peptides in the skin secretion of this frog are well known, and the cDNAs encoding their precursors have been described [9]. The most potent antibacterial peptide is esculentin, a 46-residue peptide with two cysteines close to the C-terminus, which form an intramolecular disulfide bridge [9]. Related molecules, such as brevinins [10], ranalexin [11] and gaegurins/rugosins [12,13], are present in the skin secretion of other *Rana* species.

2. Materials and methods

2.1. Frogs and collection of skin secretion

Specimens of *R. esculenta* (adults of 20–30 g each) were collected in the wild near Rome. They were maintained in individual cages and fed larvae of *Tenebrio molitor*. Skin secretion was collected after giving the frog a mild electrical stimulation, usually 10 V for 5 s at three equal intervals [14]. The secretion was washed off with 0.05% acetic acid and freeze-dried. Typically, a single stimulation of a frog produced 10–20 mg of freeze-dried crude secretion.

2.2. HPLC fractionation of frog skin secretion

About 2 mg of secretion was fractionated by HPLC on a Beckman System Gold chromatographer using a C_8 reverse-phase column (Aquapore RP-300, 4.6×250 mm, Applied Biosystems) eluted with a 30-min gradient of 5–60% acetonitrile/2-propanol 4/1 in 0.2% (v/v) trifluoroacetic acid, at a flow rate of 0.8 ml/min. Elution of the peptides was monitored using a diode array Beckman model 168 detector.

2.3. Bacterial counts

Bacteria were collected from the mouth of frogs by introducing 20 μl LB medium and subsequently withdrawing 5 μl. Bacteria present in these samples of 'saliva' were spread in 3 ml soft agar on LB plates. Colonies were counted after overnight incubation at 30°C and the numbers of viable bacteria are given as colony forming units, cfu/5 μl of saliva.

2.4. Glucocorticoid treatment of frogs

Test frogs were first given an electrical stimulation in order to discharge the peptides stored in their skin glands and then carefully washed as described above. About 30 min later, a cream (0.25 g/g body weight) with a systemically acting glucocorticoid (Clobesol, Glaxo, containing 0.05 g clobetasol propionate/100 g) was applied to the whole skin surface of the frogs. Alternatively, frogs were injected, every second day for a week, either intraperitoneally or subcutaneously, with 75 μg/g body weight of a water-soluble, locally acting, GC derivative (Solu-Medrol, Upjohn), a sodium succinate ester of methylprednisolone.

2.5. Northern blot analysis

Total skin RNA was isolated from 5 g of tissue by acidic guanidinium thiocyanate-phenol-chloroform extraction [15]. RNA samples (10 μg) were fractionated by electrophoresis in 1.2% agarose gel containing 0.8 M formaldehyde [16] and then blotted directly onto a nylon membrane (Sartorius). A cDNA coding for the esculentin pre-

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cursor (clone Escl-17-1, [9]), labeled by random priming, was used as probe.

2.6. Preparation of frog skin crude cytosol

Frog skins (5 g) were minced with scissors and homogenized with Ultra-Turrax in 25 ml ice-cold 20 mM HEPES, containing 2 mM EDTA and 1 mM PMSF. The lysates were centrifuged at 4500 rpm for 10 min a 4°C. The soluble fractions were further centrifuged at 12000 rpm for 30 min at 4°C. The supernatants were concentrated with a Centricon 30 membrane (Amicon) to 5 ml. Protein concentrations of each preparation were measured using a Bio-Rad protein assay kit.

2.7. Immunoblotting

Total proteins (100 μg) were separated by 12% SDS polyacrylamide gels (Bio-Rad), electroblotted on nitrocellulose (Sartorius) and then blocked in TBST (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 20) containing 0.5% BSA. The membranes were incubated for 16 h at 4°C with 1:1000 dilution of polyclonal rabbit antibodies against a synthetic peptide corresponding to residues 26–39 from the N-terminus of human IκBα (New England Biolabs Inc., Beverly, MA, USA). The blots were washed and incubated with 1:2000 dilution of anti-rabbit Ig-peroxidase conjugate in TBST. After several washes, antibody-reactive bands were visualized by enhanced chemiluminescence (New England Biolabs Inc.).

3. Results

The influence of glucocorticoids on the production of antimicrobial peptides by skin glands of *R. esculenta* was first demonstrated by the effects on the number of aerobic bacteria present in the frog mouth. Frogs, kept in cages for 2 weeks after capture, normally carried 70–140 cfu/5 μl LB saliva.

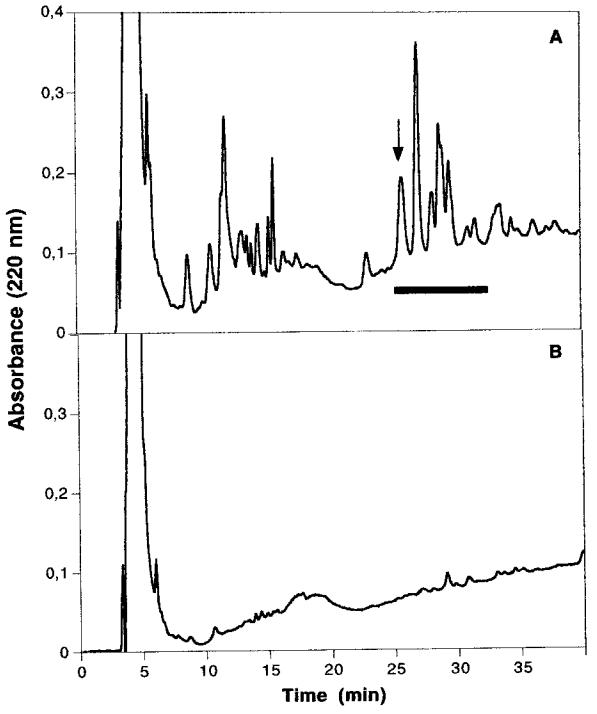

Fig. 1. Reversed-phase HPLC fractionation of the total skin secretion from two *R. esculenta* specimens. After stimulation, the frogs were washed with 6 ml 0.05% acetic acid. Of the acetic acid used, 5 ml was recovered, freeze-dried and redissolved in 3 ml 20% ethanol. One sixth of this solution was analyzed by HPLC. A: HPLC chromatogram of the total skin secretion of the control frog. The horizontal bar indicates fractions with known antibacterial activity; the arrow indicates the esculentin peak. B: The corresponding chromatogram from the GC-treated frog.

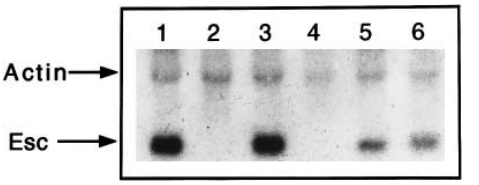

Fig. 2. Northern blot analysis of total RNA from skin of *R. esculenta*. A cDNA encoding the esculentin precursor (Esc) was used as probe. Lane 1, wild frog; lanes 2 and 4, frogs treated with glucocorticoid for a week; lane 3, control frog kept in a cage for a week; lanes 5 and 6, two frogs given a GC injection under the dorsal skin or intraperitoneally, respectively. A probe encoding human actin was used as control. This experiment was repeated three times.

When frogs were given an electrical stimulation to discharge the peptides stored in the glands, no bacteria could be recovered from the mouth 30 min later. After a stimulation, the skin secretion visibly enters the mouth of the animal either through the mouth itself or through the orifices located close to the eyes. Frogs receiving a single dose of glucocorticoid cream on the skin surface showed after 48 h an increase in mouth bacteria to around 4000 cfu/5 μl saliva. Three days after the electrical stimulation, the amount of bacteria was restored to 40 in the untreated frog, and decreased to 400 in the GC-treated animal, thus approaching the normal values.

In the next set of experiments, two different frog specimens were electrically stimulated and carefully washed. One of the frogs received for a week a daily treatment with the GC cream, the other was an untreated control. After 7 days, the skin secretions of both frogs were collected and their antibacterial activity against *Escherichia coli* D21 was recorded with the inhibition zone assay [17]. The secretion of the GC-treated frog showed only 7% of the activity of the untreated control. Moreover, the secretions of the two frogs were analyzed by HPLC. The secretion of the control frog (Fig. 1A) gave a chromatographic pattern typical for the occurrence of the various families of antibacterial peptides of *R. esculenta* [9], whereas the secretion of the GC-treated frog gave no significant peptide peaks (Fig. 1B). Thus, the GC treatment resulted in the disappearance of all known antibacterial peptides in the skin secretion.

To date, no gene structures are available that encode antibacterial peptides from *Rana* species. However, a Northern blot analysis of total RNA from skin of *R. esculenta*, using as probe a cDNA encoding the precursor of esculentin [9], shows clearly that GC treatment of the frog skin blocked the transcription of the esculentin gene (Fig. 2). Since the preproregions of the precursors of all antimicrobial peptides present in the skin secretion of *R. esculenta* are very similar [9], and since our hybridization conditions were not highly stringent, we believe that no antimicrobial peptide genes are transcribed, as supported by the results shown in Fig. 1. Fig. 2 also shows total RNA from two frogs injected with a soluble glucocorticoid, one in the dorsal sac under the skin, the other intraperitoneally. In both cases, the esculentin probe gave weak signals. The fact that the signals did not completely disappear may be due either to the lack of systemic action or to a too low dose of the glucocorticoid.

In order to establish whether in *R. esculenta* the glucocorticoid treatment was associated with an increase in IκBα levels, a cytoplasmic extract of proteins from *R. esculenta* skin was analyzed by immunoblotting with IκBα antibodies. Fig. 3

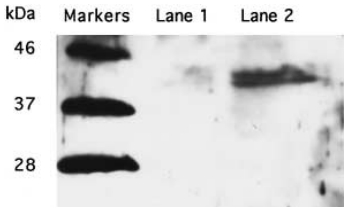

Fig. 3. Immunoblot analysis of total cytoplasmic proteins from *R. esculenta* skin with polyclonal rabbit antibodies against a synthetic peptide corresponding to residues 26–39 from the N-terminus of human IκBα (New England Biolabs Inc.). Proteins were separated by 12% SDS polyacrylamide gels and then electroblotted on nitrocellulose. Immunostaining of the peroxidase-linked antibodies was according to the manufacturer's instructions with minor modifications. Markers, biotinylated proteins with the M_r given as kDa; lane 1, control frog; lane 2, frog treated with glucocorticoid cream for a week. This experiment was repeated four times (attempting to minimize the background).

shows significant levels of IκBα in the skin of GC-treated frogs, while the amount in the control is below the level of detection. Electrophoretic mobility of IκBα derivatives can vary a great deal depending on the number of ankyrin repeats, the extension of the N-terminal portion and the degree of phosphorylation (factors not yet known). However, the size of around 40 kDa (estimated from the markers in Fig. 3) is in good agreement with the size of human IκBα. Migration as a doublet was observed also for human IκBα [18]. Moreover, microscopic analysis of skin glands of GC-treated *R. esculenta* showed that the secretory granules, known to contain the stored peptides in normal glands, were lacking (M. Simmaco and T. Renda, unpublished results).

4. Discussion

There is a vast and recent literature on the transcriptional control of NF-κB in both mammals [19] and insects [20], including the induction of IκBα in cell cultures incubated with GC [4,5]. In insects, NF-κB is also involved in the control of both immunity and body development [21]. It was first shown that the expression of immune genes in *Cecropia* was controlled by Cif, a transcription factor immunologically cross-reacting with mammalian NF-κB [22]. Then, different genes for peptide antibiotics in *Drosophila*, *Sarcophaga* and *Anopheles* were found to be regulated by NF-κB/IκB-like interactions [23–25]. However, so far no immunological effector molecules in live vertebrates have been shown to be under NF-κB/IκB control and affected by GC treatment. Here, we demonstrate by HPLC analysis (Fig. 1) that GC treatment of *R. esculenta* causes a complete loss of all antimicrobial peptides in the skin secretion, presumably by blocking the transcription of the genes encoding these peptides (Fig. 2). This explains the increase of viable mouth bacteria initially observed in GC-treated frogs. Moreover, immunoblotting of skin proteins shows that GC treatment induces significant levels of IκBα, while this factor can hardly be detected in untreated skin (Fig. 3). The genes for peptide antibiotics in both human and insects [6–8,20,21] have binding sites for NF-κB, and the results described can be expected to apply to many different species. Since human skin was recently found to produce two different antibacterial peptides, LL-37 [26] and a β-defensin [27], it seems possible that GC treatment of human skin (or other epithelia) could also cause some undesirable infection. Thus, the results here presented may have clinical relevance in several situations.

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