Adrenomedullin signals through NF-κB in epithelial cells

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Abstract Adrenomedullin is a peptide found in a variety of cells and tissues and involved in a multitude of biological processes. Recently, adrenomedullin has been identified as a host defense peptide and as such it plays a role in the inflammatory response. The transcription factor NF-κB is a major regulator of genes involved in the inflammatory response and the aim of this study was to determine whether NF-κB played a role in the inflammatory process triggered by adrenomedullin. Skin epithelial cells (HaCaTs) were used as our model in vitro. Western blot analysis from adrenomedullin-stimulated HaCaT cells revealed a rapid degradation of NF-κB inhibitor α and β followed by the translocation of free NF-κB to the nucleus, where it was detected by Texas Red immunostaining after incubation with adrenomedullin for 15 min. Electromobility shift assay showed that NF-κB present in the nucleus was active, since it bound to a probe containing an NF-κB binding site. Supershift assays indicated that p50 and p65, members of the NF-κB family, were both part of the NF-κB dimmers involved in adrenomedullin cell signaling. HaCaTs secreted interleukin-6 in response to AM, which was significantly attenuated by the NF-κB inhibitor SN-50. Taken together, the data lend support for an immunoregulatory role for AM.

Keywords: Adrenomedullin; Skin epithelial cell; NF-κB; Interleukin-6; HaCaT cell; Inflammation

1. Introduction

Adrenomedullin (AM) is a multifunctional peptide first isolated from extracts of human phaeochromocytoma and noted, primarily, for its potent vasorelaxant effects [1]. The 52 amino acid peptide is produced by a wide variety of cells and tissues, amongst which the endothelium is prominent [2]. Recently, studies from our laboratory, and others, have noted that AM is also expressed in key mucosal surfaces and is emerging as an important effector molecule in mucosal defense [3–7].

AM is found in low picomolar concentrations in the circulation and is reported to increase in a variety of disorders including sepsis, congestive failure, renal failure and hypertension for review see [2] and references within. AM has been demonstrated to accumulate in the apical regions of normal bronchial epithelium, and in the gut, skin and oral mucosa [6–10]. Cameron and Fleming [11] demonstrated the localization of AM mRNA in epithelial cells lining the uterus, bronchioles and gastrointestinal tract in rodents. It has also been demonstrated that AM is expressed throughout all epithelial components of normal and malignant human skin [6,12]. Data from our laboratory, and others, implicate AM as a possible growth regulatory factor of the skin and contributes as an antimicrobial agent in the integument's protective barrier [3,5,6,10,12,13].

The recorded activities of some antimicrobial peptides extend beyond antimicrobial action to functions indicating roles in regulating host defense mechanisms. Most activities are involved in modulating/enhancing the inflammatory response. A key example of this is the observation that AM can stimulate the release of proinflammatory cytokines such as interleukin (IL)-6 from fibroblasts [14]. Studies from our own laboratory have shown that AM can increase expression of cell adhesion molecules such as ICAM-1 and E-selectin and this may be mediated via an NF-κB-dependent mechanism [15–17].

The ubiquitously expressed transcription factor nuclear factor κB (NF-κB) is a key player in the regulation of immune and inflammatory responses, including those of proinflammatory cytokines and AM [18,19], and numerous findings have indicated that NF-κB also plays a pivotal role in epidermal biology. The translocation and activation of NF-κB in response to various stimuli, such as LPS and cytokine, occur at the molecular level. In its inactive state, the heterodimeric NF-κB, which is mainly composed of 2 subunits p50 (NF-κB$_1$) and p65 (Rel A), is present in the cytosol associated with its inhibitor protein Iκ-B [20]. Upon stimulation, Iκ-B undergoes phosphorylation, thereby unmasking the nuclear localization signal on the p65 subunit and allowing the nuclear translocation of the complex [21].

We, and other workers, have reported the effects of AM on epidermal cells in relation to its effect on cell proliferation and its secretion in response to a range of agents, such as cytokines and steroids [6,10]. We also know that AM has immunomodulatory effects in keratinocytes [17] and taken together it is tempting to suggest that under some conditions AM can directly couple to a signaling pathway relevant to keratinocyte function, in particular hyperproliferation and inflammatory mediator production. The aim of this study was to determine whether AM exerts its inflammatory role in keratinocytes through the activation of NF-κB and IL-6 secretion.

2. Materials and methods

2.1. Materials

Human adrenomedullin peptide was purchased from Phoenix Europe GmbH (Germany). HaCaT cells were used throughout and

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originated from normal adult skin keratinocytes by spontaneous transformation [22]. These cells were a generous gift from Professor N. Fusenig (Heidelberg, Germany). HeLa cell nuclear extracts were used as a positive control for the supershift assays and were obtained from Promega (UK). DMEM and all supplements were obtained from Invitrogen (Paisley, UK). Lipopolysaccharide (LPS; *E. coli* serotype O26:B6) was from Sigma–Aldrich (Dorset, UK). Interleukin-6 ELISA kit was purchased from R&D Europe Systems (Oxon, UK). IκBα, IκBβ, NF-κB-p50 and -p65 antibodies, Texas Red and secondary antibody goat anti-rabbit were purchased from Santa Cruz Biotechnologies (Autogen Bioclear, Calne, UK). The ECL kit and Hyperfilm for Western blotting was obtained from Amersham Biosciences (Amersham, UK). The gel shift assay core system and oligonucleotides for NF-κB and SP1 were from Promega. All other chemicals were of the best grade available and obtained from Sigma–Aldrich.

2.2. Cell culture and treatments

HaCaT cells were in DMEM supplemented with 10% fetal bovine serum (FBS) and routine antibiotics in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Twenty-four hours before experiments were carried out, 80% confluent 60 × 15 mm tissue culture dishes were rendered quiescent by placing them in serum-free medium. On the day of experiments, cells were washed in sterile PBS and incubated with adrenomedullin at a final concentration of 10^{-8}–10^{-7} M or LPS at 10 ng/ml to 1 μg/ml.

In other experiments, cells were treated with 10^{-8} M AM for 4 h in the absence or presence of 10^{-5} M SN-50 a specific NF-κB Inhibitor [52]. After this time, culture supernatants were harvested and stored at −20 °C until ready for assay. IL-6 was measured by ELISA.

2.3. Western blotting

After treatment cells were lysed by adding 0.5 ml of 1× SDS–PAGE sample buffer (2% SDS, 10% glycerol, and 5% 2-mercaptoethanol, 0.002% bromophenol blue, and 62.5 mM Tris–HCl, pH 6.8). Twenty μl of cell lysate was separated on a 12% SDS–PAGE gel. The proteins were then transferred to a PVDF membrane that was blocked overnight with PBS-Tween (PBS-T) containing 5% dried skimmed milk (PBS-TM). The membrane was then incubated with the primary antibodies against IκBα and β for 2 h at 1:1000 dilution in PBS-TM at room temperature and after 3 × 5 min washes in PBS-T, the membrane was incubated with the secondary antibody (goat anti-rabbit) for 1h at 1:2000 dilution in PBS-TM at room temperature. After 3 × 5 min washes in PBS-T, ECL reaction was performed following the manufacturer's instructions. Membranes were exposed to X-ray Hyperfilm ECL.

2.4. Electrophoretic mobility shift and supershift assays

Nuclear extracts were prepared as previously described [23]. Five μg of nuclear extracts was used in the binding reaction with 35pmol of labeled NF-κB oligonucleotide and 2 μl of 5× binding buffer (Promega). The oligonucleotide was labeled with γ^{32}-P in a reaction catalyzed by the T4 polynucleotide kinase following the manufacturer's instructions. Positive controls were performed by using HeLa nuclear extracts and competition assays were carried out to verify the specificity of the probe by adding cold NF-κB probe (specific competitor) or AP2 probe (non-specific competitor). The resulting protein–DNA complexes were separated on a 5% polyacrylamide gel. The gel was dried and exposed to an X-ray Hyperfilm MP at −70 °C.

For supershift assays, 1 μl of antibody against p50 or p65 was incubated with the nuclear extracts at room temperature for 30 min prior addition of the labeled NF-κB probe.

2.5. Immunocytochemistry

Ten thousand cells were grown on sterile glass coverslips placed in a 24-well plate. After 48 h, cells were serum starved for 24 h and then treated with 10^{-7} M of AM for 15 or 30 min in triplicate. Cells were washed with PBS and fixed with 3.5% paraformaldehyde for 15 min at 4 °C. After washes with PBS, 1% Triton (v/v in PBS) was added for 5 min and then washed with PBS. Cells were incubated with blocking buffer (10% FBS in PBS) for 30 min prior to overnight incubation with 1 μg/ml of primary antibody in either p50 or p65 made up in PBS with 1.5% FBS. Cells were washed and incubated with 1 μg/ml of secondary antibody conjugated with Texas Red in PBS containing 3% FBS. After several washes, nuclear staining was carried out by incubating cells with 1:1000 DAPI in PBS for 5 min. Cells were washed again and the coverslips were mounted cell side down onto Immu-Mount on fresh slides. The coverslips were sealed and examined using a fluorescence microscope.

3. Results

3.1. AM stimulates IκB degradation

In normal conditions, NF-κB is found in the cytoplasm of cells as inactive form bound to its inhibitor IκB. After an inflammatory stimulus IκB kinases (IKK) phosphorylates IκB, which will be degraded releasing NF-κB that will be translocated to the nucleus to induce transcription of genes involved in the early onset of the inflammatory response. Western blot analysis detected rapid degradation of IκBα only after 5 min of treating the cells with 10^{-7} M AM (Fig. 1A). Shortly after, the levels started to rise back reaching normal levels 2 h post-stimulation. IκBβ experienced a more discrete decrease after 5 min of treatment with AM (Fig. 1A), however, no statistical difference was observed (Fig. 1B). Fig. 1B in the lower panel quantifies data pooled from 3 Western blot experiments.

3.2. AM stimulates the nuclear translocation of NF-κB subunits p50 and p65

HaCaT cells were treated with 10^{-7} M AM for 15 or 30 min and immunocytochemistry was performed to detect p50 and p65, predominant members of the NF-κB family of transcription factors. Fig. 2 illustrates that in non-stimulated cells staining for p50 or p65 was located in the cytoplasm, whereas after 15 min of treatment with AM the staining concentrated in the nucleus of cells, indicating translocation of p50 and p65 (Fig. 2). Cells treated for 30 min did not show nuclear staining in concordance with the fact that once the initial stimulus

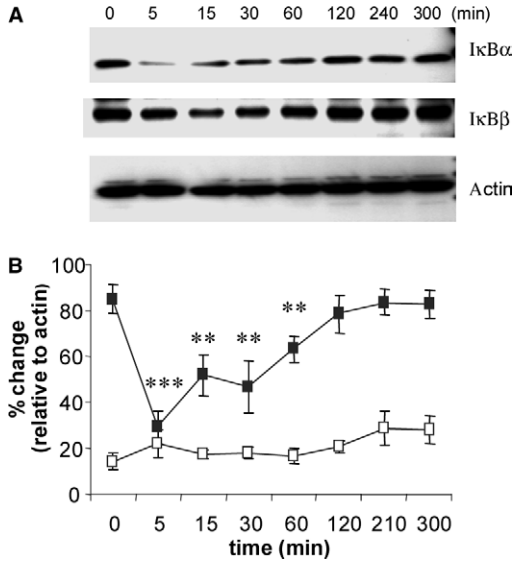

Fig. 1. Effect of AM on IκBα/β degradation. Protein was extracted from HaCaT cells treated with 10^{-7} M of AM for various lengths of time and immunoblotted for IκBα, IκBβ or actin. (A) Upper panel is a representative Western blot image of IκBα levels; middle panel is an image of IκBβ levels; lower panel is a representative Western blot image of actin levels. (B) Quantification of pooled data from three experiments; open squares represent IBα levels, filled squares IκBβ levels. Values are means ± S.E.M., $n = 3$. $^{**}P < 0.05$, $^{***}P < 0.01$ compared to control (Tukey's multiple comparison test).

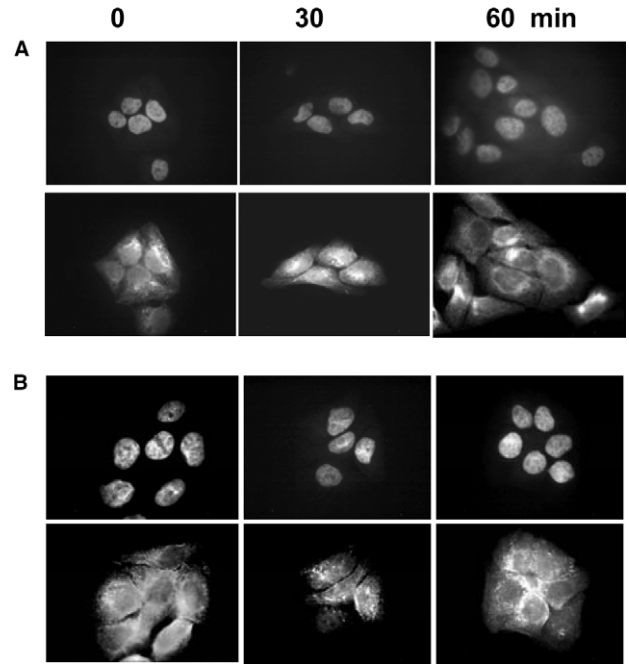

Fig. 2. Immunostaining of p50 in HaCaT cells. (A) HaCaT cells were stimulated with 10^{-7} M AM for 15 or 30 min and immunostained for DAPI nuclear staining (upper panel) or p50 (lower panel). (B) Immunostaining for DAPI nuclear staining (upper panel) or p65 (lower panel).

disappears the nuclear NF-κB is sequestered by newly synthesized IκBs (Figs. 2 and 3).

Electrophoretic mobility shift assay using nuclear extracts from HaCaT cells incubated with 10^{-7} M AM demonstrated that the NF-κB detected in the nucleus by immunostaining was indeed active, since it was capable of binding to a DNA oligoprobe containing an NF-κB binding site (Fig. 3A, upper panel). The time course showed that the nucleus of non-treated cells contained some basal levels of active NF-κB, but the levels peaked between 10 and 15 min of AM treatment followed by a steady decrease in activity (Fig. 3A upper panel). The lower panel quantifies data pooled from 3 such experiments.

Supershift assays using antibodies against two of the most common NF-κB family members, p50 and p65, were performed to corroborate that both members were involved in AM cell signaling. The addition of antibodies against p50 and p65 created supershifts formed by antibody/NF-κB/probe, indicating that p50 and p65 are part of the NF-κB dimmers activated by AM (Fig. 3B upper panel).

3.3. AM stimulates IL-6 secretion from HaCaTs

HaCaT cells were treated with a range of AM concentrations. Fig. 4 upper panel illustrates that AM caused a dose-dependent increase in IL-6 secretion from these cells when incubated in the presence of AM for 4 h. Significant increases in IL-5 production occurred at 10^{-10} M AM and the effect was maximal at 10^{-7} M AM. Taking 10^{-8} M as the dose of AM to give a maximal IL-6 response, the lower panel figure illustrates the effect of incubating the cells with this concentration for increasing lengths of time. The figure shows that there was significant IL-6 secretion after a 30-min exposure to AM with maximal production within 4 h.

Fig. 5 illustrates the effect of 10^{-8} M AM for 4 h in the absence and presence of SN-50, an inhibitor of NF-κB. IL-6 levels were significantly attenuated when AM-treated cells were incubated in the presence of SN-50, a specific NF-κB inhibitor.

4. Discussion

The skin is the largest organ of the body and is continuous with the mucous membranes lining the body's orifices. It exerts a multitude of important functions mainly linked to dealing with external physical, chemical and biological traumas. The epidermis is a stratified epithelium and keratinocytes are the principal cells providing both structural and functional roles to skin. The skin was, until recently, regarded as a simple physical barrier preventing bacterial invasion and the escape of body fluids. However, it is clear that the various cells found within the epidermis, particularly keratinocytes, are highly dynamic cells with the ability to react to changes within their environment. Keratinocytes can interact with other cells and are important during processes such as inflammation and wound healing [24,25] and respond by secreting a range of growth factors and cytokines [25,26]. However, keratinocytes have received little attention in the world of immunology even though the wide range of factors secreted by them have a major role in processes such as proliferation, differentiation and migration [27]. Studies from our laboratory, and others, have demonstrated that AM stimulates proliferation of cultured skin keratinocytes and that these cells express a functional receptor for AM [6,10]. Recent studies by our group and others have suggested that AM plays an important role as a host defense peptide and inflammation [3–5,28].

AM is a 52 amino acid peptide with a disulfide bridge originally extracted from a phaeochromocytoma and is part of the calcitonin family of peptides [1]. AM has a wide tissue and cell distribution and though it was initially described as a vasodilatory peptide, the current literature confirms that AM has many functions beyond vasoactivity. AM is also found in all body fluids studied so far. Studies by Caron's group highlighted the importance of AM when it was reported that AM knockout mice were embryonically lethal [29]. AM has now been shown to have a role in proliferation, apoptosis, inhibition of salt appetite and water intake, host defense mechanisms, increases in steroid and cytokine secretion [2–5,10].

We, and others, have reported that AM activates the cAMP signaling pathway [14,30–34] and there are reports from others demonstrating that AM can signal through other mechanisms such as elevation of intracellular free Ca^{2+} [35–37]. However, AM has been shown to have a regulatory effect on growth and mitogenesis by inhibiting mitogen-activated protein kinase (MAPK) [33] and to be linked with K(ATP) channels through CGRP1 receptor activation [38]. The major aim of this study was to determine the role of AM and the NF-κB signaling pathway. Gathered evidence indicates that AM is involved in the regulation of inflammation and modifies secretion of cytokines such as IL-6, TNF-α and cytokine-induced neutrophil chemoattractant [39–42]. NF-κB is a family of transcription factors that play a crucial role in the activation of pro-inflammatory genes and data from our laboratory and other have provided evidence for an immunoregulatory role for AM through the activation of NF-κB.

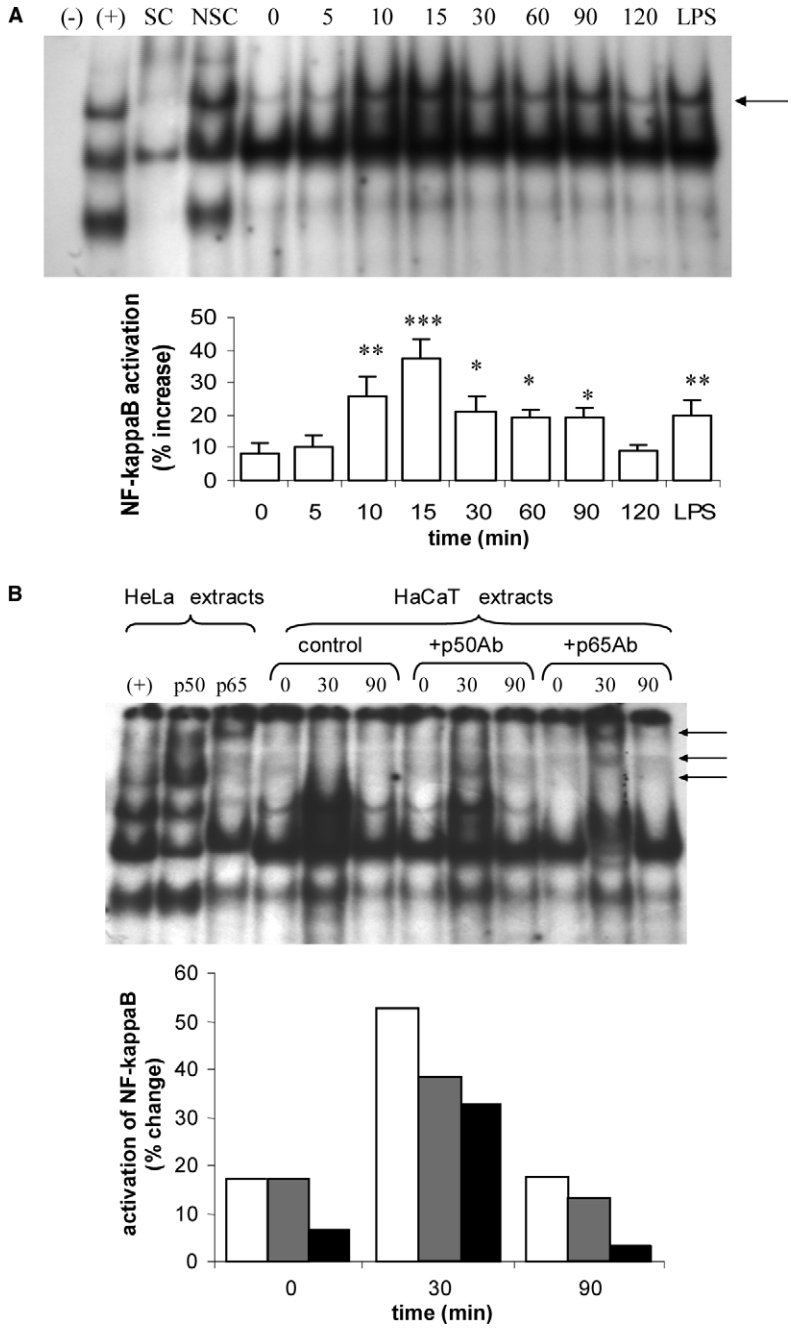

Fig. 3. Activation of NF-κB by AM. (A) Upper panel, the NF-κB probe was tested for its specificity by using no nuclear extract (−), HeLa nuclear extracts (+), HeLa extracts and cold NF-κB probe (SC-specific competitor), or cold AP2 probe (NSC-non-specific competitor). The lanes represent the shifts of HaCaT nuclear extracts obtained from a time course of treatment with 10^{-7} M AM for various lengths of time (in mins) or 10 ng/ml LPS for 60 min. The arrow marks the shift generated by NF-κB bound to probe. Lower panel, quantification of pooled data from three experiments. (B) Upper panel, HaCaT cells were incubated with 10^{-7} M AM for 0, 30 or 90 min and nuclear protein extracted for testing the presence of p50 and p65 in the NF-κB–DNA complex (supershift assay). HeLa nuclear extracts were used as a positive control. The arrows point at the immunocomplexes generated by the addition of antibodies against p50 and p65. Lower panel, quantification of pooled data from three experiments; open bars, NF-κB; gray bars, p65; filled bars, p50 levels. Values are means ± S.E.M., $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to 0 (no treatment; analysis of variance and Tukey's multiple comparison test).

Members of the NF-κB/Rel family of transcription factors are also pleiotropic regulators of cellular growth and homeostasis [43]. Recently, NF-κB was also implicated as a pivotal regulator of epidermal proliferation and differentiation for reviews see [44,45], even though the precise function of NF-κB in epidermal homeostasis remains enigmatic. Some emerging studies have suggested that NF-κB has a protective role in premature apoptosis [46,47].

In normal conditions, NF-κB is found in the cytoplasm of cells bound to its inhibitor (IκB). Several stimuli, such as pro-inflammatory cytokines and bacterial infection trigger activation of IKK that will phosphorylate NF-κB inhibitors. This

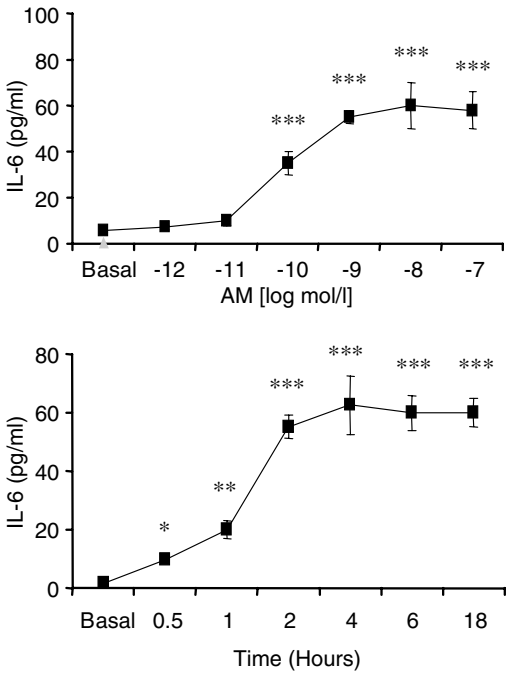

Fig. 4. Effect of AM and time on IL-6 secretion. Upper panel, cells were incubated with increasing concentrations of AM. Lower panel, cells were incubated with 10^{-8} M AM for increasing lengths of time. Values are means ± S.E.M., $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to basal (ANOVA and Tukey's multiple comparison test).

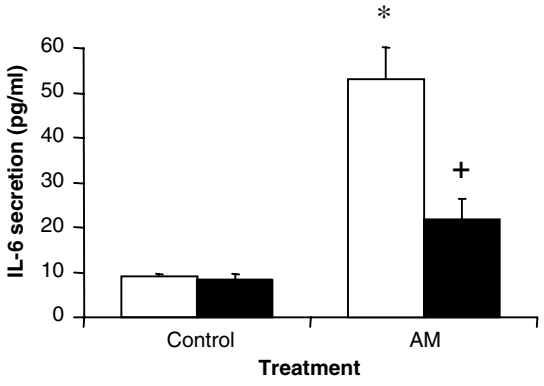

Fig. 5. Measurement of IL-6 secretion from HaCaT cells. Cells were incubated for 4 h with control or 10^{-8} M AM in the absence (empty bars) and presence of 10^{-5} M SN-50 (filled bars), an NF-κB inhibitor. Values are means ± S.E.M., $n = 3$. $^{*}P < 0.05$ compared to control (ANOVA and Tukey's multiple comparison test), $^{+}P < 0.05$ compared to AM-stimulated IL-6 levels (Student's t test).

step will release NF-κB, which will be translocated into the nucleus where it will bind to NF-κB binding sites in the promoter of certain genes inducing their transcription. Once IκB is phosphorylated, it will be ubiquitinized and degraded (hence, measurement of IκB degradation is a good indication of NF-κB activation). Several groups questioned the need for two NF-κB inhibitors, IκBα and IκBβ, and that led to a search for any differences in their degradation pattern. IκBα is degraded by all known inducers of NF-κB as opposed to IκBβ being degraded by stimuli that cause persistent activation of NF-κB, such as IL-1 and LPS. However in cases of persistent activation, once the original pool of IκBβ is degraded, new IκBβ is synthesized with an altered conformation and binds to NF-κB not to inhibit it but to protect it from IκBα inhibition. NF-κB bound to IκBβ is still active and capable of inducing gene transcription [48]. IκBα and IκBβ also play different roles in apoptosis in mice pre-B-cells. Research showed that the apoptotic drug 3-CPA inhibited the degradation of IκBα, whereas the anti-apoptotic drug Na-3-CPA inhibited degradation of IκBβ [49]. These indicate that both inhibitors have distinct roles in the regulation of various NF-κB activities.

Combining the results obtained, it can be concluded that NF-κB is involved in the AM cell signaling. Degradation of IBs was observed after 5 min of treatment with AM. IκBα degradation was noticeably higher than IκBβ degradation, indicating that AM does not induce a persistent activation of NF-κB as required by either IL-1 or LPS. The steady decrease in IκBs corresponds in time with the detection by immunostaining of free translocated NF-κB in the nuclei of cells. The nuclear staining decreases after 30 min of treatment with AM and the staining returns to the cytoplasm, indicating that nuclear NF-κB has been sequestered by the newly synthesized IκBs and transported back to the cytoplasm.

Electromobility shift assays showed that the nuclear NF-κB detected in the cells by immunostaining was active and capable of binding to NF-κB binding sites in the DNA within 30 min. NF-κB consists of homo- or heterodimers of the different members of the family (p65, c-Rel, RelB, p50/p105, and p52/p100). P50 and p65 are the predominant proteins in the NF-κB complex, so to assess whether their activation was induced by AM, antibodies against them were incubated with nuclear protein extracts and NF-κB probe. Supershifts were observed when antibodies against p50 and p65 were used, indicating the presence of both members in the NF-κB complex activated by AM treatment in HaCaT cells. These supershift assays were not carried out at 10 min, however, it should be noted that degradation of IκBα and IκBβ occurring, as measured within 30 min, does not exclude the subsequentNF-κB subunits binding to the regulatory elements during this time.

Binding of NF-κB to promoter sequences induces transcription of genes involved in the early onset of the inflammatory response. Several pro-inflammatory cytokines (TNF-α, IL-1α, IL-6, etc.) are synthesized and secreted as a result. We measured the secretion of IL-6 after treatment with AM, as an indication of its inflammatory role, and a significant increase was observed in HaCaT cells, suggesting an immunomodulatory role for AM in these cells. Other studies in HaCaT cells challenged with bacteria or LPS showed no secretion of IL-6, IL-1 or TNF-α compared to the controls, but showed increased expression of MHC class II [50], however that may have been influenced by cell density, since cytokine synthesis and secretion by HaCaT cells increases when cells are proliferating [51].

To conclude, we have shown that AM induces degradation of IκBs and as a result, NF-κB (p50/p65) is translocated to the nucleus of HaCaT cells where it will induce expression of genes such as IL-6. There are several aspects of the AM signaling through NF-κB that are yet to be understood, such as the differential involvement of IκBα and IκBβ and their kinases IKKα and IKKβ and what genes are up or downregulated as a consequence of NF-κB activation.

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