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Glucocorticoids Mediate Differential Anti-Apoptotic Effects in Human Fibroblasts and Keratinocytes via Sphingosine-1-Phosphate Formation

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Abstract Glucocorticoids are potent anti-inflammatory and immunomodulatory drugs which also induce growth inhibition in a variety of cell types. For this reason long-term treatment of inflammatory skin diseases may result in irreversible skin atrophy. To elucidate whether the antiproliferative action of glucocorticoids in fibroblasts is accompanied by induction of apoptosis we investigated the influence of dexamethasone (DEX) on both parameters. Interestingly, we revealed that growth inhibitory concentrations of this glucocorticoid did not induce fibroblast apoptosis. Moreover, DEX protected these cells from apoptosis induced by tumor necrosis factor α (TNFα)/actinomycin, UV-irradiation, and cell permeable ceramides. These findings are in contrast to the lack of anti-apoptotic effects detected in keratinocytes. Although DEX inhibited TNFα mediated nuclear factor-kappa (NF-κB) activity in fibroblasts, this mechanism was not involved in its cytoprotection as it was verified by specific NF-κB inhibitors. Therefore, we looked for alternative intracellular mediators. Coincubation of fibroblasts with the sphingosine kinase inhibitor *N,N*-dimethylsphingosine, which blocks formation of the sphingolipid degradation product sphingosine-1-phosphate (S1P), abrogated the protective glucocorticoid effect almost completely. As preincubation with S1P reduced the number of apoptotic cells after stimulation with TNFα/actinomycin and moreover DEX increased the intracellular S1P content a role of this sphingolipid in the cytoprotection by DEX is suggested. J. Cell. Biochem. 91: 840–851, 2004.

Key words: apoptosis; proliferation; keratinocytes; fibroblasts; dexamethasone; sphingosine-1-phosphate; TNFα, NF-κB

Glucocorticoids are well characterized in their anti-inflammatory and immunomodulatory effects [De Bosscher et al., 2000]. Therefore, they are some of the most widely used drugs in the treatment of inflammatory skin diseases. The potent suppression of inflammation, however, is associated with an antiproliferative effect in fibroblasts which may result in skin atrophy following prolonged glucocorticoid treatment [Ponec et al., 1980; Schäfer-Korting et al., 1996; Ramalingam et al., 1997; Lange et al., 2000; Korting et al., 2002]. Several lines of evidence suggest that antiproliferative effects of glucocorticoids are often accompanied by an induction of apoptosis [Amsterdam and Sasson, 2002]. Indeed, methylprednisolone ameliorates mesangial hypercellularity in glomerulonephritis by decreasing proliferating cells and increasing apoptosis [Maruyama et al., 2001]. Moreover, glucocorticoids are efficacious in the treatment of leukemia as they are inductors of apoptosis in many lymphocytes [Distelhorst, 2002]. This proapoptotic effect on thymocytes and peripheral blood monocytes is in accordance with its strong anti-inflammatory behavior [Sikora et al., 1996]. But there is also incidence for a complementary role of glucocorticoids in protecting cells of tissues in which the inflammation takes place. Thus, it has been indicated that in hepatocytes as well as bovine glomerular endothelial, ovarian follicular, and mammary gland cells glucocorticoids are acting as cytoprotective substances [Messmer et al., 1999; Evans-Storms and Cidlowski, 2000; Moran et al., 2000; Webster et al., 2002]. Currently,

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the influence of glucocorticoids on apoptosis of skin cells has been studied to a minor extent only.

Most of the effects of glucocorticoids are mediated by interaction with cytosolic glucocorticoid receptors (GR) which then dimerize and translocate into the nucleus. There they bind to glucocorticoid response elements (GRE) of glucocorticoid responsive genes, resulting in increased transcription of target genes. Some of the expressed target genes are involved in the regulation of apoptosis and suppression of inflammation [Bamberger et al., 1996]. Most important, however, is the inhibited transcription of multiple inflammatory genes (cytokines, enzymes, receptors, and adhesion molecules) [Adcock and Caramori, 2001] due to a direct interaction between activated GR and transcription factors, such as activator protein-1 and nuclear factor-kappa B (NF-κB) [McKay and Cidlowski, 1999; Almawi and Melemedjian, 2002]. Depending on the cell type NF-κB itself is involved in both, pro- and anti-apoptotic processes [Van Antwerp et al., 1996; Lipton, 1997; Kasibhatla et al., 1999; Li et al., 1999].

The important proinflammatory and pro-apoptotic cytokine tumor necrosis factor α (TNFα) binds to its p55 receptor followed by a recruitment of associative proteins leading to a final activation of caspases and cell death [Wallach et al., 1999]. A novel apoptotic signal transduction pathway of TNFα referred to as the sphingomyelin pathway is initiated by activation of a neutral sphingomyelinase that hydrolyzes sphingomyelin of membranes to caspase inducing ceramide [Dbaibo et al., 2001; Luberto et al., 2002]. Interestingly, ceramides are not only inductors of apoptosis as their degradation product sphingosine is also substrate of the cytosolic sphingosine kinase [Xia et al., 1999]. This enzyme catalyzes the phosphorylation of sphingosine leading to formation of anti-apoptotic sphingosine-1-phosphate (S1P). Indeed, activation of sphingosine kinase have been reported by diverse anti-apoptotic stimuli [Kleuser et al., 1998; Kleuser et al., 2001; Manggau et al., 2001].

Here, we show glucocorticoid effects on proliferation and apoptosis of human fibroblasts and keratinocytes alone and in the presence of various apoptosis inducers. Surprisingly, we found that the glucocorticoid dexamethasone (DEX) at growth inhibitory concentrations protects human fibroblasts from apoptosis induced by TNFα, radiation, and cell-permeable ceramides. Despite the NF-κB-inhibition by DEX in these cells, this mechanism is not involved in its cytoprotective action. Moreover, data presented here suggest for the first time that the protective effect of glucocorticoids against apoptosis is likely to be mediated by formation of S1P.

MATERIALS AND METHODS

Materials

N,N-dimethylsphingosine (DMS), S1P, and the cell-permeable ceramide *N*-acetylsphingosine (C_2-Cer) were purchased from Calbiochem (Bad Soden, Germany), [methyl-^{3}H]thymidine from Amersham (Buckinghamshire, UK). Annexin V-fluoresceine isothiocyanate (Annexin V-FITC) and propidium iodide (PI) were obtained from Alexis (Grünberg, Germany). DEX, RU486, TNFα, actinomycin, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (LLnL), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), sulfasalazine, fatty acid free bovine serum albumin (BSA), reagents and media except where noted were purchased from Sigma (Deisenhofen, Germany). Keratinocyte basal medium, epidermal growth factor, insulin, hydrocortisone, bovine pituitary extract, gentamicin sulfate, and amphotericin B were purchased from Clonetics (San Diego, CA).

A stock solution of DEX in ethanol (10^{-3} M) was prepared and diluted in medium immediately before the experiments. S1P was dissolved in methanol (5×10^{-4} M). For experiments aliquots of the S1P stock solution were used. Therefore, methanol was evaporated and the dried lipid was resolved in phosphate-buffered saline (PBS) supplemented with 0.4% BSA by rigorous vortexing and sonication on ice for 5 min. Stock solutions of C_2-Cer and DMS were prepared in DMSO (both 5×10^{-3} M), from which aliquots were used for the experiments.

Cell Culture

To isolate human keratinocytes, juvenile foreskin from surgery was incubated at 37°C for 2 h in a solution of 0.25% trypsin and 0.2% ethylenediamine tetraacetic acid (EDTA). Trypsinization was terminated by the addition of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Cells were washed with PBS and centrifuged at 250*g* for 5 min. The pellet was resuspended in keratinocyte growth medium that was prepared

from keratinocyte basal medium by the addition of 0.1 ng per ml recombinant epidermal growth factor, 5.0 μg insulin per ml, 0.5 μg hydrocortisone per ml, 0.15 mM Ca^{2+}, 30 μg bovine pituitary extract per ml, 50 μg gentamicin sulfate per ml, and 50 ng amphotericin B per ml.

For isolation of human fibroblasts the remaining skin was trypsinized for another 30 min at 37°C. The enzymatic reaction was stopped as described above and after centrifugation cells were seeded in fibroblast growth medium prepared from DMEM by the addition of 10% FCS, 2 mM L-glutamine, 50 ng amphotericin B per ml, and 20 μg gentamicin sulfate per ml. Keratinocytes and fibroblasts from at least three donors were pooled and cultured at 37°C in 5% CO_2. Cells of the second to fourth passage were used for the experiments.

NF-κB Repression

Subconfluent fibroblasts were incubated with a transfection mixture of 1 μg of the NF-κB responsive luciferase reporter plasmid pNFκB-Luc (Stratagene, La Jolla, CA) and 1.5 μl Fugene™ transfection reagent (Roche Diagnostics, Mannheim, Germany) per ml of growth medium. For normalization, cells were cotransfected with 0.2 μg of the phRL-SV40 vector (Promega, Mannheim, Germany). Media were changed 24 h after transfection.

After another 24 h transfected fibroblasts were splitted into 24-well plates. Cells were stimulated with DEX (0.001–1 μM) for 2 h and grown in the presence of TNFα (10 ng/ml) for another 22 h. After harvesting the fibroblasts, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Mannheim, Germany) according to the manufacturer's specifications. The ratio of luminescence signals from the reaction mediated by firefly luciferase to those from the reaction mediated by Renilla luciferase was examined with a Luminometer (Berthold, Bad Wildbad, Germany).

Thymidine Incorporation

Fibroblasts and keratinocytes (10^4 cells per well) were grown in 24-well plates in growth medium for 24 h. Medium was replaced by fresh growth medium and cells were incubated with different concentrations of DEX for 48 h. Then cells were allowed to incorporate [methyl-^{3}H]thymidine (1 μCi/well) for another 23 h and afterwards washed twice each with PBS and ice-cold trichloroacetic acid (3%). The precipitated material was dissolved in 200 μl of 0.3 N NaOH/well and shaken for 1 h/300 rpm. An aliquot of 100 μl was mixed thoroughly with 1.5 ml of OptiPhase SuperMix scintillation fluid (Wallac, Freiburg, Germany), and incorporated [methyl-^{3}H]thymidine was measured by liquid scintillation counting (MicroBeta Plus, Wallac, Freiburg, Germany).

Annexin V Binding and PI Dye Exclusion by Flow Cytometry

Keratinocytes (1.7×10^5 cells per well) were cultured in keratinocyte basal medium and fibroblasts (8×10^4 cells per well) in fibroblast basal medium (DMEM with 2 mM L-glutamin, 50 ng/ml amphotericin B, and 20 μg/ml gentamicin sulfate). After incubation with the indicated agents cells were trypsinized and washed twice with binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Apoptosis was determined by bivariate flow cytometric detection (FACS Calibur, Becton Dickinson, Heidelberg) of phosphatidylserine translocation using Annexin V-FITC (0.5 μg/ml) and the nonvital dye PI (1 μg/ml) as described [Manggau et al., 2001].

Mass Measurement of S1P

S1P levels were determined as described recently [Ruwisch et al., 2001]. Briefly, after stimulation, 2×10^6 cells were washed with PBS and scraped into 1 ml methanol containing 2.5 μl concentrated HCl. As internal standard dihydro-S1P (50 pmol) was added and lipids were extracted by addition of 1 ml chloroform and 200 μl 4 M NaCl. For alkalisation, 100 μl 3 N NaOH were added. The alkaline aqueous phase was transferred into a siliconised glass tube, and the organic phase was reextracted with 0.5 ml methanol, 0.5 ml 1 M NaCl, and 50 μl 3 N NaOH. The aqueous phases were combined, acidified with 100 μl concentrated HCl and extracted twice with 1.5 ml chloroform. The organic phases were evaporated, and the dried lipids dissolved in 275 μl methanol/0.07 M K_2HPO_4 (9:1). A derivatisation mixture of 10 mg *o*-phthaldialdehyde, 200 μl ethanol, 10 μl 2-mercaptoethanol, and 10 ml 3% boric acid was prepared and adjusted to pH 10.5 with KOH. Twenty five microliter of the derivatisation mixture were added to the resolved lipids for 15 min at room temperature. The derivatives were analysed by a Merck Hitachi LaChrom

HPLC system (Merck Hitachi, Darmstadt, Germany) using a RP 18 Kromasil column (Chromatographie Service, Langerwehe, Germany). Separation was done with a gradient of methanol and 0.07 M K_2HPO_4. Resulting profiles were evaluated using the Merck system manager software. The recovery of S1P was calculated using dihydro-S1P as standard [Ruwisch et al., 2001].

Protein Kinase C Activity

Cells were incubated in the absence or presence of DMS (5 μM) for 3 h followed by treatment without or with TPA (200 nM) for 15 min. Cells were washed twice with PBS, scraped from dishes, and suspended in kinase buffer [0.1 M Tris-HCl (pH 7.4), 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 10 mg leupeptin per ml and aprotinin each, 1 mM phenylmethylsulfonylchloride, 0.5 mM deoxypyridoxine]. Fibroblasts were lysed by freeze-thawing and centrifuged at 140,000*g* for 30 min. The supernatant, containing the cytosolic fraction, was collected, whereas the pellet was resuspended by passing through a 27 gauge needle 10 times in kinase buffer containing 0.1% Triton X-100. After centrifugation at 140,000*g* for 30 min, the supernatant was saved and designated as the membrane fraction. Protein kinase C (PKC) activity was assayed in the cytosolic and membrane fractions using a commercial kit from Upstate Biotechnology (Lake Placid, NY).

Statistical Analysis

Data are the mean from triplicate assays and are expressed as mean ± SD. All experiments were repeated at least three times independently. Statistics were performed using Student's *t*-test with $P < 0.05$ considered significant.

RESULTS

Glucocorticoid Effects on Proliferation and Apoptosis of Human Fibroblasts

It is well known that glucocorticoids inhibit fibroblast proliferation [Ramalingam et al., 1997; Lange et al., 2000]. In a variety of cell types like thymocytes, T-lymphocytes and monocytes growth inhibition induced by glucocorticoids is connected with an increase of apoptosis [Chauhan et al., 1997; Ramdas and Harmon, 1998; Smets et al., 1999; Distelhorst, 2002]. But in contrast, in some cells of endothelial and epithelial origin there is increasing evidence for a complementary role as glucocorticoids protect these cells from apoptosis [Messmer et al., 1999; Evans-Storms and Cidlowski, 2000; Moran et al., 2000; Webster et al., 2002]. Therefore, we examined the action of glucocorticoids in primary human fibroblasts and keratinocytes. In agreement with many previous studies DEX inhibited fibroblast growth (Fig. 1A), whereas in human keratinocytes no antiproliferative action was detected (data not shown). The inhibition of fibroblast growth became visible in the concentration of 0.1 μM of DEX. To evaluate, whether growth arrest is accompanied by an increase of apoptotic cells phosphatidylserine translocation was measured. As it is presented in Figure 1B DEX up to at least 10 μM did not enhance apoptosis of fibroblasts indicating that the antiproliferative property of DEX is not a consequence of cytotoxicity.

Protective Effect of Glucocorticoids in Human Fibroblasts

To reveal potential anti-apoptotic effects of glucocorticoids, fibroblasts were treated with TNFα, from which a pro-apoptotic role is well known. Indeed, exposure of fibroblasts to TNFα in the presence of actinomycin resulted in a major increase of apoptotic cells. But when fibroblasts were preincubated with DEX, the subsequent treatment with TNFα/actinomycin failed to induce apoptosis confirming an anti-apoptotic action of the glucocorticoid (Fig. 2A). To prove whether the survival effect of DEX is specific for fibroblasts, we also measured a possible protective role on human keratinocytes. Indeed, in these cells, which were isolated from the same donors as the fibroblasts, glucocorticoids neither induced apoptosis nor possessed a protective effect as it is shown in Figure 2B.

Recently it has been suggested that the anti-apoptotic effect of glucocorticoids in osteoblasts depends on the apoptotic stimuli [Chae et al., 2000]. In these cells glucocorticoids rescued from TNFα induced apoptosis, whereas they did not affect apoptosis caused by ceramides. In contrast to these results we found, that glucocorticoids prevented apoptosis in fibroblasts independent from the apoptotic stimulus. UV-exposure as well as treatment with C_2-Cer increased the number of apoptotic cells, which was almost completely blocked by DEX (Fig. 3A).

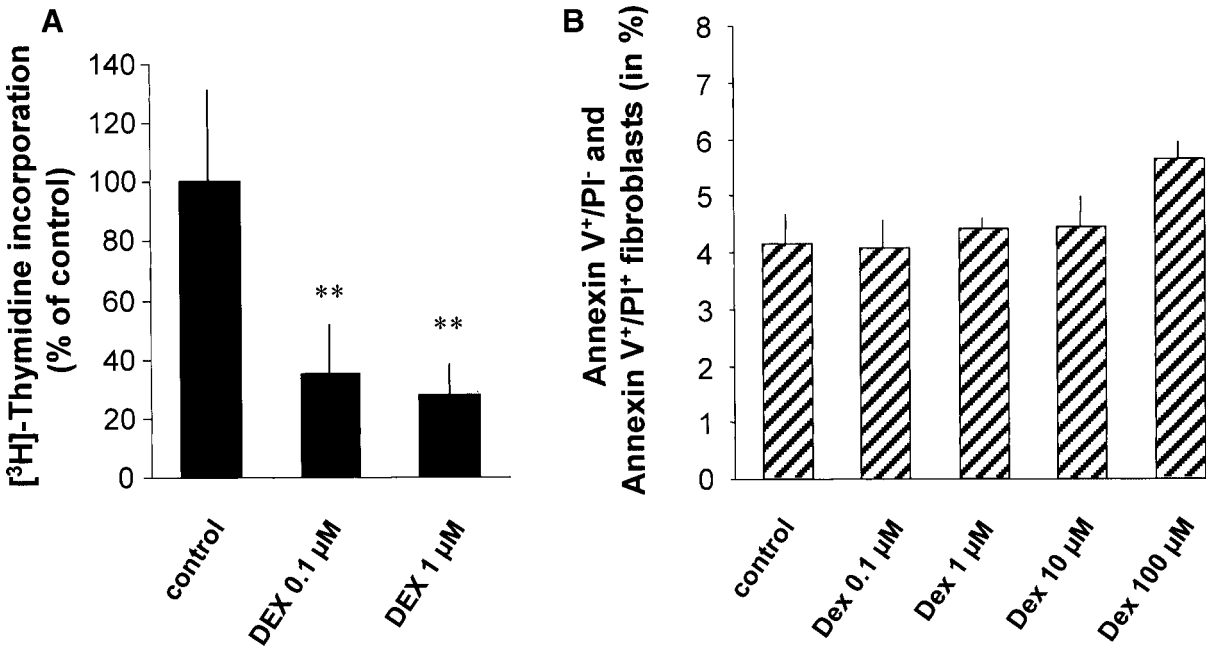

Fig. 1. Effects of dexamethasone (DEX) on proliferation and apoptosis in human fibroblasts. Fibroblasts were treated with the indicated concentrations of DEX for 72 h. **A**: Proliferation was measured by [methyl-^{3}H]thymidine incorporation as described in "Materials and Methods". **B**: Annexin V^+/PI^+ and Annexin V^+/PI^- cells were determined by flow cytometric detection of phosphatidylserine translocation and propidium iodide (PI) uptake.

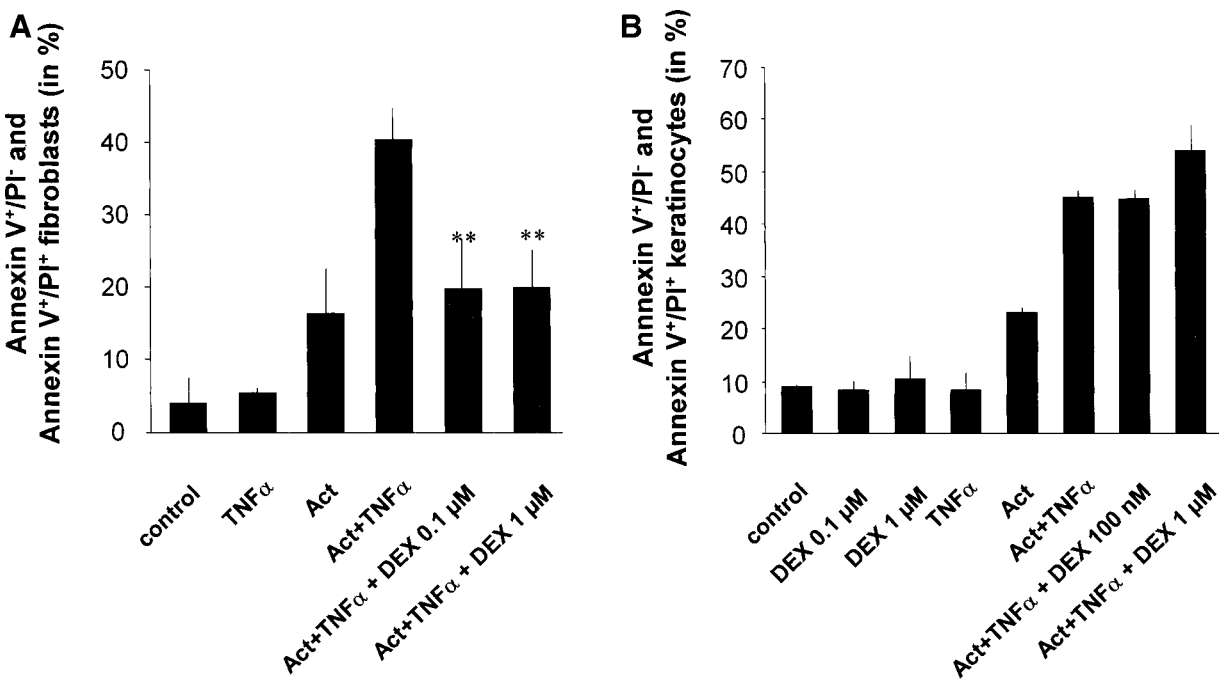

Fig. 2. Effects of DEX on TNFα/actinomycin (Act) induced apoptosis in human skin cells. Fibroblasts (**A**) and keratinocytes (**B**) were preincubated in the presence or absence of DEX for 24 h. Then 100 ng/ml Act and 20 ng/ml TNFα were added for 16 h. Apoptosis was determined by Annexin V-FITC/PI double staining as described in "Materials and Methods".

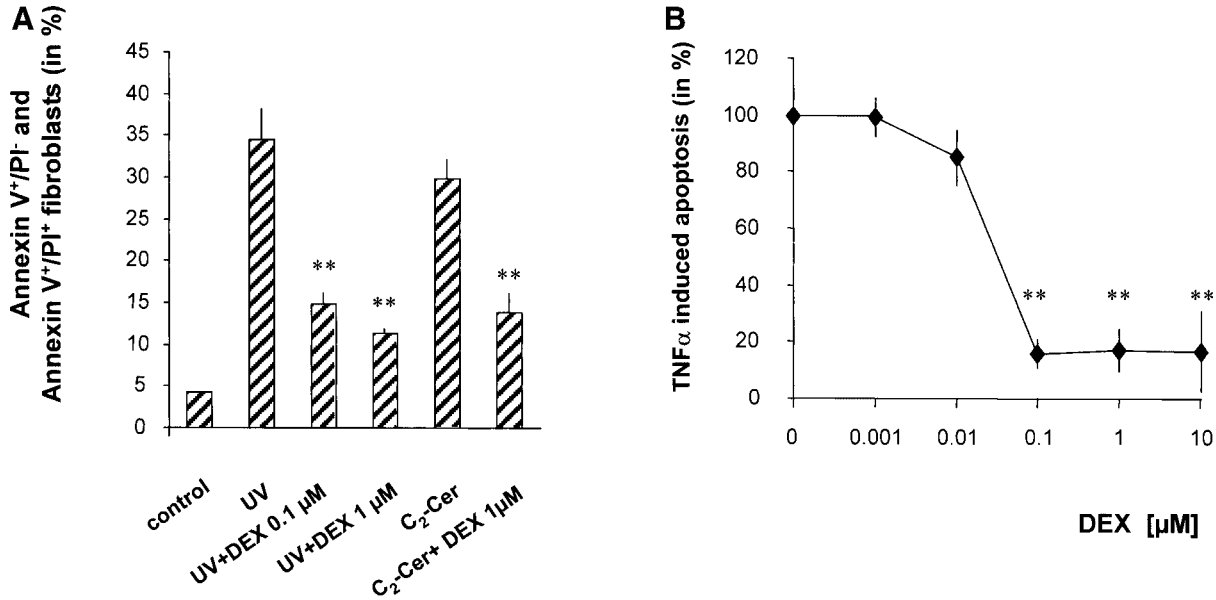

Fig. 3. Cytoprotective effects of DEX against different apoptotic stimuli. Fibroblasts were pretreated with the indicated concentrations of DEX for 24 h. Apoptosis was induced by UVB irradiation (11.76 mJ/cm^2) for 24 h, 25 µM cell-permeable ceramides (C_2-Cer) for 3 h (**A**), or TNFα/actinomycin (Act) (20 ng/ml/100 ng/ml) for 24 h (**B**). Annexin V-FITC/PI double staining was performed as described in "Materials and Methods".

But it should be mentioned that the preincubation time as well as the concentration of the glucocorticoid were crucial points concerning its preventive capacity. A relevant resistance was measured with a concentration of 0.01 µM of DEX whereas 0.1 µM almost completely prevented cells from TNFα mediated apoptosis (Fig. 3B). Regarding the preincubation period, a protection occurred only when cells were treated at least 4 h and for maximal protection at least 8 h of glucocorticoid pretreatment were needed (Fig. 4A) suggesting an action via intracellular receptors. Indeed, preincubation with the glucocorticoid receptor antagonist RU486 completely abrogated the cytoprotective effect of DEX (Fig. 4B), which confirmed an involvement of the glucocorticoid receptor.

Independence of NF-κB in Glucocorticoid Mediated Survival

It has been indicated that NF-κB is involved in both, induction as well as suppression of apoptosis [Van Antwerp et al., 1996; Lipton, 1997; Kasibhatla et al., 1999; Li et al., 1999]. Therefore, we investigated whether glucocorticoids interfere with the repression of NF-κB activation in human fibroblasts and the relevance of this effect on apoptosis. Fibroblasts were transfected with a NF-κB responsive luciferase reporter plasmid. Indeed, as it is shown in Figure 5A stimulation of transfected cells with TNFα increased luciferase activity considerably. Diminished luciferase activity following DEX treatment revealed that this glucocorticoid inhibits NF-κB activation in fibroblasts. To address an involvement of NF-κB inhibition on the cytoprotective glucocorticoid effect, sulfasalazine, a specific inhibitor of NF-κB activation [Wahl et al., 1998], as well as the proteasome inhibitor LLnL, which anticipates the degradation of IκBα and therefore NF-κB activation [Kouba et al., 2001], were used. Indeed, as expected TNFα-induced luciferase activities were significantly diminished in the presence of sulfasalazine and LLnL (data not shown). Next, the effect of these inhibitors on TNFα-induced apoptosis was examined. Most interestingly treatment of fibroblasts with sulfasalazine or LLnL augmented apoptosis, which was further increased by TNFα. Moreover, in the presence of sulfasalazine or LLnL, TNFα induced apoptosis in the absence of the protein synthesis inhibitor actinomycin (Fig. 5B). These results clearly indicate that inhibition of NF-κB activity did not protect human fibroblasts from apoptosis, in fact decreased levels of NFκB promoted programmed fibroblast cell death. However, the anti-apoptotic effect of DEX was evident in the presence of sulfasalazine and LLnL (Fig. 5B). To summarize, although NF-κB has been sug-

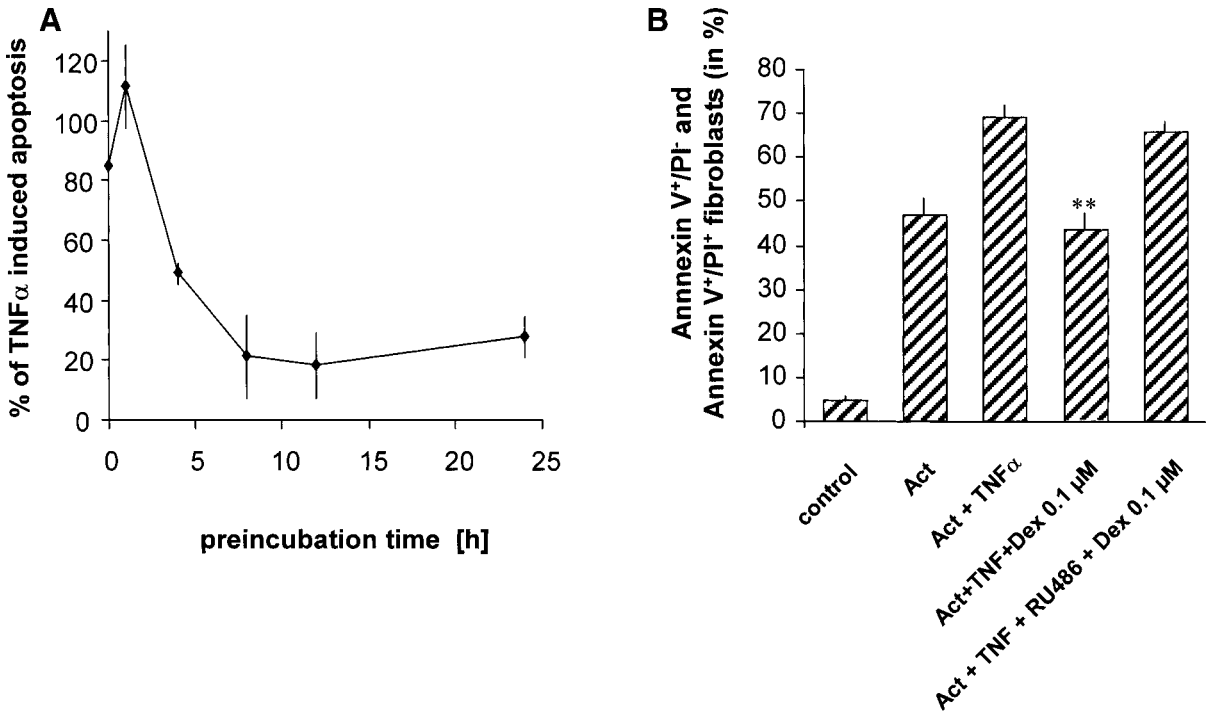

Fig. 4. Effect of the DEX preincubation time and the glucocorticoid antagonist RU486 on protection against TNFα induced apoptosis. Human fibroblasts were pretreated with 0.1 μM of DEX for the indicated time periods (**A**) and in the presence or absence of 10 nM RU486 for 24 h (**B**). Apoptosis was induced by adding 100 ng/ml actinomycin (Act) and 20 ng/ml TNFα for 24 h. Annexin V-FITC and PI double staining was performed as described in "Materials and Methods".

gested to be involved in the action of glucocorticoids present results indicate no significance of NF-κB for the cytoprotective effect of DEX.

S1P in the Anti-apoptotic Effects of Glucocorticoids

Besides increasing NF-κB, TNFα activates the release of ceramide, which in turn acts as a pro-apoptotic mediator [Dbaibo et al., 2001; Colell et al., 2002]. In osteoblasts, DEX inhibited the formation of ceramide, which may explain its anti-apoptotic effect. In these studies, the glucocorticoid prevented TNFα induced apoptosis, whereas ceramide mediated cell death appeared unaltered [Chae et al., 2000]. To investigate the function of glucocorticoids on the suppression of ceramide formation in fibroblasts, cells were stimulated with C_2-Cer. As described above, this treatment increased apoptosis and pretreatment with DEX opposed ceramide induced apoptosis significantly (Fig. 3A). In contrast to osteoblasts, this result suggests a regulatory role of glucocorticoids downstream of ceramide formation. Most recently we found in human keratinocytes that vitamin D_3 has a protective effect as it favors ceramide metabolism to S1P which opposes ceramide induced apoptosis [Manggau et al., 2001]. To study the possible involvement of S1P in the cytoprotective effect of glucocorticoids we investigated whether treatment of fibroblasts with DEX is associated with alterations of S1P mass levels. Indeed, contents of S1P raised in response to DEX. A significant increase of S1P by almost 25% was measured after treatment with DEX for 12 h (Fig. 6C). Moreover, we examined the cytoprotective effect of S1P in human fibroblasts. Actually, S1P reduced TNFα induced apoptosis (Fig. 6B), which is in accordance with a cytoprotective role of S1P in several fibroblast cell lines [Grey et al., 2002]. To further substantiate the crucial role of S1P in the cytoprotective effect of DEX in fibroblasts, we used DMS, a well known inhibitor of sphingosine kinase [Edsall et al., 1998]. As DMS has also been described to inhibit PKC activity, we measured the influence of DMS on TPA-induced membrane-associated PKC activity. The addition of 5 μM did not reduce membrane-associated PKC activity. In contrast, DEX induced S1P formation was completely inhibited indicating that DMS is a specific inhibitor of

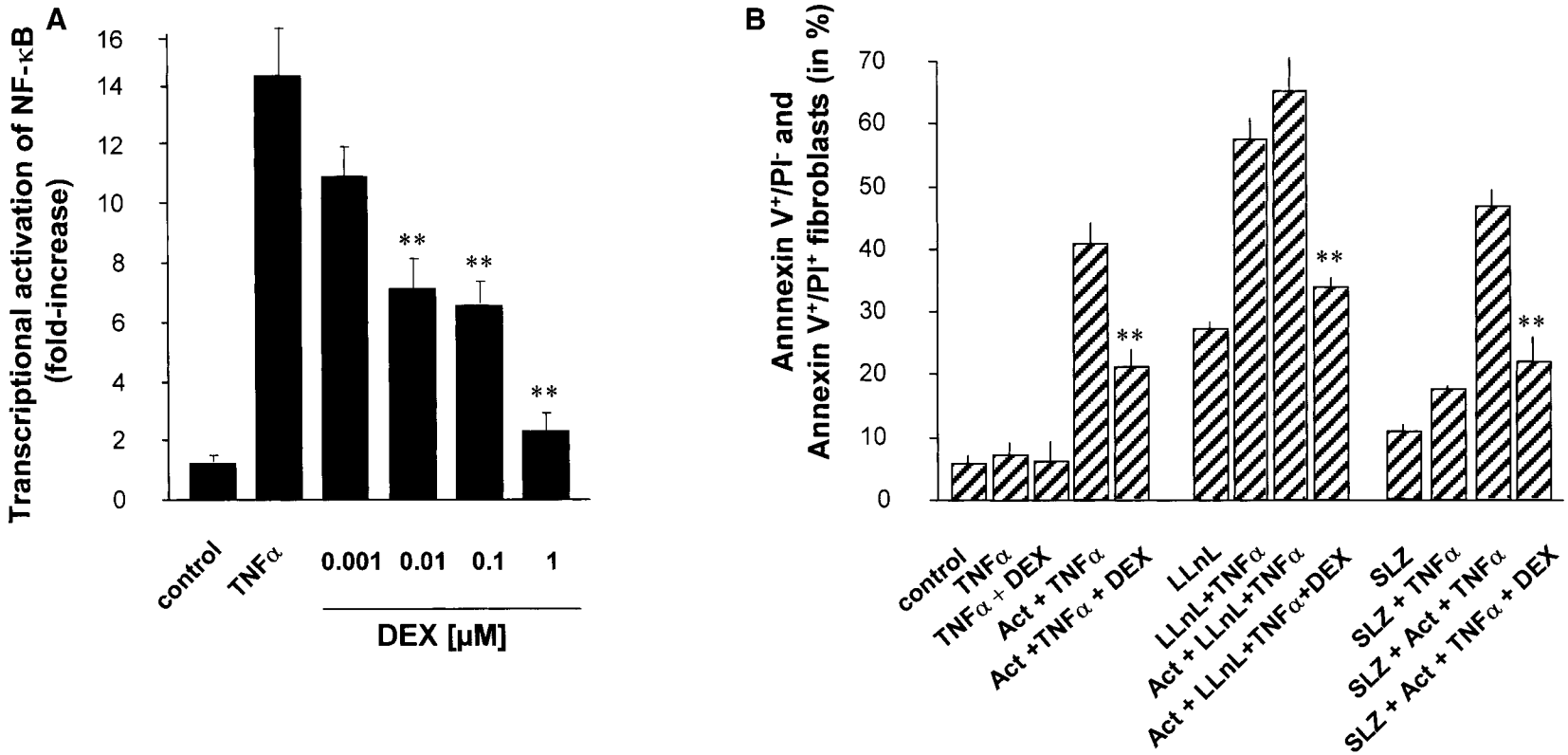

Fig. 5. Involvement of NF-κB inhibition in DEX effects on human fibroblasts. **A**: Human fibroblasts were cotransfected with the NF–κB responsive luciferase reporter plasmid pNF–κB-Luc and the phRL-SV40 vector. Then, cells were stimulated with DEX in the indicated concentrations and 10 ng/ml TNFα for 24 h. The procedures for DNA transfection and luciferase assays are described in "Materials and Methods." **B**: Cells were pretreated with 0.1 μM DEX for 24 h as indicated and NF-κB activity inhibited by the addition of 25 μg/ml *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (LLnL) (B) or 1.5 mM sulfasalazine (SLZ) (C). Then cells were treated with TNFα (20 ng/ml) alone or TNFα (20 ng/ml)/actinomycin (Act, 100 ng/ml) for 24 h. Annexin V-FITC/PI double staining was performed as described in "Materials and Methods".

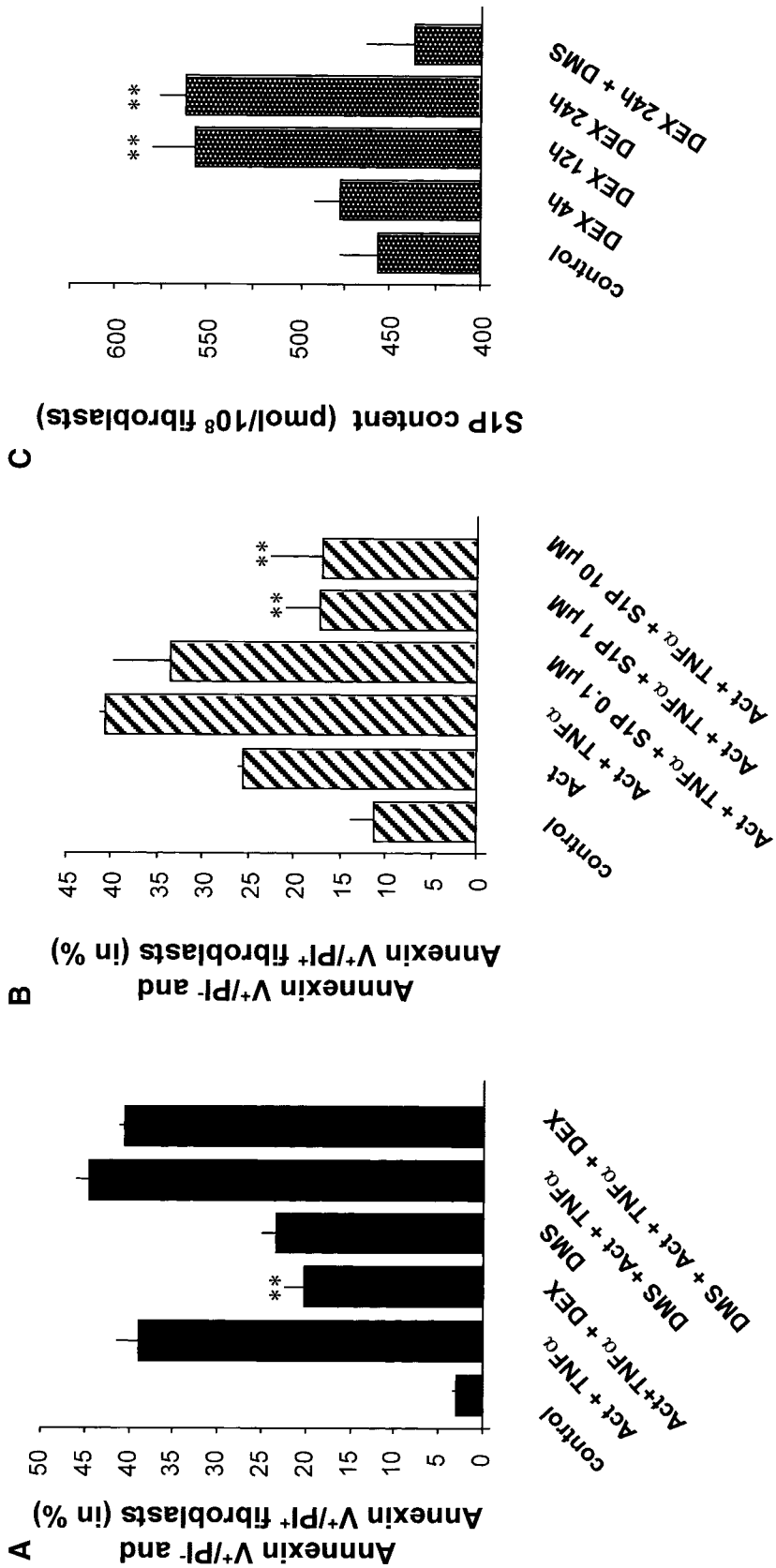

Fig. 6. Influence of sphingosine-1-phosphate (S1P) and sphingosine kinase inhibition by *N,N*-dimethyl-sphingosine (DMS) on apoptosis of human fibroblasts (**A**, **B**) and effect of DEX on S1P formation (**C**). In fibroblasts pretreated with DMS (5 μM) and DEX (1 μM) (A) or various concentrations of S1P (B) for 24 h, apoptosis was induced by TNFα/actinomycin (Act) (20 ng/ml and 100 ng/ml) for 24 h, Annexin V-FITC/PI double staining was performed. Fibroblasts were treated with DEX (1 μM) without or with DMS (5 μM) for the indicated time periods. Then S1P contents were measured as described (C).

sphingosine kinase (Fig. 6C). Treatment of fibroblasts with DMS alone resulted in enhanced apoptosis which was further increased with TNFα and actinomycin (Fig. 6A). When cells were pretreated with DEX in the presence of DMS, the anti-apoptotic action was almost completely abolished, which proved the essential role of S1P in the cytoprotective effect of DEX.

DISCUSSION

Glucocorticoids belong to the most widely used compounds for the treatment of inflammatory skin diseases. But the potent suppression of inflammation is associated with an antiproliferative effect in fibroblasts, which may result in skin atrophy following long-term treatment [Ponec et al., 1980; Schäfer-Korting et al., 1996; Lange et al., 2000; Korting et al., 2002]. Here, we show that glucocorticoids maintain viability of primary human fibroblasts by promoting anti-apoptotic processes independent of proliferation indicating that cell growth arrest and cytoprotection are regulated by different signaling pathways. Indeed, glucocorticoids have been identified to mediate strikingly different cellular responses such as cell death or survival depending on the cell type [Amsterdam and Sasson, 2002]. Hematopoietic cells such as monocytes, macrophages, T-lymphocytes, and lymphoma cells are prone to undergo apoptosis after exposure to glucocorticoids. Therefore, glucocorticoids are used as chemotherapeutics against many leukemias [Schwartzman and Cidlowski, 1994; Distelhorst, 2002]. In contrast, glucocorticoids protect some cells of endothelial and epithelial origin from apoptosis induced by agents such as TNFα, UV, or serum deprivation [Messmer et al., 1999; Evans-Storms and Cidlowski, 2000; Moran et al., 2000; Webster et al., 2002].

A crucial route related to the anti-inflammatory effect of glucocorticoids is the decrease of NF-κB activation. Indeed, NF-κB has also been identified to influence apoptosis. Enigmatically, both pro- and anti-apoptotic effects have been described, but best evidence supports an anti-apoptotic action of NF-κB on TNFα induced apoptosis [Van Antwerp et al., 1996; Lipton, 1997; Sugiyama et al., 1999]. Data presented here indicate that fibroblasts became susceptible to TNFα mediated cell death in the presence of different NF-κB inhibitors (Fig. 5B). In hepatoma cells it has been demonstrated that the anti-apoptotic action of DEX is associated with increased nuclear translocation of NF-κB [Evans-Storms and Cidlowski, 2000]. In contrast to hepatocytes, DEX suppressed NF-κB transactivation in fibroblasts (Fig. 5B). The cytoprotective effect of the glucocorticoid, however, was not affected by the presence of NF-κB inhibitors, which is well in accordance with results from mouse L929-cells [Costas et al., 2000]. Thus, both DEX and NF-κB protect human fibroblasts from apoptosis. But as DEX decreases NF-κB activation different signaling events seem to mediate these cytoprotective actions.

There is an increasing evidence that ceramides are involved in TNFα induced apoptosis [Dbaibo et al., 2001; Colell et al., 2002; Luberto et al., 2002]. By activating the neutral and acidic sphingomyelinase TNFα enhances formation of ceramide, which acts as an apoptotic stimulus as well [Kolesnick and Kronke, 1998]. Downregulation of the endogenous sphingomyelinase activity as well as incubation with inhibitors of this enzyme prevented TNFα induced apoptosis suggesting the crucial role of ceramides in the actions of TNFα [Xia et al., 1999; Luberto et al., 2002]. Indeed, it has been proposed that glucocorticoids oppose the apoptotic effect of TNFα by the inhibition of ceramide formation at least in MC3T3E1 osteoblasts, since DEX prevented TNFα, but not ceramide induced apoptosis [Chae et al., 2000]. This is controversial to our results in fibroblasts as DEX opposed both TNFα as well as ceramide induced cell death in these cells (Figs. 2A, 3A). This result suggests that key regulatory components downstream of ceramide formation are influenced by glucocorticoids.

Calcitriol (1α,25-dihydroxyvitamin D_3, 1,25-$(OH)_2D_3$) is another member of the steroid hormone superfamily with well characterized cell type specific pro- and anti-apoptotic properties. It shows a strong apoptotic effect in diverse melanoma and breast cancer cell lines as well as insulinoma cells which is partly mediated by the formation of ceramide [Okazaki et al., 1989]. In keratinocytes, however, 1,25-$(OH)_2D_3$ acts as a cytoprotective agent with a newly characterized mechanism. Although 1,25-$(OH)_2D_3$ increases ceramide levels via an autocrine mechanism by upregulation of TNFα secretion [Geilen et al., 1997], it also enhances sphingosine kinase activity [Kleuser et al., 1998; Manggau et al.,

2001]. This cytosolic enzyme is crucial for a phosphorylation of the ceramide degradation product sphingosine leading to a concomitant increase of S1P. Indeed, S1P prevents the appearance of the hallmarks of apoptosis in keratinocytes and therefore mediates the cytoprotective effects of 1,25-$(OH)_2D_3$ [Manggau et al., 2001]. Importantly, in endothelial cells, which are resistant to TNFα induced apoptosis, this cytokine not only induces sphingomyelin hydrolysis resulting in ceramide accumulation but also activates sphingosine kinase to generate S1P that protects endothelial cells from apoptosis [Xia et al., 1999].

A central finding of this report is that glucocorticoids may mediate their protective role in fibroblasts due to the formation of S1P as DEX increased S1P contents and coincubation of DEX with DMS almost completely diminished the cytoprotective effects of this glucocorticoid against TNFα induced apoptosis (Fig. 6A,C). DMS has been identified as a specific competitive inhibitor of sphingosine kinase and therefore serves to investigate the biologic role of S1P [Edsall et al., 1998]. Moreover, we revealed that S1P possesses anti-apoptotic properties also in human fibroblasts, supporting an involvement of S1P in the cytoprotective action of glucocorticoids (Fig. 6B). S1P may mediate its protective role in fibroblasts by stimulation of G-protein coupled S1P-receptors or act as an intracellular mediator. The present data indicate that glucocorticoids increase intracellular S1P mass levels (Fig. 6C). But it can not be excluded, that intracellular formed S1P is transported into the extracellular medium acting in an autocrine fashion via S1P-receptors.

In conclusion data presented here suggest that although glucocorticoids inhibit cell growth of human fibroblasts they even make these cells resistant against apoptosis. Increased knowledge of these mechanisms of glucocorticoids in controlling cell death may allow their more comprehensive use in controlling inflammation and various types of cancer and possibly preventing apoptosis in some degenerative diseases.

REFERENCES

Adcock IM, Caramori G. 2001. Cross-talk between pro-inflammatory transcription factors and glucocorticoids. Immunol Cell Biol 79:376–384.

Almawi WY, Melemedjian OK. 2002. Negative regulation of nuclear factor-kappaB activation and function by glucocorticoids. J Mol Endocrinol 28:69–78.

Amsterdam A, Sasson R. 2002. The anti-inflammatory action of glucocorticoids is mediated by cell type specific regulation of apoptosis. Mol Cell Endocrinol 189:1–9.

Bamberger CM, Schulte HM, Chrousos GP. 1996. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocr Rev 17:245–261.

Chae HJ, Chae SW, Kang JS, Bang BG, Cho SB, Park RK, So HS, Kim YK, Kim HM, Kim HR. 2000. Dexamethasone suppresses tumor necrosis factor-alpha-induced apoptosis in osteoblasts: Possible role for ceramide. Endocrinology 141:2904–2913.

Chauhan D, Pandey P, Ogata A, Teoh G, Treon S, Urashima M, Kharbanda S, Anderson KC. 1997. Dexamethasone induces apoptosis of multiple myeloma cells in a JNK/SAP kinase independent mechanism. Oncogene 15:837–843.

Colell A, Morales A, Fernandez-Checa JC, Garcia-Ruiz C. 2002. Ceramide generated by acidic sphingomyelinase contributes to tumor necrosis factor-alpha-mediated apoptosis in human colon HT-29 cells through glycosphingolipids formation. Possible role of ganglioside GD3. FEBS Lett 526:135–141.

Costas MA, Muller Igaz L, Holsboer F, Arzt E. 2000. Transrepression of NF-kappa B is not required forglucocorticoid-mediated protection of TNF-alpha-induced apoptos is on fibroblasts. Biochim Biophys Acta 1499: 122–129.

Dbaibo GS, El-Assaad W, Krikorian A, Liu B, Diab K, Idriss NZ, El-Sabban M, Driscoll TA, Perry DK, Hannun YA. 2001. Ceramide generation by two distinct pathways in tumor necrosis factor alpha-induced cell death. FEBS Lett 503:7–12.

De Bosscher K, Vanden Berghe W, Haegeman G. 2000. Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: Negative interference of activated glucocorticoid receptor with transcription factors. J Neuroimmunol 109:16–22.

Distelhorst CW. 2002. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. Cell Death Differ 9:6–19.

Edsall LC, Van Brocklyn JR, Cuvillier O, Kleuser B, Spiegel S. 1998. *N*,*N*-Dimethylsphingosine is a potent competitive inhibitor of sphingosine kinase but not of protein kinase C: Modulation of cellular levels of sphingosine 1-phosphate and ceramide. Biochemistry 37: 12892–12898.

Evans-Storms RB, Cidlowski JA. 2000. Delineation of an antiapoptotic action of glucocorticoids in hepatoma cells: The role of nuclear factor-kappaB. Endocrinology 141: 1854–1862.

Geilen CC, Wieder T, Orfanos CE. 1997. Ceramide signalling: Regulatory role in cell proliferation, differentiation, and apoptosis in human epidermis. Arch Dermatol Res 289:559–566.

Grey A, Chen Q, Callon K, Xu X, Reid IR, Cornish J. 2002. The phospholipids sphingosine-1-phosphate and lysophosphatidic acid prevent apoptosis in osteoblastic cells via a signaling pathway involving G(i) proteins and phosphatidylinositol-3 kinase. Endocrinology 143:4755–4763.

Kasibhatla S, Genestier L, Green DR. 1999. Regulation of fas-ligand expression during activation-induced cell death in T lymphocytes via nuclear factor kappa B. J Biol Chem 274:987–992.

Kleuser B, Cuvillier O, Spiegel S. 1998. 1α,25-dihydroxyvitamin D_3 inhibits programmed cell death in HL-60 cells by activation of sphingosine kinase. Cancer Res 58: 1817–1824.

Kleuser B, Maceyka M, Milstien S, Spiegel S. 2001. Stimulation of nuclear sphingosine kinase activity by platelet-derived growth factor. FEBS Lett 503:85–90.

Kolesnick RN, Kronke M. 1998. Regulation of ceramide production and apoptosis. Annu Rev Physiol 60:643–665.

Korting HC, Unholzer A, Schäfer-Korting M, Tausch I, Gassmueller J, Nietsch KH. 2002. Different skin thinning potential of equipotent medium-strength glucocorticoids. Skin Pharmacol Appl Skin Physiol 15:85–91.

Kouba DJ, Nakano H, Nishiyama T, Kang J, Uitto J, Mauviel A. 2001. Tumor necrosis factor-alpha induces distinctive NF-kappa B signaling within human dermal fibroblasts. J Biol Chem 276:6214–6224.

Lange K, Kleuser B, Gysler A, Bader M, Maia C, Scheidereit C, Korting HC, Schäfer-Korting M. 2000. Cutaneous inflammation and proliferation in vitro: Differential effects and mode of action of topical glucocorticoids. Skin Pharmacol Appl Skin Physiol 13:93–103.

Li D, Saldeen T, Mehta JL. 1999. Gamma-tocopherol decreases ox-LDL-mediated activation of nuclear factor-kappaB and apoptosis in human coronary artery endothelial cells. Biochem Biophys Res Commun 259: 157–161.

Lipton SA. 1997. Janus faces of NF-kappa B: Neurodestruction versus neuroprotection. Nat Med 3:20–22.

Luberto C, Hassler DF, Signorelli P, Okamoto Y, Sawai H, Boros E, Hazen-Martin DJ, Obeid LM, Hannun YA, Smith GK. 2002. Inhibition of tumor necrosis factor-induced cell death in MCF7 by a novel inhibitor of neutral sphingomyelinase. J Biol Chem 277:41128–41139.

Manggau M, Kim DS, Ruwisch L, Vogler R, Korting HC, Schäfer-Korting M, Kleuser B. 2001. 1α,25-dihydroxyvitamin D_3 protects human keratinocytes from apoptosis by the formation of sphingosine-1-phosphate. J Invest Dermatol 117:1241–1249.

Maruyama K, Kashihara N, Yamasaki Y, Sato M, Sugiyama H, Okamoto K, Maeshima Y, Odawara M, Sasaki J, Makino H. 2001. Methylprednisolone accelerates the resolution of glomerulonephritis by sensitizing mesangial cells to apoptosis. Exp Nephrol 9:317–326.

McKay LI, Cidlowski JA. 1999. Molecular control of immune/inflammatory responses: Interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocr Rev 20:435–459.

Messmer UK, Winkel G, Briner VA, Pfeilschifter J. 1999. Glucocorticoids potently block tumour necrosis factor-alpha- and lipopolysaccharide-induced apoptotic cell death in bovine glomerular endothelial cells upstream of caspase 3 activation. Br J Pharmacol 127:1633–1640.

Moran TJ, Gray S, Mikosz CA, Conzen SD. 2000. The glucocorticoid receptor mediates a survival signal in human mammary epithelial cells. Cancer Res 60:867–872.

Okazaki T, Bell RM, Hannun YA. 1989. Sphingomyelin turnover induced by vitamin D_3 in HL-60 cells. Role in cell differentiation. J Biol Chem 264:19076–19080.

Ponec M, De Kloet ER, Kempenaar JA. 1980. Corticoids and human skin fibroblasts: Intracellular specific binding in relation to growth inhibition. J Invest Dermatol 75:293–296.

Ramalingam A, Hirai A, Thompson EA. 1997. Glucocorticoid inhibition of fibroblast proliferation and regulation of the cyclin kinase inhibitor p21Cip1. Mol Endocrinol 11:577–586.

Ramdas J, Harmon JM. 1998. Glucocorticoid-induced apoptosis and regulation of NF-kappaB activity in human leukemic T cells. Endocrinology 139:3813–3821.

Ruwisch L, Schäfer-Korting M, Kleuser B. 2001. An improved high-performance liquid chromatographic method for the determination of sphingosine 1-phosphate in complex biological materials. Naunyn Schmiedebergs Arch Pharmacol 363:358–363.

Schwartzman RA, Cidlowski JA. 1994. Glucocorticoid-induced apoptosis of lymphoid cells. Int Arch Allergy Immunol 105:347–354.

Schäfer-Korting M, Schmid MH, Korting HC. 1996. Topical glucocorticoids with improved risk-benefit ratio. Rationale of a new concept. Drug Saf 14:375–385.

Sikora E, Rossini GP, Grassilli E, Bellesia E, Salomoni P, Franceschi C. 1996. Interference between DNA binding activities of AP-1 and GR transcription factors in rat thymocytes undergoing dexamethasone-induced apoptosis. Acta Biochim Pol 43:721–731.

Smets LA, Salomons G, van den Berg J. 1999. Glucocorticoid induced apoptosis in leukemia. Adv Exp Med Biol 457:607–614.

Sugiyama H, Savill JS, Kitamura M, Zhao L, Stylianou E. 1999. Selective sensitization to tumor necrosis factor-alpha-induced apoptosis by blockade of NF-kappaB in primary glomerular mesangial cells. J Biol Chem 274: 19532–19537.

Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. 1996. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science 274:787–789.

Wahl C, Liptay S, Adler G, Schmid RM. 1998. Sulfasalazine: A potent and specific inhibitor of nuclear factor kappa B. J Clin Invest 101:1163–1174.

Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP. 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol 17:331–367.

Webster JC, Huber RM, Hanson RL, Collier PM, Haws TF, Mills JK, Burn TC, Allegretto EA. 2002. Dexamethasone and tumor necrosis factor-alpha act together to induce the cellular inhibitor of apoptosis-2 gene and prevent apoptosis in a variety of cell types. Endocrinology 143: 3866–3874.

Xia P, Wang L, Gamble JR, Vadas MA. 1999. Activation of sphingosine kinase by tumor necrosis factor-alpha inhibits apoptosis in human endothelial cells. J Biol Chem 274:34499–34505.