Aims: Nitric oxide (NO) has been increasingly implicated in inflammatory skin diseases, namely in allergic contact dermatitis. In this work, we investigated the effect of dexamethasone on NO production induced by the epidermal cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF) in a mouse fetal skin dendritic cell line. Methods: NO production was assessed by the method of Griess. Expression of the inducible isoform of nitric oxide synthase (iNOS) protein was evaluated by western blot analysis and immunofluorescence microscopy. Western blot analysis was also performed to evaluate cytosolic IkappaB-alpha (IxB-a) protein levels. The electrophoretic mobility shift assay was used to evaluate the activation or inhibition of nuclear factor kappa B (NF-kB). Results: GM-CSF induced iNOS expression and NO production, and activated the transcription factor NF-kB. Dexamethasone inhibited, in a dose-dependent manner, NO production induced by GM-CSF. Addition of dexamethasone to the culture, 30 min before GM-CSF stimulation, significantly inhibited the cellular expression of iNOS. Dexamethasone also inhibited GM-CSF-induced NF-kB activation by preventing a significant decrease on the IxB-a protein levels, thus blocking NF-kB migration to the nucleus. Conclusions: The corticosteroid dexamethasone inhibits GM-CSF-induced NF-kB activation, iNOS protein expression and NO production. These results suggest that dexamethasone is a potent inhibitor of intracellular events that are involved on NO synthesis, in skin dendritic cells.

Key words: Granulocyte–macrophage colony-stimulating factor, Nitric oxide; Transcription factors, Dexamethasone; Skin dendritic cell

Dexamethasone prevents granulocyte–macrophage colony-stimulating factor-induced nuclear factor-kB activation, inducible nitric oxide synthase expression and nitric oxide production in a skin dendritic cell line

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Introduction

Nitric oxide (NO), synthesized in large quantities by the inducible isoform of NO synthase (iNOS), has a dual role in human health, behaving as a ‘double edge sword’. This free radical acts as a cytotoxic agent against exogenous microorganisms, by stimulating macrophage-induced inflammation and death of infected cells. On the other hand, it favors exaggerated tissue injury in sepsis, hypersensitivity or autoimmunity, by promoting inflammation or cytotoxicity of innocent bystander cells. In the skin, NO is produced by iNOS in several cells, namely in keratinocytes, fibroblasts, Langerhans cells and other dendritic cells. Apart from its potential beneficial activity against external aggressors, it is increasingly reported to be involved in skin inflammatory and immune responses, namely contact dermatitis, both allergic contact dermatitis and irritant contact dermatitis, atopic dermatitis, and psoriasis. Among these diseases, which show a good clinical response to topical glucocorticosteroids (GCs), a topical iNOS inhibitor has also been shown to be effective in atopic dermatitis and psoriasis.

After stimulation by microorganisms and epidermal cytokines, like interferon-γ, tumor necrosis factor-α, interleukin 1-β and granulocyte–macrophage colony-stimulating factor (GM-CSF), Langerhans and other dendritic skin antigen presenting cells produce NO, which may be important in the process of antigen presentation to T cells. We have previously shown, using a mouse fetal skin dendritic cell line (FSDC) in culture, that GM-CSF, a cytokine produced by allergen-stimulated keratinocytes that promotes Langerhans cell maturation and antigen presentation, induces NO production. This dependent on the expression of iNOS and is associated with the activation of the transcription nuclear factor kappa B (NF-kB). This transcription factor is present in a latent (inactive) state in the cytoplasm, bound to an inhibitor, IkappaB (IxB). Exposure of cells to a variety of physiological and non-physiological stimuli induces phosphorylation, and subse-
quent degradation, of IκB protein. This process releases NF-κB from this inhibitor, enabling it to translocate to the nucleus, where it increases the expression of specific genes, including those involved in iNOS expression.

Glucocorticoids, which exert a multifunctional anti-inflammatory activity in several skin diseases and affect Langerhans cells maturation, have been shown, both in vivo and in vitro, to interfere with the activity of iNOS and NF-κB in some pathological processes and in some cell lines.

In this work, we investigated the effect of dexamethasone on a FSDC cell line stimulated by GM-CSF, evaluating NO production, iNOS expression and NF-κB activation. Our results showed that dexamethasone prevents GM-CSF-induced reduction of the cytosolic levels of the NF-κB inhibitor, IκB-α, and the subsequent translocation of the NF-κB proteins into the nucleus, where it is likely to mediate the observed inhibition of iNOS expression and NO production.

**Materials and methods**

**Materials**

The rabbit anti-mouse iNOS polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY, USA) and the rabbit anti-IκB-α polyclonal antibody from New England BioLabs Inc. (Beverly, MA, USA). The NF-κB consensus oligonucleotide was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas the mouse monoclonal antibody against actin and the protease inhibitor cocktail were obtained from Roche (Carnaxide, Portugal). The fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulin was from DAKO (Copenhagen, Denmark). The mounting medium for fluorescence, Vectashield, was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). The 32P-labeled γ-ATP, T4 polynucleotide kinase, poly(dI–dC),poly(dI–dC), the ECL western blotting analysis system, the horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin and the X-ray films were from Amersham Biosciences (Carnaxide, Portugal). The horseradish peroxidase-conjugated goat anti-mouse immunoglobulin was from Pierce (Rockford, IL, USA). The mouse rGM-CSF was from R&D systems (Minneapolis, MN, USA), fetal calf serum was from Biochrom KG (Berlin, Germany) and trypsin was from Invitrogen (Paisley, UK). Dexamethasone was obtained from Sigma-Aldrich Química (Sintra, Portugal). Triton®X 100 and paraformaldehyde were from WWR International/Merck Eurolab (Lisboa, Portugal). All other reagents were from Sigma Chemical Co (St Louis, MO, USA).

**Cell culture**

The fetal mouse skin dendritic cell line FSDC, kindly supplied by Dr G. Girolomoni (Laboratory of Immunology, Instituto Dermopatico dell’Immacolata, IRCCS, Rome, Italy), is a skin dendritic cell precursor with antigen presenting capacity. The cells were cultured in endothetox free Iscove’s Modified Dulbecco’s Medium, supplemented with 10% (v/v) fetal calf serum, 1% (w/v) glutamine, 3.02 g/l of sodium bicarbonate, 100 μg/ml of streptomycin and 100 U/ml of penicillin. For western blot and electrophoretic mobility shift assay (EMSA) analysis, FSDC were plated at 2 x 10^6 cells/well, in six-well culture plates, whereas for nitrite measurements the cells were plated at 0.2 x 10^5 cells/well, in 48-well culture plates. For immunofluorescence analysis, FSDC cells were grown on Lab-Tek chamber slides with a cover (0.2 x 10^5 cells/slide).

**Nitrite measurement**

The production of NO was accessed as the accumulation of nitrite (NO_2^-) in the culture supernatants, using a colorimetric reaction with the Griess reagent, as described previously. Briefly, cells were stimulated with GM-CSF (100 ng/ml) for 48 h, alone (control) or in the presence of increasing concentrations of dexamethasone (0.0025–1 μM), added to the cell culture 30 min before GM-CSF. In another set of experiments, the cells were incubated for 48 h with the same concentrations of dexamethasone, in the absence of other stimuli.

At 48 h, culture supernatants were collected and diluted with equal volumes of the Griess reagent (0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride, 1% (w/v) sulfanilamide and 5% (w/v) H_3PO_4). After 10 min incubation, the absorbance was measured at 550 nm in an automated plate reader (SLT Spectra; Salzburg, Austria). The nitrite concentration was determined from a sodium nitrate standard curve.

**Western blot analysis**

For immunodetection of iNOS, cells were treated with culture medium alone (control) or with GM-CSF (100 ng/ml) for 24 h, in the absence or in the presence of the corticosteroid dexamethasone (0.5 μM), added to the cell culture 30 min before the GM-CSF. As a second control, cells were also treated with dexamethasone (0.5 μM) alone for the same time period. After treatment, cells were washed twice with phosphate-buffered saline (PBS) and total cell lysates were obtained after harvesting the cells in a sonication buffer containing 0.32 M sucrose, 10 mM Tris–HCl (pH 7.5), 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol, 0.1 mM phenylmethyl-
sulfonylfluoride and the protease inhibitor cocktail. Then, the lysates were incubated on ice for 30 min and sonicated to disrupt the cells. The protein concentration was determined using the bicinchoninic acid method.

For immunodetection of IκB-α, FSDC cells were maintained in the culture medium alone (control) or were treated with GM-CSF (100 ng/ml) for 15, 45 and 60 min. To evaluate the effect of dexamethasone, cells were stimulated for 45 min with GM-CSF (100 ng/ml) after pre-incubation with dexamethasone (0.5 μM), for 30 min. Proteins of the cytosolic fraction were obtained after harvesting the cells in 10 mM NaCl, 3 mM MgCl2, 0.5% (v/v) Nonidet P-40, 1 mM dithiothreitol, 10 mM Tris–HCl (pH 7.5), 0.1 mM phenylmethylsulfonylfuoride and protease inhibitor cocktail. The lysates were incubated on ice for 15 min, and the cytosolic proteins were isolated from the supernatant obtained after centrifugation at 2300 × g for 10 min. The supernatant containing the cytosolic proteins was collected, and the protein concentration was determined using the bicinchoninic acid solution.

Protein samples were separated on 10% (v/v) (for iNOS detection) or 15% (v/v) (for IκB-α detection) sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% (w/v) dry milk in Tris-buffered saline with 0.1% (v/v) Tween 20 for 1 h. The levels of iNOS and IκB-α proteins were detected using, respectively, a rabbit polyclonal anti-IκB-α antibody (1:1000) for 1 h, and a rabbit polyclonal anti-mouse iNOS antibody (1:2000) and a protease inhibitor cocktail. The lysates were incubated on ice for 15 min, and the cytosolic proteins were isolated from the supernatant obtained after centrifugation at 2300 × g for 10 min. The supernatant containing the cytosolic proteins was collected, and the protein concentration was determined using the bicinchoninic acid solution.

Electrophoretic mobility shift assay

FSDC cells were kept in the culture medium alone (control), or were kept with dexamethasone (0.5 μM) or were treated with GM-CSF (100 ng/ml) for 15, 45 and 60 min. In another set of experiments, cells stimulated for 45 min with GM-CSF (100 ng/ml) were also incubated with dexamethasone (0.5 μM), added to the culture 30 min before the GM-CSF. The cells were then washed with PBS and lysed in 10 mM NaCl, 3 mM MgCl2, 0.5% (v/v) Nonidet P-40, 1 mM dithiothreitol, 10 mM Tris–HCl (pH 7.5), 0.1 mM phenylmethylsulfonylfuoride and protease inhibitor cocktail. The lysates were incubated on ice for 15 min and centrifuged at 2300 × g for 10 min. The pellet obtained was resuspended in 300 mM NaCl, 5 mM MgCl2, 20% (v/v) glycerol, 1 mM dithiothreitol, 0.2 mM EDTA, 20 mM HEPES buffer (pH 7.5), 0.1 mM phenylmethylsulfonylfuoride and protease inhibitor cocktail, incubated on ice for 1 h and centrifuged at 12,000 × g for 20 min. The supernatant containing the nuclear proteins was collected, and the protein concentration was determined using the bicinchoninic acid method.

The EMSA method used was similar to that described previously with slight modifications. The probes consisted of a double stranded oligonucleotide containing the consensus binding sequence for NF-κB (5′-AGT TGA GGC TAC TTT GCC AGG C-3′) end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. Typical binding reactions consisted of 12 μg of nuclear extract, about 100,000 cpm of [γ-32P]-labeled oligonucleotide and 100 μg/ml of poly(dI–dC).poly(dI–dC) in a buffer containing 20 mM HEPES (pH 7.9), 1 mM MgCl2, 4% (w/v) Ficoll 400, 0.5 mM dithiothreitol, 50 mM KCl, and 1 mg/ml of BSA, and were incubated at room temperature for 45 min. Binding reactions were separated on 7% (v/v) non-denaturing polyacrylamide gels, in a buffer system containing 0.044 M Tris–Base (pH 8.0), 4.45 mM boric acid and 1 mM EDTA, at a constant voltage of 150 V, for 2h 30 min at room temperature. The gels
were transferred to Whatman paper, dried, and subjected to autoradiography. In competition experiments, an excess of unlabeled oligonucleotide (100 ×) was added to the nuclear extracts for 30 min before addition of radiolabeled probe. The blots were digitally generated using a HP ScanJet 5p and processed in the Corel Photo-Paint program.

Data analysis

Results are presented as mean ± standard error of the mean of the indicated number of experiments. Mean values were compared using one-way analysis of variance and the Bonferroni’s multiple comparison test. The significance levels were *p < 0.05, **p < 0.01 and ***p < 0.001.

Results

Dexamethasone inhibits NO production in GM-CSF-stimulated FSDC in a dose-dependent manner

We have previously reported that GM-CSF causes a dose-dependent increase in nitrite concentration in culture supernatants, reflecting an increase in NO production by the FSDC.22 In the present work, 100 ng/ml of GM-CSF was used, which was previously shown to increase significantly NO production.22 Cell stimulation with GM-CSF for 48 h significantly increased nitrite production, from 2.7 ± 1.1 μM when FSDC were incubated with culture medium alone to 4.2 ± 1.5 μM when FSDC were incubated with 100 ng/ml of GM-CSF. To investigate the effect of the dose of dexamethasone on GM-CSF-induced NO production, the corticosteroid was added in increasing concentrations (0.0025 to 1 μM) to the culture, 30 min before stimulation with GM-CSF. Dexamethasone inhibited nitrite production induced by GM-CSF in a dose-dependent manner (Fig. 1), with an IC₅₀ value of 3.2 ± 10⁻³ μM, as calculated by the Hill plot. A concentration of 0.5 μM dexamethasone, which completely inhibited NO production, was used in subsequent experiments to study its mechanism of action. Dexamethasone, in the absence of GM-CSF stimulation, had no effect on NO production (data not shown).

Cellular viability was evaluated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and by the Trypan blue assay, as described previously.21 The results indicated that there was no significant toxicity of dexamethasone in the concentrations used (data not shown).

Dexamethasone inhibits iNOS protein expression in FSDC stimulated with GM-CSF

Western blot analysis was used to characterize iNOS protein levels in non-treated cells (control), in cells treated with GM-CSF, in the presence or in the absence of dexamethasone, and in cells treated only with dexamethasone. As indicated in Fig. 2A, control cells (i.e. non-activated cells) expressed a residual level of iNOS protein (130 kDa) (lane 1). In cells stimulated with GM-CSF, the expression of iNOS protein was increased by 106% relative to control cells (lane 2). In GM-CSF-stimulated cells treated with dexamethasone, the expression of iNOS protein was significantly inhibited, decreasing to 42% of the GM-CSF-stimulated cell levels (lane 4) and to 85% of the iNOS levels expressed by control cells. When the effect of dexamethasone was tested alone, we observed a reduction of iNOS expression to 68% of the control, which did not reach statistical significance (lane 3). This decrease in iNOS protein expression correlated well with the inhibitory effect of dexamethasone on NO production, as shown in Fig. 1.

Immunofluorescence assay was performed to confirm these results, under the same conditions used for western blot. Fig. 2C shows that control cells (i.e. cells maintained in culture medium) expressed a residual level of iNOS protein. In cells treated with GM-CSF, the fluorescence was markedly increased, showing a significant increase in iNOS protein expression. GM-CSF-stimulated cells treated with dexamethasone exhibited a similar, although weaker, fluorescence than control. The fluorescence exhib-
Dexamethasone preventing NF-kB activation, iNOS and NO production

Dexamethasone blocks the activation of NF-κB in FSDC stimulated with GM-CSF

The effect of dexamethasone on NF-κB activation induced by GM-CSF in FSDC cells was evaluated in nuclear extracts by EMSA analysis. The cells were incubated with GM-CSF for 15, 45 and 60 min to determine the effect of cytokine on the activation of NF-κB, or were pre-treated with dexamethasone for 30 min and then stimulated with GM-CSF for 45 min in order to investigate the effect of the corticosteroid. As shown in Fig. 3A,B, GM-CSF induced NF-κB binding to DNA, exhibiting a maximal effect at 45 min when the NF-κB binding activity was increased by 101% relative to control cells (lanes 2 and 4). Pre-treatment with dexamethasone prevented NF-κB binding to DNA in GM-CSF-stimulated cells. Fig.
3A,B shows that pre-incubation of the cells with dexamethasone for 30 min before stimulation with GM-CSF for 45 min, in the presence of the corticosteroid, fully blocked GM-CSF-induced NF-κB binding to DNA (lane 6). Under these experimental conditions, NF-κB binding to DNA was decreased to 55% of the activity found in GM-CSF-stimulated cells and was not significantly different from the control. Dexamethasone alone had no effect on NF-κB binding to DNA (10% of difference from the control) (lane 7). As control for the gel shift assay, unlabeled oligonucleotide (i.e. non-radioactive NF-κB consensus containing oligonucleotide) was used in excess (100-fold) to demonstrate specificity of the induced bands (lane 8). (A) Nuclear extracts were subjected to EMSA analysis as described in Materials and methods. The blot shown is representative of three blots yielding similar results. (B) The bands were quantified with an image analyzer and the results are shown as means ± standard error of the mean from three independent experiments. **p < 0.01 and ***p < 0.001, as determined by one-way analysis of variance with Bonferroni’s multiple comparison test.

As shown in Fig. 4A,B, we observed that GM-CSF induced a maximal reduction in IκB-α protein levels at 45 min, when a reduction in the protein levels to 44% of the control was observed (lanes 1 and 3). This reduction was fully inhibited when FSDC were pre-incubated with dexamethasone for 30 min and then stimulated with GM-CSF for 45 min in the presence of the corticosteroid (lane 5), where an increase of 93% in IκB-α protein levels was observed comparative with GM-CSF-stimulated cells in the absence of dexamethasone. Indeed, the amount of IκB-α present in FSDC stimulated with GM-CSF in the presence of dexamethasone was not significantly different from the protein levels in control cells. These results show that there is a correlation between the inhibitory

Dexamethasone inhibition of NF-κB in GM-CSF-stimulated FSDC is mediated by modulation of cytosolic IκB-α levels

As we have previously shown, GM-CSF induces an IκB-α degradation in FSDC.22 To determine whether the suppressive effect of dexamethasone on GM-CSF-induced NF-κB activation was due to the modulation of its inhibitor, IκB-α, the levels of this protein on cytosolic cell extracts were examined by western blot analysis.
**Discussion**

The anti-inflammatory properties of GCs such as dexamethasone are due to the down-regulation of the expression of immunomodulatory genes, thereby inhibiting the expression of enzymes involved in the inflammatory process, namely iNOS, which produces NO in large amounts. GCs mediate these effects through the intracellular glucocorticoid receptor and, depending on the target gene, the complex ligand–glucocorticoid receptor may stimulate (transactivation), or alternatively inhibit (transrepression), gene transcription.

In this work, we show that, in FSDC, the glucocorticoid dexamethasone inhibits iNOS protein expression and nitrite production induced by GM-CSF. Furthermore, dexamethasone also prevents the decrease of IκB-α protein levels, and subsequent NF-κB translocation to the nucleus, induced by GM-CSF (Fig. 3 and Fig. 4).

NO has previously been involved in skin inflammatory processes, namely in psoriasis, allergic contact dermatitis, atopic dermatitis, and other inflammatory skin diseases, where topical GCs have shown to be beneficial.

GM-CSF is a multipotent cytokine that mediates the development of immunologically active DC in culture and that is involved in inflammatory processes. In the skin, the epidermal cytokine GM-CSF is produced by keratinocytes upon stimulation by allergens and it promotes Langerhans cell maturation, thus favoring antigen presentation to T cells. Allergic contact dermatitis has an inflammatory component, and therefore GM-CSF is probably involved in this pathology.

In our previous work, we observed that, in FSDC, GM-CSF induces iNOS protein expression and NO production in a process dependent on the nuclear migration of NF-κB, which occurs upon phosphorylation and degradation of their cytosolic inhibitor, IκB-α. IκB-α protein is a member of a family of inhibitory molecules that masks the nuclear localization sequence of NF-κB, inhibiting its binding to DNA. Here, we demonstrated that, in FSDC cells, dexamethasone prevents the decrease of cytosolic IκB-α protein levels induced by GM-CSF (Fig. 4), and inhibits NF-κB binding to DNA (Fig. 3). In contrast, activated NF-κB may then bind to the iNOS gene promoter region, thereby stimulating iNOS protein expression. Accordingly, the promoter region of the iNOS gene contains binding sites for the NF-κB.

The induction of allergic responses by chemical sensitizers encountered on the skin is dependent upon the action of cytokines produced locally. These skin cytokines may also serve to influence the inflammatory and immune responses provoked by chemical sensitizers and, concomitantly, the characteristics of allergic diseases. The anti-inflammatory proprieties of glucocorticoids on iNOS expression and NO production induced by the epidermal cytokine GM-CSF had not been described before. The results presented here show that the inhibitory effect of dexamethasone on GM-CSF-induced iNOS expression and NO production can account for the anti-inflammatory effect of this glucocorticoid.

A better knowledge of the signaling pathways activated in DC that induce iNOS expression and the mechanism of action of drugs, like GCs, useful in the treatment of cutaneous inflammatory processes, could help in the development of new drugs.
can open a window to our understanding of these diseases and their treatment.

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