available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Potencies of topical glucocorticoids to mediate genomic and nongenomic effects on human peripheral blood mononuclear cells

Lydia Naumann [a], *Eugen Feist* [a], *Rainer H. Straub* [b], *Gerd-Rüdiger Burmester* [a], *Frank Buttgereit* [a,*]

[a] *Department of Medicine (Rheumatology and Clinical Immunology), Charité University Hospital, Schumannstrasse 20/21, 10117 Berlin, Germany*

[b] *Department of Internal Medicine I, University Hospital, 93042 Regensburg, Germany*

ARTICLE INFO

Article history:
Received 10 August 2005
Accepted 20 October 2005

Keywords:
Human peripheral blood mononuclear cells
Systemic and topical glucocorticoids
Energy metabolism
Apoptosis
IL-6
Genomic and nongenomic glucocorticoid effects

Abbreviations:
GC, glucocorticoid
cGR, cytosolic glucocorticoid receptor
mGR, membrane-bound glucocorticoid receptor
Con A, concanavalin A
PHA, phytohaemagglutinin
IL, interleukin
mab, monoclonal antibody
PBMC, peripheral blood mononuclear cells

ABSTRACT

Several different genomic and nongenomic mechanisms are known to mediate the important anti-inflammatory and immunomodulatory effects of glucocorticoids (GC). Genomic effects are the most important while the clinical relevance of nongenomic actions is still a matter of debate. We therefore investigated whether beclometasone and clobetasol are particularly suitable for topical application because of their specific spectrum of genomic and nongenomic actions.

For these purposes we compared effects on oxygen consumption as measured with a Clark electrode (nonspecific nongenomic glucocorticoid effects), on interleukin-6 synthesis by means of ELISA (genomic effects) and on apoptosis using flow cytometry (nongenomic and genomic effects) in quiescent and mitogen-stimulated PBMC.

Beclometasone and clobetasol indeed had stronger effects on the oxygen consumption of quiescent and stimulated cells at lower concentrations (10^{-10} and 10^{-8} M) but were less potent at higher concentrations (10^{-5} and 10^{-4} M) in comparison with dexamethasone. Also in terms of genomic potency, topical GC were more effective than dexamethasone at 10^{-10} and 10^{-8} M but gave similar results at higher concentrations. The ability of all three GC to induce apoptosis was found to be concentration-dependent and similar at concentrations between 10^{-8} and 10^{-5} M. But, compared with 10^{-4} M dexamethasone, topical GC at 10^{-4} M were significantly more effective at inducing apoptosis in both PBMC and Jurkat T-cells.

These results show that topical GC have different concentration – (genomic/nongenomic) effect – ratios compared with dexamethasone: besides to the well-known genomic effects there are also significant nongenomic effects of topical glucocorticoids that already at low concentrations might be more therapeutically relevant in certain clinical conditions than currently assumed.

* *Corresponding author*. Tel.: +49 30 450 513125; fax: +49 30 450 513917.
E-mail addresses: lydia.naumann@charite.de (L. Naumann), frank.buttgereit@charite.de (F. Buttgereit).

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.10.045

1. Introduction

Glucocorticoids (GC) are important immunosuppressive and anti-inflammatory drugs used clinically for more than five decades. Their actions are mediated by two qualitatively different mechanisms: *genomic* and *nongenomic* mechanisms of action [1,2].

The *genomic effects* have been well-investigated [3]. After diffusion through the cell membrane and binding to the cytosolic GC receptor α (GRα), the activated glucocorticoid/receptor complex translocates into the cell nucleus, binds there to glucocorticoid responsive elements of the DNA and thus induces the synthesis of regulatory proteins (transactivation). Another action is the inhibition of transcription factors such as AP-1 or NF-κB, leading to reduced synthesis of certain regulatory proteins (transrepression). The onset of these genomic effects is seen after a latent period of at least 30 min. Moreover, it has recently been shown that binding to the GRα can also trigger rapid and transcription-independent effects [4,5]. Genomic effects are seen even at very low concentrations of GC (10^{-10} M). At higher concentrations the receptor occupancy and hence the genomic effects increase. However, all GRα are saturated with doses of 200–300 mg prednisolone equivalent per day in adults [2] and moreover, GC therapy results in a downregulation of glucocorticoid receptors [6,7]. For these reasons and for their fast onset within seconds or minutes, the rapid effects of high doses of GC for systemic therapy or local administration (e.g. by intra-articular injection) can only be explained by other – so called nongenomic-mechanisms.

The rapid *nongenomic effects* are considered to be mediated through binding to membrane-bound GC receptors (mGR) (*specific* nongenomic effects) and/or generated by unspecific interactions (intercalation) with cell membranes (*unspecific* nongenomic effects) [1]. In either case however, their onset is so rapid that they cannot be explained by the classical genomic pathway. We could recently demonstrate the existence of mGR on human monocytes and B-cells. The function of these receptors is still unclear. It has been suggested that binding of GC at medium concentrations (10^{-8} to 10^{-6} M) to mGR triggers rapid signal cascades resulting in the induction of apoptosis, for example [8,9]. With high concentrations (10^{-5} to 10^{-4} M), intercalation of GC molecules in the membrane increases quantitatively, affecting the membrane fluidity, membrane-associated proteins and cation cycling [10,11]. Measurable results include the reduction of cation transport ATPase activity, for example.

In an earlier study, we found clear differences in the ability of systemic GC to trigger these unspecific nongenomic effects. The potency hierarchy established clearly differs from the known hierarchy for genomic effects [12]. These results were the basis for the investigations presented here. We analysed the genomic and nongenomic potencies of the topically applied GC beclometasone and clobetasol. As a well-known comparator we used dexamethasone. Our initial hypothesis was that these topical GC have a strong nongenomic efficacy as they achieve high local concentrations.

2. Materials and methods

2.1. Preparation of human PBMC

Peripheral blood mononuclear cells (PBMC) from buffy coats of healthy donors were isolated by density centrifugation using the Ficoll-Hypaque method (Pharmacia Biotech AB, Uppsala, Sweden). Each buffy-coat preparation was diluted with PBS, and density gradient centrifugation was performed at 400 rpm for 20 min. The PBMC-enriched interphase was isolated, washed with saline, and resuspended. Viability of freshly isolated cells was >95% as determined by trypan blue exclusion. The PBMC suspension consisted of lymphocytes (about 90%) and monocytes (about 10%) as determined by FACS analysis.

2.2. Culture and incubation of human PBMC

The culture medium used for Clark electrode analysis was a 1.5:1 mixture of Eagle's basal medium with a salt mixture according to Hanks (+0.15 M Tris/HCl, -glutamine, -$NaHCO_3$, -glucose, pH 7.4) supplemented by 19 L-amino acids each at 0.2 mmol/l final concentration as previously described [12]. This medium was chosen as in the case of Eagle's basal medium the contribution of glycolysis to ATP production was negligible. Thus ATP production was determined by oxidative phosphorylation and quantifiable by measurement of oxygen consumption [13–15]. Cells were resuspended at 2×10^7 cells/ml and then kept on ice for a maximum of 3 h in wide-necked plastic flasks to ensure aeration until measurement. GC and/or Con A were added at the start of each measurement. There was no incubation time because of the rapid onset of effects on oxygen consumption within seconds.

The incubation medium used for ELISA and FACS analysis was RPMI 1640 (Biochrom KG, Berlin, FRG) with 0.5 g/l glutamine, 2.0 g/l $NaHCO_3$ buffered at pH 7.4 with 25 mM HEPES and supplemented with fetal calf serum (1:10, Sigma–Aldrich, Steinheim, FRG). For ELISA analysis, duplicates of the cell suspension (1×10^6 cells/ml) were cultured in 96-well, round-bottom microtitre plates either as controls or stimulated with concanavalin A (Con A; 5 μg/ml) or phytohaemagglutinin (PHA; 10 μg/ml). To this end, the cell suspensions were first cultured for 2 h (37 °C, 5% CO_2). Thereafter, nonadherent cells were recollected and incubated for a further 24 h with/without Con A or PHA and GC, respectively. The medium was renewed after 12 h to avoid an IL-6 peak by monocytes/macrophages [16]. After two wash steps aliquots of cell culture supernatant were then removed and stored at −20 °C until measurement. For measurements by FACS analysis, cell suspensions (1×10^6 cells/ml) were cultured in 24-well, round-bottom microtitre plates (Corning, NY, USA). GC were added at the start of cell culture. Cells were incubated for 20 h (37 °C, 5% CO_2). Incubation of cells with GC did not change cell viability or cell number.

2.3. Cell culture and incubation of Jurkat T cells

Jurkat cells (clone E6-1), a human ALL T cell line in suspension were obtained from the American Type Culture Collection (catalogue no. TIB-152). Culture and incubation were carried

out in cell culture bottles (Costar) with a total volume of 30 ml at 37 °C, 5% CO_2. The culture medium was RPMI-1640 supplemented with 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin. Change of the medium and cell transfer were carried out at a density of approximately 5×10^5 cells/ml after 2–3 days. Cell vitality was checked by trypan blue exclusion.

2.4. Measurement of oxygen consumption

Oxygen consumption was measured amperometrically with a Clark-electrode in a 0.7 ml aliquot of cell suspension. The cell suspension in the Perspex incubation chamber of the electrode was magnetically stirred and thermostatically maintained at 37 °C. Con A was added at 3.75 μg/10^6 cells. This concentration has been shown in previous experiments to give reproducible cell stimulation persisting over time of measurement. Respiration was measured up to 3 min to evaluate basal respiration rate (cell suspension without GC or Con A) and another 3 min directly after each addition of concanavalin A or GC as described previously [17,18]. The onset of maximal and constant effects was achieved within seconds (Fig. 1).

2.5. Measurement of IL-6

IL-6 was determined in cell culture supernatants using a classical two-site sandwich ELISA (Milenia IL-6 Endpoint Enzyme Immunometric Assay, DPC, Germany). Diluted standard samples, diluted supernatant samples (with and without Con A or PHA) and positive as well as negative control samples (with and without glucocorticoids), each at 100 μl/well were incubated in microtitre plates pre-coated with (mouse-) anti-IL-6 mab for 2 h at room temperature. After three wash steps samples were incubated with 200 μl of horseradish peroxidase-conjugated polyclonal (rabbit-) anti-IL-6 mab for 2 h at room temperature. After washing, 3,3′,5,5′-tetramethyl benzidine was added as substrate. The reaction was stopped after 30 min by the addition of 2N HCl. Optical density at 450 nm was read and a standard curve constructed for each plate. The lower detection limit of this method was 4 pg/ml.

2.6. Measurement of apoptosis

FACS analysis of GC-induced apoptosis were performed with an annexin V-PI assay (Immunotech, Marseille, France) and a DNA-staining procedure. After incubation with GC cells were washed and the cell pellets were resuspended with 450 μl PBS. Cells were stained with 5 μl of annexin V-FITC solution and 5 μl of propidium iodide solution for 10 min at 4 °C in the dark, following the manufacturer's instructions. DNA-staining was performed using an appropriate assay (Institute for Immunology and Genetics, Kaiserlautern, Germany). Following cell permeabilization with 200 μl of a triton buffer solution for 15 min, 500 μl of FITC/PI buffer solution was added and cells were stained for 15 min at 4 °C in the dark. Apoptosis was finally measured by FACS analysis (Coulter, Miami, USA). For each analysis, 10,000 events were recorded. Controls stained with annexin V and PI alone were used for compensation. Annexin V-FITC and PI double negative cells were defined as living cells. Annexin V-FITC positive and PI negative cells were defined as early apoptotic cells. Annexin V-FITC and PI double positive cells were defined as secondary necrotic cells.

The annexin V/PI staining occurs at an very early step in the apoptosis cascade, whilst staining of DNA fragments does not take place until a much later stage. Both for Jurkat cells and human PBMC there was good correlation between the results

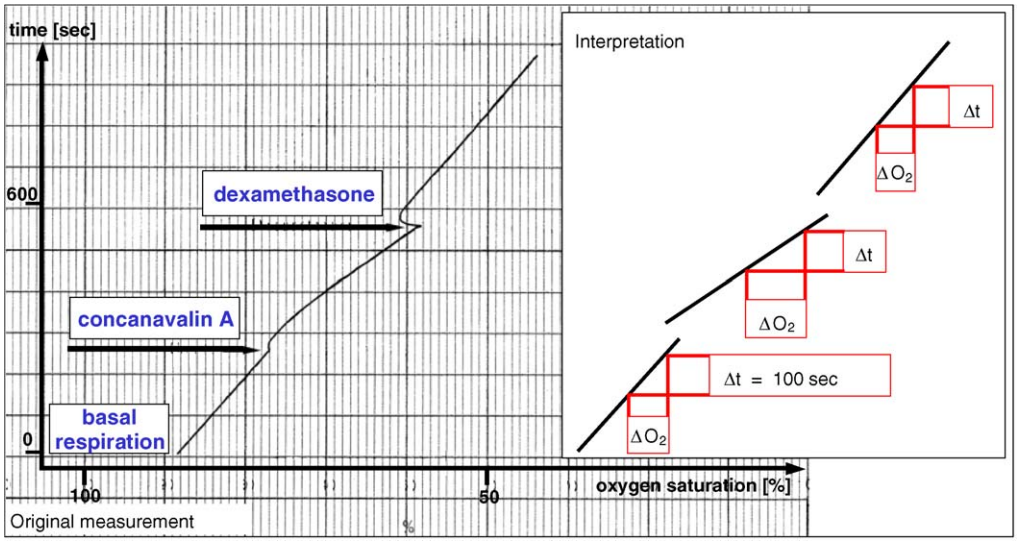

Fig. 1 – Oxygen consumption of human PBMC as stimulated by Con A and inhibited by dexamethasone. This figure shows an original trace of oxygen consumption measured amperometrically with a Clark electrode. The first part of the curve reflects the unstimulated (basal) rate of oxygen consumption, calculated by dividing the change in oxygen saturation (ΔO_2) by the time (Δt). Addition of Con A leads to a significantly greater rate of oxygen consumption within seconds as reflected by the changed slope of the line. Addition of dexamethasone at 10^{-4} M is accompanied by an artifact due to simultaneous addition of oxygen dissolved in the solvent, immediately followed by a slower steady rate of oxygen consumption. Effects of Con A and dexamethasone occur within seconds.

of the two methods. Results of the annexin V/PI staining are given as detailed results.

2.7. Statistical methods

Statistical tests were performed with the statistics programme SPSS, Version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Values are given as the mean ± standard error of the mean (S.E.M.) of n cell preparations determined as duplicates or triplicates. Statistical differences were evaluated by Student's t-test, Wilcoxon test or Mann–Whitney U-test. p values < 0.05 were accepted as significant. The Shapiro–Wilk test was used to verify a normal distribution.

2.8. Materials

Dexamethasone (Merck, Darmstadt, Germany) was dissolved in water. Clobetasol propionate and beclometasone-17,21-dipropionate (both GlaxoWellcome, Uxbridge, Middlesex, UK) were dissolved in DMSO to give a stock solution of 0.1 mM. Further dilutions of clobetasol and beclometasone were made that way to give the corresponding end concentration with 1 μl; thus the end concentration of DMSO in all experiments was never greater than 1%. All GC were aliquoted, stored at −20 °C and used at final concentrations between 0.1 nM and 0.1 mM. Concanavalin A (Con A, ICN ImmunoBiologicals, Lisle, Israel) and phytohaemagglutinine (PHA, Biochrom AG, Hannover, FRG) were dissolved in water and added at final concentrations of 3.75 μg/10^6 cells and 10 μg/10^6 cells, respectively. RU 486 (mifepristone, a GR antagonist, Sigma–Aldrich, Steinheim, FRG) was dissolved in water to give a stock solution of 0.1 mM and used at final concentrations between 0.01 nM and 0.1 mM.

3. Results

3.1. Effects on oxygen consumption

3.1.1. Basal and Con A-stimulated oxygen consumption rates of human PBMC

The basal oxygen consumption of quiescent PBMC was found to be 5.84 ± 0.22 nmol O_2/min/10^7 cells ($n = 52$). Addition of Con A at a concentration of 3.75 μg Con A/10^6 cells caused a significant increase in basal respiration rate of 55.4 ± 4.6% to 8.89 ± 0.45 nmol O_2/min/10^7 cells within seconds ($p < 0.001$, $n = 40$, see also Fig. 1). This total oxygen consumption reflects mainly the requirement of ATP-consuming processes (coupled respiration), but also uncoupled and extramitochondrial oxygen consumption.

Quiescent cells. At all concentrations, dexamethasone caused significant inhibition of oxygen consumption in quiescent PBMC. Dexamethasone at 10^{-4} M led to a significant reduction of oxygen consumption by 15.2 ± 1.9% to 4.95 ± 0.11 nmol/min/10^7 cells ($p < 0.001$, $n = 9$). Also at concentrations of 10^{-5}, 10^{-6}, 10^{-8} and 10^{-10} M, dexamethasone reduced the oxygen consumption significantly by 14.4 ± 1.7% ($p < 0.001$, $n = 6$), 5.7 ± 1.4%, 5.7 ± 1.6% and 5.4 ± 0.7% ($p < 0.01$, $n = 7$–9) respectively (Fig. 2).

The effects of clobetasol differed from those of dexamethasone significantly. Clobetasol at a concentration as low as 10^{-10} M led to a significant reduction of oxygen consumption of 10.3 ± 1.7% to 5.24 ± 0.1 nmol/min/10^7 cells ($p < 0.001$, $n = 7$) and thus led to a significantly greater inhibition of oxygen consumption as compared to dexamethasone at 10^{-10} M ($p < 0.01$). The extent of the respiratory inhibition by clobetasol remained almost constant up to 10^{-4} M (about 8–11% for all concentrations used, Fig. 2). A similar course of the inhibitory effect was shown by beclometasone but reaching a higher level of inhibition overall. Beclometasone at a concentration as low as 10^{-10} M caused oxygen consumption to be reduced by 15.6 ± 3.6% to 4.93 ± 0.23 nmol/min/10^7 cells ($p < 0.001$, $n = 6$). In comparison with dexamethasone at 10^{-10} M, the difference in inhibition was significant ($p < 0.01$). Beclometasone at a concentration of 10^{-4} M gave a reduction of 18.3 ± 2.4% to 4.77 ± 0.11 nmol/min/10^7 cells ($p < 0.001$, $n = 6$) (Fig. 2).

It should be noted that clobetasol and beclometasone were dissolved in DMSO whereby the final DMSO concentration never exceeded 1%. DMSO itself had no significant effect on oxygen consumption of quiescent PBMC (5.67 ± 0.25 nmol/min/10^7 cells, $p > 0.05$).

Con A-stimulated cells. To establish the GC induced changes in energy metabolism in activated immune cells, the PBMC were

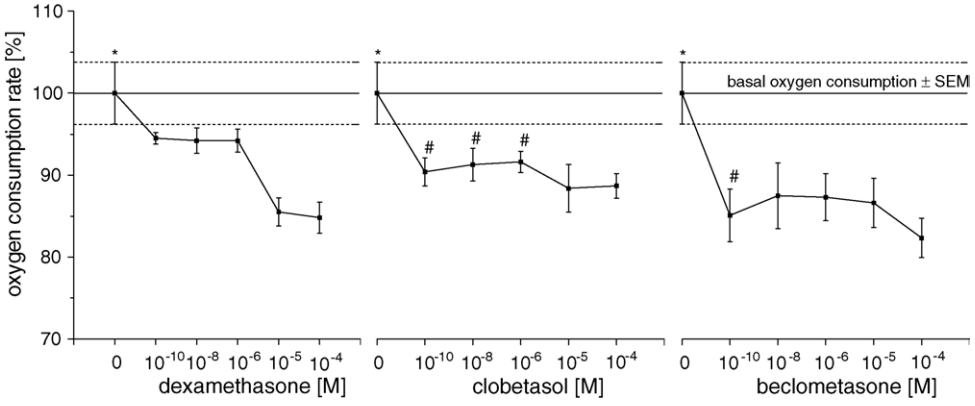

Fig. 2 – Effects of dexamethasone, clobetasol and beclometasone on oxygen consumption of quiescent human PBMC. Values are given as the mean ± S.E.M. of 6–9 cell preparations. *Significantly different vs. all concentrations of dexamethasone, clobetasol and beclometasone, #significantly different vs. corresponding dexamethasone concentration ($p < 0.05$).

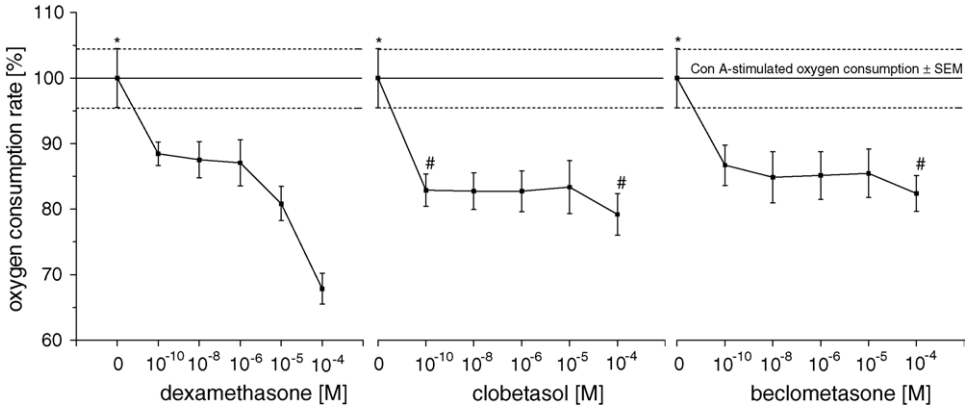

Fig. 3 – Effects of dexamethasone, clobetasol and beclometasone on oxygen consumption of Con A-stimulated human PBMC. Values are given as the mean ± S.E.M. of 6–8 cell preparations. *Significantly different vs. all concentrations of dexamethasone, clobetasol and beclometasone, #significantly different vs. corresponding dexamethasone concentration ($p < 0.05$).

pre-incubated with the mitogen Con A. In all experiments the GC were added after a constant incubation time with Con A of 3 min. Inhibitory effects of GC were seen within seconds (Fig. 1).

Dexamethasone led to a statistically significant concentration-dependent inhibition of oxygen consumption. At 10^{-4} M dexamethasone showed absolute inhibition down to 5.95 ± 0.21 nmol/min/10^7 cells ($p < 0.001$, $n = 8$, Figs. 1 and 3), corresponding to a percentage inhibition of $33.1 \pm 2.4\%$. Dexamethasone at 10^{-5} M led to inhibition of $19.2 \pm 2.6\%$ ($p < 0.001$, $n = 6$). As in the quiescent cells, concentrations of 10^{-6}, 10^{-8} and 10^{-10} M all showed similar results with statistically significant inhibition by $12.9 \pm 3.5\%$, $12.5 \pm 2.8\%$ and $11.5 \pm 1.8\%$, respectively ($p < 0.01$, $n = 7$–8).

In the stimulated cells as in the quiescent ones, the effects of both clobetasol and beclometasone were found to be concentration-independent. Clobetasol at a concentration of even 10^{-10} M caused a significant reduction in oxygen consumption of $17.1 \pm 2.5\%$ to 7.37 ± 0.22 nmol/min/10^7 cells ($p < 0.001$, $n = 6$). This level of inhibition was nearly constant with higher concentrations up to 10^{-4} M (about 16–21%; Fig. 3). Beclometasone showed very similar behaviour, giving rise to inhibition of $13.3 \pm 3.1\%$ to 7.71 ± 0.27 nmol/min/10^7 cells ($p < 0.001$, $n = 6$) at a concentration as low as 10^{-10} M. This inhibition remained almost constant to 10^{-4} M (about 14–17% for all concentrations used, Fig. 3). In comparison with dexamethasone, very low concentrations of clobetasol (10^{-10} M) gave rise to a statistically significant inhibition of stimulated oxygen consumption ($p < 0.05$), while beclometasone only showed a tendency in this direction.

As shown in quiescent cells, the solvent DMSO itself (1 μl) had no effect on the oxygen consumption of Con A-stimulated PBMC either (8.84 ± 0.22 nmol/min/10^7 cells, $p > 0.05$).

Interference of glucocorticoids with Con A stimulation. To investigate whether the GC interfere with Con A stimulation, the cell suspensions were first preincubated with the different concentrations of GC for 3 min and then stimulated with Con A. We found that if incubated with dexamethasone at a concentration of 10^{-4} M the cells could be significantly less stimulated by Con A and oxygen consumption after stimulation reached only 6.79 ± 0.34 nmol/min/10^7 cells ($p < 0.001$, $n = 9$), being $23.6 \pm 3.8\%$ less than when there was no dexamethasone present (see above). Dexamethasone at 10^{-5} to 10^{-10} M did not significantly affect Con A stimulation. At all concentrations, clobetasol did not interfere with the Con A effect ($p > 0.05$, $n = 7$). In contrast, beclometasone caused a significant reduction in Con A stimulation. This effect was independent of the concentration used and corresponded to a reduction of about 18% in relation to the oxygen consumption of stimulated cells in the absence of beclometasone ($p < 0.05$, $n = 5$).

3.2. *Effects on IL-6 synthesis*

3.2.1. *Basal and stimulated IL-6 synthesis*

The basal IL-6 production of quiescent PBMC was 9.3 ± 2.5 pg/ml ($n = 6$). Con A and PHA stimulated IL-6 synthesis significantly ($p < 0.001$) to 3434.1 ± 818.3 pg IL-6/ml and 2494.5 ± 496.9 pg IL-6/ml, respectively. Following results represent measurements on at least four preparations each determined as duplicates.

Quiescent cells. None of the three GC had a significant effect on the very low IL-6 synthesis of quiescent PBMC. Following the addition of dexamethasone at 10^{-4} M, the IL-6 synthesis was 8.7 ± 3.0 pg IL-6/ml ($p = 0.4$), following clobetasol at 10^{-5} M it was 11.5 ± 4.1 pg IL-6/ml ($p = 0.6$) and after beclomethasone at 10^{-5} M it was 10.9 ± 1.5 pg IL-6/ml ($p = 0.5$, data not shown).

Con A-stimulated cells. The addition of 10^{-4}, 10^{-6} and 10^{-8} M dexamethasone caused a significant reduction of IL-6 synthesis to 684.8 ± 238.2 pg/ml ($80.1 \pm 4.1\%$; $p < 0.05$), 893.0 ± 256.9 pg/ml ($74.0 \pm 4.2\%$; $p < 0.05$) and 1913.3 ± 490.0 pg/ml ($44.3 \pm 0.1\%$; $p < 0.05$; Fig. 4), respectively. About 10^{-10} M had no significant effect on the IL-6-synthesis, with results of 3140.5 ± 832.0 pg/ml ($8.5 \pm 5.1\%$; $p = 0.2$).

With respect to their effects on IL-6 synthesis of Con A-stimulated cells, the two topical GC showed almost an identical behaviour (Fig. 4). Even a concentration as low as 10^{-10} M gave rise to marked inhibition of IL-6-synthesis:

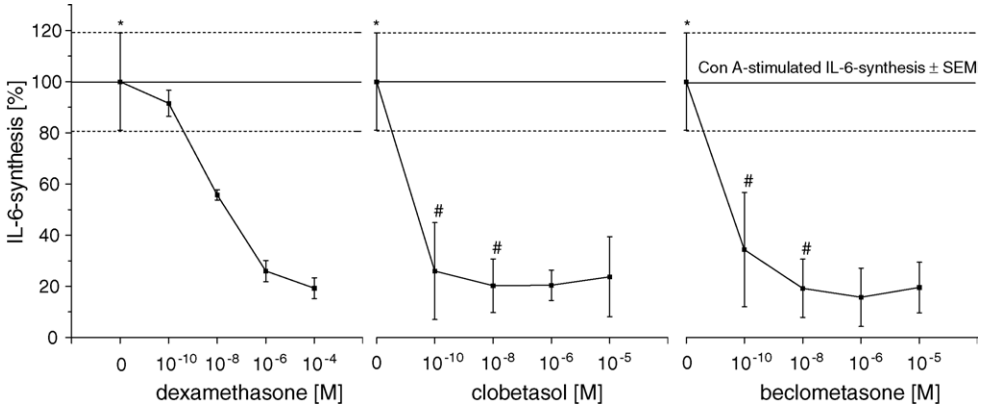

Fig. 4 – Effects of dexamethasone, clobetasol and beclometasone on IL-6 synthesis of Con A-stimulated human PBMC. Values are given as the mean ± S.E.M. of at least four cell preparations. *Significantly different vs. all concentrations of dexamethasone, clobetasol and beclometasone except dexamethasone 10^{-10} M, #significantly different vs. corresponding dexamethasone concentration ($p < 0.05$).

74.0 ± 18.9% ($p < 0.05$) for clobetasol and 65.6 ± 22.3% ($p < 0.05$) for beclometasone. At this low concentration, the extent of inhibition by clobetasol and beclometasone was significantly greater than that by dexamethasone at 10^{-10} M ($p < 0.05$). The effect of clobetasol and beclometasone was concentration-independent and at 10^{-8} to 10^{-5} M IL-6 synthesis was inhibited by about 76–84% ($p < 0.05$, Fig. 4).

PHA-stimulated cell. The extent and the concentration-dependent course of dexamethasone's inhibition of IL-6 synthesis in PHA-stimulated cells showed no significant difference from the results found for Con A-stimulated cells (data not shown).

Also in terms of clobetasol, 10^{-10} M led to a statistically significant IL-6 synthesis inhibition of about 84% ($p < 0.05$). Compared to dexamethasone at 10^{-10} M, there was a significant difference ($p < 0.05$). The level of inhibition at higher concentrations was almost in the same range (73–87%). The results for beclometasone were also similar to those obtained with Con A-stimulated cells (data not shown).

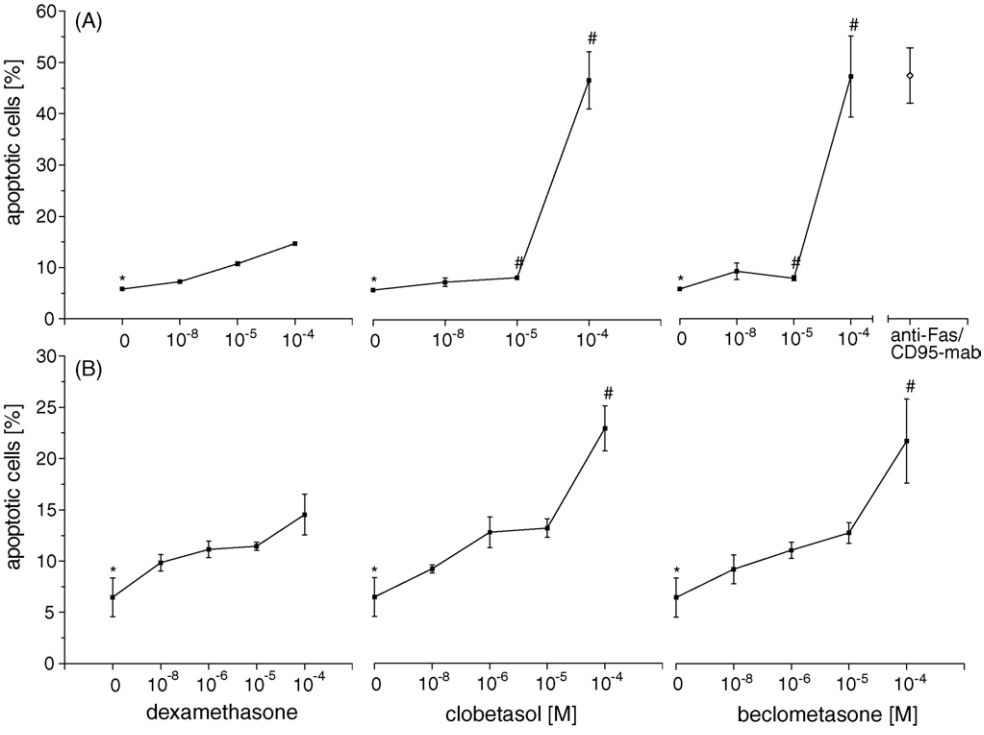

Fig. 5 – Effects of dexamethasone, clobetasol and beclometasone on apoptosis of (A) human Jurkat T cells after incubation for 16 h and of (B) human PBMC after incubation for 20 h. Values are given as the mean ± S.E.M. of three cell preparations. *Significantly different vs. all concentrations of dexamethasone, clobetasol and beclometasone except beclometasone 10^{-8} M in human PBMC, #significantly different vs. corresponding dexamethasone concentration ($p < 0.05$).

3.3. *Effects on apoptosis*

3.3.1. *Jurkat T cell line*

Jurkat T cells were incubated for 16 h with each of the GC at concentrations of 10^{-4}, 10^{-5} and 10^{-8} M. Anti-Fas/CD95 mab (1 μl/ml) was used as positive control (Fig. 5A). After 16 h the proportion of spontaneous apoptotic cells was 5.8 ± 0.2% ($n = 3$).

Dexamethasone at 10^{-8} M had no effect, while 10^{-5} and 10^{-4} M led to a significant increase in apoptotic cells to 10.7 ± 0.4% ($p < 0.05$) and 14.7 ± 0.1% ($p < 0.05$), respectively.

At 10^{-8} and 10^{-5} M, the topical GC had no effect on apoptosis. Only at 10^{-4} M, there was a significant increase in apoptotic cells to 46.7 ± 5.6% (clobetasol, $p < 0.05$) and 47.3 ± 7.9% (beclometasone, $p < 0.05$), respectively. These results at 10^{-4} M corresponded to the apoptosis rate seen after incubation with anti-Fas/CD95 antibodies for 16 h (47.5 ± 5.4%; $p < 0.05$) but differed significantly from those seen with dexamethasone at 10^{-4} M ($p < 0.05$).

3.3.2. *Human PBMC*

Initial experiments showed that an incubation with GC for 20 h lead to maximum apoptosis in PBMC (unpublished data). The cells were therefore incubated with the GC at 10^{-4}, 10^{-5}, 10^{-6} and 10^{-8} M for 20 h. All three GC induced concentration-dependent apoptosis after 20 h. The proportion of cells undergoing spontaneous apoptosis was 5.6 ± 2.0% ($n = 3$).

Only dexamethasone at 10^{-4} M led to a significant increase in the number of apoptotic cells to 14.5 ± 2.0% ($p < 0.05$). However, increases in the percentage of apoptotic cells following incubation with dexamethasone at 10^{-8} M (9.8 ± 0.8%), at 10^{-6} M (11.1 ± 0.8%) and at 10^{-5} M (11.5 ± 0.4%) were not significant (Fig. 5B).

In comparison, incubation with clobetasol or beclometasone led to a clear increase in apoptotic cells. At 10^{-5} M, there was a significant increase in the apoptotic cells to 13.2 ± 0.9% (clobetasol) and to 12.8 ± 1.0% (beclometasone) ($p < 0.05$). The maximum increase in apoptotic cells was seen after the addition of 10^{-4} M to 21.5 ± 1.3% and 20.1 ± 4.1%, respectively ($p < 0.05$). At this very high concentration, the results of the topical GC were significantly different from those of dexamethasone ($p < 0.05$).

4. Discussion

Up to the present there has been little work in which the efficacy of different GC has been compared in standardised systems [2,12,19]. In the work presented here, we investigated the effects of the two topically applied GC clobetasol and beclometasone and those of dexamethasone as reference substance which is applied both topically and systemically. The working hypothesis was that topical GC have more potent nongenomic effects than dexamethasone, which predestines them for topical use with high local concentrations. Clobetasol and beclometasone were chosen as examples of topical GC as they are two of the most potent topical GC in dermatology and pneumology, and as their therapeutic effects are well-known and well-documented [20,21]. As experimental model we selected human PBMC because they are important target cells for GC due to their central position in the cellular and humoral immune responses in autoimmune and inflammatory diseases. However, it has to be considered that other cells than PBMC, e.g. mast cells derived from human lung parenchymal tissue, intestine or skin [22] differently respond to GCs and that, therefore, it cannot be extrapolated from PBMC to a general hypothesis for all immune cells. The effects on oxygen consumption as an important parameter of cell energy metabolism were measured because the unspecific nongenomic mechanism has successfully been quantified with this method in previous studies [10,12,18]. The effect on stimulated IL-6 synthesis in human PBMC was investigated as a standard means of quantifying genomic effects [23]. It is assumed that the glucocorticoid-induced apoptosis is mediated by all three mechanisms – genomic, specific nongenomic and unspecific nongenomic [24,3,8].

4.1. *Unspecific nongenomic mechanism*

As we could show in previous work GC give rise to immediate inhibition of oxygen consumption in PBMC depending on the concentration [2,12]. It is assumed that the GC molecules intercalate immediately in the cell membranes and thus affect the physicochemical properties of the membrane and the metabolism of membrane-associated proteins within seconds. The inhibition of calcium and sodium cycling across the plasma membrane and the decrease of intracellular free calcium by high but clinically-relevant doses of methylprednisolone were caused by these direct effects on cellular membranes and not by a reduction of ATP synthesis and therefore determined to be unspecific nongenomic effects [10]. In addition, direct effects on the inner mitochondrial membrane cause uncoupling of oxidative phosphorylation by lowering the membrane permeability.

In both quiescent and activated PBMC, dexamethasone led to a concentration-dependent inhibition of oxygen consumption within seconds. Even though the effect was already significant at low concentrations, strongly marked inhibition ≥15% was observed only at higher concentrations of 10^{-5} and 10^{-4} M. In contrast, the inhibitory effects of both topical GC were already highly significant (≥15%) even at very low concentrations of 10^{-10} and 10^{-8} M.

For a clearer description of the observed differences, we have given the *nongenomic potency* of dexamethasone a value of 1, and calculated the relative potency of the topical GC in comparison with dexamethasone. Our results indicate that topical GC at very low concentrations (10^{-10} M) showed greater nongenomic potency than dexamethasone: clobetasol 1.9 > beclometasone 1.5 > dexamethasone 1.0, although they were less potent than dexamethasone at very high concentrations (10^{-4} M): dexamethasone 1.0 > clobetasol 0.5 > beclometasone 0.4.

Topical GC are more lipophilic than dexamethasone due to their esterification at position 17α and thus better able to diffuse and to intercalate into biological membranes. Because of this lipophilia, we suggest that the saturation point of the intercalation capacity of the topical GC is reached at very low concentrations of $<10^{-10}$ M (clobetasol) and 10^{-9} M (beclometasone). Beclometasone tends to inhibit less than clobetasol, as there is a negative steric effect due to the long side chains with double esterification at C17α and C21. Dexamethasone is hydroxylated at the 17α and 21 positions and no C atom is esterified, which leads to a reduction in the lipophilic

properties. We therefore presume that the intercalating capacity does not reach its saturation range until a concentration of 10^{-5} M [25].

For the systemic GC investigated to date, it has been established that clear immediate effects on the oxygen consumption occur only at very high concentrations. The inhibitory effects of dexamethasone exceeded 15% only at very high concentrations of 10^{-5} and 10^{-4} M. Since at these high concentrations all GRα are already saturated, nongenomic effects by dexamethasone are assumed to be mediated by additional unspecific effects via intercalation into cell membranes. Since the topical GC have marked immediate effects seen already at very low concentrations, underlying physicochemical membrane effects but also rapid nongenomic mechanisms either via cGRs or mGRs have to be considered. The first evidence of nongenomic, cGR-mediated effects came from cell culture experiments [26]. Croxtall et al. showed that the rapid MAPK- and $cPLA_2$-dependent modulation of cytokine-stimulated arachidonic acid production by dexamethasone started within minutes and could be blocked by RU 486 (a cGR antagonist with high binding affinity but without intrinsic effects) but not by protein synthesis inhibitors [5]. In further experiments we have also used RU 486 to inhibit oxygen consumption. At the highest concentration of 10^{-4} M there was an inhibitory effect of $8.1 \pm 1.4\%$ ($p \leq 0.05$, $n = 5$) in CD4-positive T cells (for comparison: dexamethasone at 10^{-4} M produced an inhibition of $21.5 \pm 4.4\%$ ($p \leq 0.01$, $n = 5$)). This rapid effect is weak but indicates further that physicochemical interactions are causing the effects observed. Alternatively, also RU 486 could act via mGR and/or via the mechanism suggested by Croxtall et al.

4.2. Genomic mechanism

The important anti-inflammatory and immunomodulatory effects of GC are mediated predominantly by *genomic* mechanisms. These slow (not seen within less than 30 min) mechanisms are physiologically relevant and therapeutically effective at all dosages. Between 10 and 100 genes per cell are directly regulated by GC, but many genes are regulated indirectly through an interaction with transcription factors and co-activators. This results in inhibition of the expression of many immunoregulatory and inflammatory cytokines such as Il-6 [27,3]. Relative potencies (or ''equivalent dosages'') describe the potency of systemic GC in producing these classical genomic effects. These values have been in use for decades although experimental and clinical evidence for their precision is weak. Moreover, there are no such values available for topical GC such as clobetasol and beclometasone. So far, there are only a few reports comparing the relative genomic potencies of topical and systemic GC. Seeto et al. investigated the effects of beclometasone at concentrations of 10^{-14} to 10^{-9} M on IL-5 synthesis in human PBMC and showed that this topical GC was more potent than dexamethasone in this concentration range [28]. We compared the effects of clobetasol and beclometasone on mitogen-stimulated IL-6 synthesis of human PBMC with those of dexamethasone, because IL-6 plays a central role in the regulation of the immune response and acute phase reaction. We could show that topical GC significantly inhibited IL-6 synthesis of mitogen-stimulated PBMC even at very low concentrations of 10^{-10} M and independently of concentration. At 10^{-10} M they showed clearly greater *genomic potency* in comparison with dexamethasone (clobetasol 8.7 > beclometasone 7.7 > dexamethasone 1.0), while at very high concentrations they were almost the same as dexamethasone (dexamethasone 1.0 = clobetasol 1.0 = beclometasone 1.0).

The two topical GC have a receptor binding affinity to cGRα many times greater than that of dexamethasone. Esterification at the 17α position of clobetasol and beclometasone is responsible for this phenomenon [29]. The ligand-binding domains of GRα have a steric cavity in the region of position 17α of the steroid molecule. Under the formation of van der Waal's forces this cavity allows 17α-esters to take up a configuration energetically favourable for them. The fact that beclometasone does not achieve the inhibitory effects of clobetasol may be explained by the additional esterification at position 21. In the steric proximity of position 21, the size of the ligand-binding domain is considerably more restrictive and does not have a cavity comparable to the one at position 17α. In order to bind to the GRα, 21-esters are forced to take up an energetically more unfavourable configuration [30]. Significant inhibition of IL-6 synthesis by dexamethasone was not seen until a concentration of 10^{-8} M and above, since, besides the lack of the ester group on position 17α, hydroxylation at position 17α leads to a reduction in the binding affinity [23].

4.3. Apoptosis as a complex, trimodular mediated process

4.3.1. Transcription-dependent mechanism via cGRα (genomic)

Both dexamethasone and the topical GC induced apoptosis in a concentration-dependent manner. If it is taken into consideration that glucocorticoid-induced apoptosis is mainly a genomically-mediated process, then the same structural similarities are basically responsible for the significantly different apoptosis-inducing effects of the topical GC, as discussed above regarding the effects on IL-6 synthesis. In the study presented here, clear apoptosis was induced after incubation for 20 h. It may be assumed that apoptosis represents at least secondarily a strictly genomically-dependent process [31,32,3]. The present model of genomically mediated apoptosis thus stems from a multi-stranded process [33].

4.3.2. Transcription independent mechanism

On the basis of the kinetics of lymphocytic apoptosis and the effectiveness of high-dose glucocorticoids in corresponding clinical situations, nongenomic mechanisms must also be responsible for the induction of apoptosis [34,35,22]. In addition there is evidence of rapid, cGR-mediated but transcription-independent mechanisms that can lead to apoptosis [36,37].

Unspecific nongenomic mechanism. Changes in the Ca^{2+} transport across cell membranes are functionally relevant for the glucocorticoid-induced inhibition of MAPK-phosphorylation in the process of apoptosis signal transduction [38,2]. Mitochondria are a central site of action for GC. At high concentrations, a direct, unspecific glucocorticoid effect on the inner mitochondrial membrane is responsible for the loss of membrane potential and the subsequent uncoupling of

oxidative phosphorylation [10]. The resultant diminution in cellular ATP supply is observed at an early stage of apoptosis, even before DNA fragmentation. The choice between apoptosis and necrosis depends amongst other things on the cellular ATP supply [39]. Because of the disturbed integrity of the outer mitochondrial membrane, mitochondrial intermembrane proteins such as cytochrome *c*, AIF or procaspase 2, 3 and 9 may leak out [40,41].

Specific nongenomic mechanism via mGR. Specific, membrane-bound GC receptors on human cells were first demonstrated by Gametchu et al. on a human leukaemic cell line [42]. Gametchu et al. voiced the suspicion early on that mGR could contribute to the induction of apoptosis. mGR expression on human CCRF-CEM cells is regulated by cell cycling and correlates with the induction of apoptosis [43]. The precise mechanisms that lead to mGR-induced apoptosis have not yet been elucidated completely. Bartholome et al. showed that, under physiological conditions, up to 9.2% of monocytes and up to 12.3% of B lymphocytes, but no T lymphocytes, are mGR-positive [9]. In the investigations presented here, a proportion of mGR-mediated, nongenomic mechanisms of glucocorticoid-induced apoptosis can therefore be assumed. Under the given experimental conditions, however, no firm conclusions can be reached on the proportion of these nongenomically-mediated glucocorticoid effects.

5. Conclusion

There is a large discrepancy between the clinical importance of glucocorticoids and the current state of elucidation of their mechanisms of action. Genomically-mediated effects still form the basis for comparison of the efficacy of different glucocorticoids today. The specific and unspecific nongenomic effects have not been explained sufficiently and remain largely ignored in everyday clinical practice. D-ring substituents are of crucial importance for the extent of receptor binding, transcriptional potency and benefit/risk ratio. In addition, esterification and especially the ester groups of the C atom in position 17α of the steroid structure are a distinct advantage with respect to lipophilia and receptor binding affinity, which clearly improve the benefit/risk ratio under experimental conditions and in routine clinical practice. The chemical structural properties of topical glucocorticoids may be of interest for the drug design of systemic glucocorticoids and thus of clinical benefit with regard to the central role of PBMC as target cells for GCs in autoimmune and inflammatory diseases.

Acknowledgement

This study was supported by a grant from GlaxoWellcome.

REFERENCES

[1] Buttgereit F, Straub RH, Wehling M, Burmester GR. Glucocorticoids in the treatment of rheumatic diseases. An update on mechanisms of action. Arthritis Rheum 2004;50(11):3408–17.
[2] Buttgereit F, Brand MD, Burmester GR. Equivalent dose and relative drug potencies for non-genomic glucocorticoid effects: a novel glucocorticoid hierarchy. Biochem Pharmacol 1999;58:363–8.
[3] Barnes PJ. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci (Lond) 1998;94(6):557–72.
[4] Solito E, Mulla A, Morris JF, Christian HC, Flower RJ, Buckingham JC. Dexamethasone induces rapid serine-phosphorylation and membrane translocation of annexin 1 in a human folliculostellate cell line via a novel non-genomic mechanism involving the glucocorticoid receptor, protein kinase C, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase. Endocrinology 2003;144(4):1164–74.
[5] Croxtall JD, Chudhury Q, Flowers RJ. Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor dependent, transcription independent mechanism. Br J Pharm 2000;130(2):289–98.
[6] Sanden S, Tripmacher R, Weltrich R, Rohde W, Hiepe F, Burmester GR, et al. Glucocorticoid dose dependent downregulation of glucocorticoid receptors in patients with rheumatic diseases. J Rheumatol 2000;27(5):1265–70.
[7] Berki T, Tavakoli A, Nagy KK, Nagy G, Nemeth P. Alterations of glucocorticoid receptor expression during glucocorticoid hormone therapy in renal transplant patients. Transpl Int 2002;15(2–3):132–8.
[8] Gametchu B. Glucocorticoid receptor-like antigen in lymphoma cell membranes: correlation to cell lysis. Science 1987;236(4800):456–61.
[9] Bartholome B, Spies CM, Gaber T, Schuchmann S, Berki T, Kunkel D, et al. Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. FASEB J 2004;18(1):70–80.
[10] Buttgereit F, Krauss S, Brand MD. Methylprednisolone inhibits uptake of Ca^{2+} and Na^{+} ions into Con A stimulated thymocytes. J Biochem 1997;326:329–32.
[11] Buttgereit F, Brand MD, Müller M. Effects of methylprednisolone on the energy metabolism of quiescent and Con A-stimulated thymocytes of rats. Biosci Rep 1993;13:41–52.
[12] Schmid D, Burmester GR, Tripmacher R, Kuhnke A, Buttgereit F. Bioenergetics of human peripheral blood mononuclear cell metabolism in quiescent, activated and glucocorticoid-treated states. Biosci Rep 2000;20:289–302.
[13] Buttgereit F, Brand MD. A hierarchy of ATP-consuming processes in mammalian cells. J Biochem 1995;312:163–7.
[14] Rolfe DF, Brown GC. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol Rev 1997;77(3):731–58.
[15] Muller M, Siems W, Buttgereit F, Dumdey R, Rapoport SM. Quantification of ATP-producing and consuming processes of Ehrlich ascites tumour cells. Eur J Biochem 1986;161(3):701–5.
[16] Navarro S, Debili N, Bernaudin JF, Vainchenker W, Doly J. Regulation of the expression of IL-6 in human monocytes. J Immunol 1989;142(12):4339–45.
[17] Buttgereit F, Grant A, Müller M, Brand MD. The effects of methylprednisolone on oxidative phosphorylation in Con A-stimulated thymocytes: top down elasticity analysis and control analysis. Eur J Biochem 1994;223:513–9.
[18] Kuhnke A, Burmester GR, Krauss S, Buttgereit F. Bioenergetics of immune cells to assess rheumatic disease activity and efficacy of glucocorticoid treatment. Ann Rheum Dis 2003;62(2):133–9.

[19] Croxtall JD, van Hal PTW, Choudhury Q, Gilroy DW, Flower RJ. Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A549 cells. Br J Pharmacol 2002;135(2):511–9.
[20] Griffiths CE. Keeping psoriasis under wraps. J Dermatolog Treat 2001;12(3):133.
[21] Pfister R, Menz G. Glucocorticosteroid therapy in bronchial asthma. Pneumologie 1995;49(5):293–305.
[22] Cohan VL, Undem BJ, Fox CC, Adkinson Jr NF, Lichtenstein LM, Schleimer RP. Dexamethasone does not inhibit the release of mediators from human mast cells residing in airway, intestine, or skin. Am Rev Respir Dis 1989;140(4):951–4.
[23] Buttgereit F, Brink I, Thiele B, Burmester GR, Hiepe F, Hall ED. Effects of methylprednisolone and 21-aminosteroids on mitogen-induced interleukin-6 and tumor necrosis factor-alpha production in human peripheral blood mononuclear cells. J Pharmacol Exp Ther 1995;275(2):850–3.
[24] Gold R, Buttgereit F, Toyka KV. Mechanism of action of glucocorticosteroid hormones: possible implications for therapy of neuroimmunological disorders. J Neuroimmunol 2001;117(1–2):1–8.
[25] Ponec M, Kempenaar J, Shroot B, Caron JC. Glucocorticoids: binding affinity and lipophilicity. J Pharm Sci 1986;75(10):973–5.
[26] Koukouritaki SB, Margioris AN, Gravanis A, Hartig R, Stournaras C. Dexamethasone induces rapid actin assembly in human endometrial cells without affecting its synthesis. J Cell Biochem 1997;65(4):492–500.
[27] Adcock IM, Caramori G. Cross-talk between pro-inflammatory transcription factors and glucocorticoids. Immunol Cell Biol 2001;79(4):376–84.
[28] Seeto C, Namkung-Matthai H, Jayram S, Foe K, Brown KF, et al. Differential potency of beclomethasone esters in-vitro on human T-lymphocyte cytokine production and osteoblast activity. J Pharm Pharmacol 2000;52:417–23.
[29] Pörtner M, Möllmann H, Rohdewald P. Glucocorticoid receptors in human synovial tissue and relative receptor affinities of glucocorticoid-21-esters. Pharm Res 1988;5:623–7.
[30] Hammer S, Spika I, Sippl W, Jessen G, Kleuser B, Höltje HD, et al. Glucocorticoid receptor interactions with glucocorticoids: evaluation by molecular modeling and functional analysis of glucocorticoid receptor mutants. Steroids 2003;68:329–39.
[31] Hulkko SM, Wakui H, Zilliacus J. The pro-apoptotic protein death-associated protein 3 (DAP3) interacts with the glucocorticoid receptor and affects the receptor function. Biochem J 2000;349(Pt 3):885–93.
[32] Reichardt HM, Tuckermann JP, Bauer A, Schutz G. Molecular genetic dissection of glucocorticoid receptor function in vivo. Z Rheumatol 2000;59(Suppl. 2:II):1–5.
[33] Distelhorst CW. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. Cell Death Diff 2002;9:6–19.
[34] Schmidt J, Gold R, Schonrock L, Zettl UK, Hartung HP, Toyka KV. T cell apoptosis in situ in experimental autoimmune encephalomyelitis following methylprednisolone pulse therapy. Brain 2000;123(Pt 7):1431–41.
[35] Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, et al. DNA binding of the glucocorticoid receptor is not essential for survival. Cell 1998;93(4):531–41.
[36] Marchetti MC, Di Marco B, Cifone G, Migliorati G, Riccardi C. Dexamethasone-induced apoptosis of thymocytes: role of glucocorticoid receptor-associated Src kinase and caspase-8 activation. Blood 2003;101(2):585–93.
[37] Cifone MG, Migliorati G, Parroni R, Marchetti C, Millimaggi D, Santoni A, et al. Dexamethasone-induced thymocyte apoptosis: apoptotic signal involves the sequential activation of phosphoinositide-specific phospholipase C, acidic sphingomyelinase, and caspases. Blood 1999;93(7):2282–96.
[38] Diem R, Hobom M, Maier K, Weissert R, Storch MK, Meyer R, et al. Methylprednisolone increases neuronal apoptosis during autoimmune CNS inflammation by inhibition of an endogenous neuroprotective pathway. J Neurosci 2003;23(18):6993–7000.
[39] Leist M. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 1997;185:1481–6.
[40] Dallaporta B, Marchetti P, de Pablo MA, Maisse C, Duc HT, Metivier D, et al. Plasma membrane potential in thymocyte apoptosis. J Immunol 1999;162(11):6534–42.
[41] Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, et al. Mitochondrial permeability transition is a central coordinating event of apoptosis. J Exp Med 1996;184(3):1155–60.
[42] Gametchu B, Watson CS, Wu S. Use of receptor antibodies to demonstrate membrane glucocorticoid receptor in cells from human leucemic patients. FASEB 1993;7(13):1283–92.
[43] Sackey FN, Watson CS, Gametchu B. Cell cycle regulation of membrane glucocorticoid receptor in CCRF-CEM human ALL cells: correlation to apoptosis. Am J Physiol 1997;273(3 Pt 1):E571–83.