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Antiproliferative and Apoptotic Potencies of Glucocorticoids: Nonconcordance with Their Antiinflammatory and Immunossuppressive Properties

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ABSTRACT

Relative antiinflammatory and immunosuppressive potencies of glucocorticoids (GC) were previously well defined. Nonetheless, GC also regulate cell proliferation and programmed death (apoptosis). The aim of this study was to determine the relative potency of different GC on the modulation of cell survival. The GC-sensitive lymphoblast cell line CEM-c7/14 was submitted to 48h-exposure to GC (dose-response curve from 10^{-8} to 10^{-5}M). Cell survival was analyzed employing the DimethylTiazol-Tetrazolium (MTT) test. For each GC at least 4 experiments were performed in quadruplicate. Responses to different GC at the same molarity were analyzed by ANOVA on Ranks. Cell responses to the same GC in different concentrations were tested by repeated measures ANOVA. The EC50 for each GC was calculated with the GraphPad Prism 3.0 software. The use of low concentrations (10^{-8} and 10^{-7}M) of hydrocortisone and methylprednisolone determined a similar effects on cell survival, which was less prominent than that observed with betamethasone, budesonide or momethasone. Momethasone was the most potent GC, inducing the most intense dexamethasone reduction on cell survival at the lowest concentration (10^{-8}M). Momethasone and methylprednisolone were the two GC with the strongest impact on cell survival. Our findings suggest that antiproliferative and apoptotic potencies of GC are different from those previously reported antiinflammatory and immunosuppressive actions. **(Arq Bras Endocrinol Metab 2005;49/3:378-383)**

Keywords: Glucocorticoid; Cell survival; Cell death; Apoptosis

RESUMO

Potências Antiproliferativa e Pró-Apoptótica dos Glicocorticóides: Discordância com as Propriedades Anti-inflamatórias e Imunossupressoras.
As potências antiinflamatória e imunossupressora dos glicocorticóides (GC) já foram bem estabelecidas previamente. No entanto, os GC também possuem atividade reguladora da proliferação celular e da morte celular programada (apoptose). O objetivo deste estudo foi determinar a potência relativa de diferentes GC na modulação da sobrevida celular. Linfoblastos cortico-sensíveis (linhagem celular CEM-C7/14) foram mantidos em cultura prolongada e submetidos ao tratamento com GC por 48h, em doses variando entre 10^{-8} e 10^{-5} molar. O índice de sobrevida celular foi quantificado pelo teste MTT (DimetilTiazol-Tetrezolium). Para cada GC avaliado, foram realizados pelo menos quatro experimentos em quadruplicata. A resposta celular aos diferentes GC foi analisada através do teste estatístico *ANOVA on Ranks*, enquanto a resposta ao mesmo GC em concentrações diferentes foi analisada pelo teste *ANOVA for repeated measures*. O EC50 de cada GC foi calculado utilizando-se o software GraphPad Prism 3.0. Durante o uso de concentrações baixas (10^{-8} e 10^{-7} molar), observou-se sobrevida semelhante dos linfoblastos após tratamento com hidrocortisona ou metilprednisolona. Nestas mesmas concentrações baixas, a sobrevida celular foi menor quando utilizou-se dexametasona, betametasona, budesonida ou mometasona. A mometasona e a metilprednisolona

foram os dois GC que determinaram maior redução da sobrevida linfoblástica. Nossos resultados sugerem que as potências antiproliferativa e pró-apoptótica dos GC sejam diferentes dos efeitos antiinflamatórios e imunossupressores previamente estabelecidos para estes GC. **(Arq Bras Endocrinol Metab 2005;49/3:378-383)**

Descritores: Glicocorticóide; Sobrevida celular; Morte celular; Apoptose

CORTISOL, THE ENDOGENOUS GLUCOCORTICOID (GC), is secreted basally and during stress and modulates the amplitude of defensive responses. Cortisol and a variety of synthetic glucocorticoid agonists are able to control carbohydrate, protein and lipid metabolism, and to regulate immune and cardiovascular functions (1,2). GC suppress innate inflammatory responses, as well the cellular immunity (3). One of the major effects of GC is their ability to exert anti-proliferative and apoptotic actions both *in vivo* as *in vitro* cell culture (4). Glucocorticoid-induced apoptosis is an active, ATP-dependent phenomenon characterized by cellular and mitochondrial membrane changes, and alterations in calcium and potassium compartmental distributions (5). Programmed cell death depends on the activation of nuclear proteases, generating DNA, RNA and protein fragmentation, genomic instability and failure of DNA repair. The antiproliferative and apoptotic actions of glucocorticoids mediate their therapeutic effects in several autoimmune and lymphoproliferative diseases.

Cell survival can be measured by the ability of live cells to metabolize MTT, a yellow tetrazolic salt, to its dark violet crystal product formazan. This conversion occurs after active enzymatic cleavage at the mitochondrial level, and the measurement of the final product can be used as a quantitative assay, reflecting the cell viability (5). Relative glucocorticoid potencies are well established for their anti-inflammatory and immunosuppressive effects. On the other hand, the pathways related to modulation of cell survival and death are unique, requiring additional studies to determine the relative potencies of new synthetic glucocorticoids. In this study, we compared the relative antiproliferative and apoptotic potencies of hydrocortisone against several other synthetic glucocorticoids.

MATERIALS AND METHODS

A GC stock-solution was prepared by diluting GC salts in absolute-ethanol to obtain a final concentration of 10^{-2}M. Working-solutions were obtained by subsequent dilution of the stock-solution, 1:9 in RPMI-1640 (GIBCO BRL Cat # 11875-093).

The cell line CEM-c7/14, derived from a patient with a glucocorticoid-sensitive lymphoblastic leukemia, was kindly offered by Dr. E. B. Thompson (the University of Texas Medical Branch at Galvestone, TX, USA). The cells were kept at growing phase in RPMI-1640 supplemented with 10% FBS (Fetal Bovine Serum, GIBCO BRL Cat # 16140-071) and 1% penicillin/ streptomycin. Cell culture was maintained at 5% pCO_2 and 37°C.

Cell viability was established in haemocytometer in a 1:1 solution of trypan blue (Sigma, Cat # T0776), resuspended to $4x10^6$ viable cells/mL, and cultured in quadruplicate in a 24-well microplate (Fisher, Cat # 07-200-84). The first well of the assay-plate received only RPMI-1640 medium, and into the subsequent wells it was applied cells without glucocorticoid and cells plus glucocorticoid in increasing final molar concentration ranging from 10^{-8} to 10^{-5} M. To achieve the final glucocorticoid concentration, 5µL of glucocorticoid was added to each well (e.g., 5µL of 10^{-3}M to 500µL of cell suspension to obtain a 10^{-5}M final concentration). At least four experiments in quadruplicate were performed for each glucocorticoid.

After a 48h-incubation period 100mL of MTT (dimethyl-Tiazol-Tetrazolium, Sigma Cat # M-2128) solution (5mg/mL) was added to each well and incubated for an additional 4h-incubation period at the same conditions previously described, to allow the MTT conversion into formazam. Dissolution of formazam-crystals was achieved in 3 volumes (1800mL) of Isopropanol-HCl (23:2) solution (Sigma, Cat # I-9516 and Merck, Cat # 100983, respectively). An aliquot of 200µL was transferred in duplicate to a 96-well microplate (Fisher, Cat # 07-200-89), with subsequent determination of the optical density (OD) of the solution at 595nm (Universal Microplate Reader Elx800, Bio-Tek Instruments, Inc, USA). These OD measured values are directly dependent on the number of alive cells. For each plate, the blank-background was represented by the OD values observed in the medium-only well. The maximum cell growth for each experiment was represented by the values observed in the wells containing cells plus RPMI-1640 without the addition of glucocorticoids and expressed as the 100% cell viability for that plate-assay.

Cell survival under GC therapy with a progressive molar concentration was expressed in percentage as a function of the maximum number of cells observed for each plate.

Statistical analysis employed the SigmaStat 2.03 software (SPSS, Inc.). Comparison of the same glucocorticoid at different molarities was performed applying the Friedman test, ANOVA for repeated measures. When significant difference was detected, the All Pairwise Multiple Comparison Procedures – Tukey Test was used to recognize each different pair concentration. For comparison among different GC at the same molarity the Kruskal-Wallis – ANOVA on ranks test was performed, followed by the All Pairwise Multiple Comparison Procedures – Dunn's Method to verify the difference among two different glucocorticoids. The EC50 was calculated employing GraphPad Prism 3.0 software.

RESULTS

The most characteristic patterns observed for all tested glucocorticoids are shown in tables 1 and 2. Hydrocortisone decreased cell survival in molar concentrations ≥ 10^{-7}M, with the maximum effect at 10^{-5}M. The same pattern of cell survival reduction was observed with methylprednisolone, but the final effect was higher than the one observed with hydrocortisone (reduction of 60.9% and 36.7%, respectively). Dexamethasone decreased cell viability at molar concentrations ≥ 10^{-8}M, with maximum effect at 10^{-5}M (49.6% of cell reduction). Betamethasone, budesonide and momethasone showed a pattern similar to that observed for dexamethasone. In a molar concentration as low as 10^{-8}M, these three GC had an effect equivalent to that observed at 10^{-5}M, and the cell survival was significantly lower than that observed for dexamethasone treated cells at 10^{-8}M.

Comparing hydrocortisone to synthetic glucocorticoids at the concentration of 10^{-8}M, we observed that all GC, but methylprednisolone, had significantly higher potency in decreasing cell survival.

The GC concentration necessary to obtain 50% of the maximal effect (EC50) was between 10^{-7} M to 10^{-6}M for hydrocortisone, 10^{-7}M for methylpred-

Table 1. Percentage of live cells expressed as mean (SD) after treatment with glucocorticoid for 48 hours.

Glucocorticoid	GC (-)	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
Hydrocortisone (n= 24)	99.5 (5.0)	93.9 (7.6)	90.5 (5.9)[a]	68.3 (10.0)[a]	62.8 (10.3)[a]
Methylprednisolone (n= 24)	105.1 (10.1)	102.1 (8.8)	76.0 (17.5)[a]	49.5 (8.0)[a]	44.2 (7.0)[a,b]
Dexamethasone (n= 40)	98.0 (9.4)	76.3 (11.0)[a]	55.7 (7.7)[a]	48.8 (8.2)[a]	48.4 (10.4)[a,b]
Betamethasone (n= 24)	104.6 (7.7)	59.4 (2.9)[a,c]	55.6 (2.3)[a]	51.6 (3.0)[a]	50.7 (8.2)[a,b]
Budesonide (n= 16)	99.4 (5.8)	66.2 (5.0)[a,c]	65.5 (7.0)[a]	54.0 (14.9)[a]	53.3 (21.0)[a]
Momethasone (n= 38)	98.3 (6.7)	45.4 (2.2)[a,c]	39.9 (13.1)[a]	39.7 (13.0)[a]	39.5 (11.3)[a]

* corticosensitive lymphoblasts (c7/14 cell line); GC(-): maximal cell survival without glucocorticoid; M= molar;
n= total number of point-experiments performed for each glucocorticoid
[a]: significant reduction when compared to basal values, p< 0.05 (Anova Repeated Measures)
[b]: significant reduction when compared to hydrocortisone 10^{-5}M, p< 0.05 (Kruskal-Wallis – Anova on Ranks test)
[c]: significant reduction when compared to dexamethasone 10^{-8}M, p< 0.05 (Kruskal-Wallis – Anova on Ranks test)

Table 2. Major pharmacologic characteristics of glucocorticoids regarding their proapoptotic properties.

Glucocorticoid	Start Effect	Max Effect	EC50	Max Cell reduction
Hydrocortisone	10^{-7}M	10^{-6}M	5 x 10^{-7}M	37%
Methylprednisolone	10^{-7}M	10^{-6}M	10^{-7}M	61%
Dexamethasone	10^{-7}M	10^{-6}M	5 x 10^{-8}M	50%
Betamethasone	10^{-8}M	10^{-6}M	< 10^{-8}M	54%
Budesonide	10^{-8}M	10^{-8}M	< 10^{-8}M	46%
Momethasone	10^{-8}M	10^{-8}M	< 10^{-8}M	59%

Start effect: minimal glucocorticoid concentration to start significant reduction on cell survival
Max effect: minimal glucocorticoid concentration able to determine a maximal reduction on cell survival
EC50: Concentration at which 50% of the glucocorticoid effect was observed
Max cell reduction: maximal cell reduction (in percentage) related to basal nontreated cells

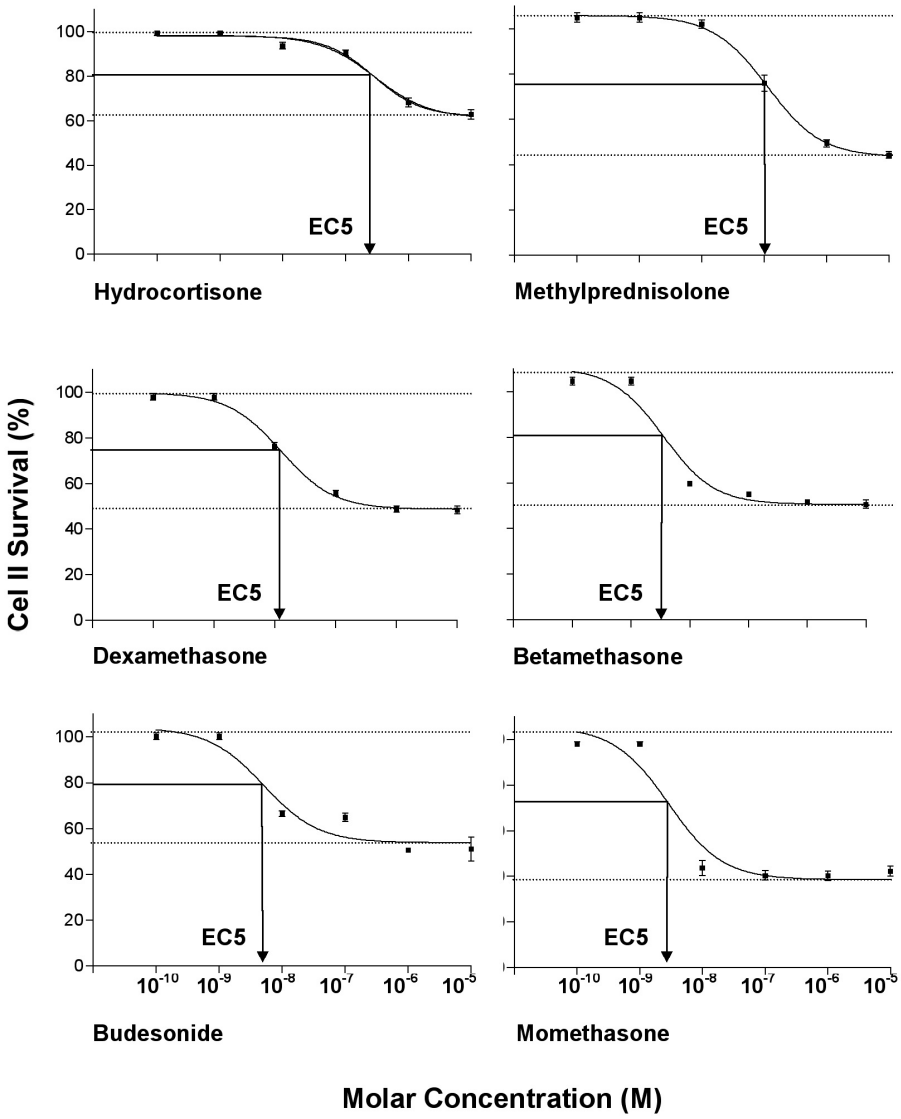

Figure 1. Dose response curve of cell survival after 48h of glucocorticoid treatment.

nisolone, between 10^{-8}M to 10^{-7}M for dexamethasone, and smaller than 10^{-8}M for betamethasone, budesonide and momethasone (figure 1).

DISCUSSION

Glucocorticoids have specific biologic effects in several organ systems, depending on their pharmacokinetic characteristics and inherent actions exerted through their specific nuclear receptors (GR). Chemical changes in cortisol molecule can enhance glucocorticoid or mineralocorticoid activities, determining improved therapeutic properties and decreased side effects. Anti-inflammatory potencies have been defined in studies based on *in vivo* and *in vitro* methods. Relative anti-inflammatory potencies were previously reported for hydrocortisone, prednisolone and dexamethasone regarding inhibition of lymphocytes when stimulated by phytohemaglutinine (6). The anti-inflammatory effect was also related to other adrenal and gonadal steroids (7). It was observed that dexamethasone had the highest anti-inflammatory potency compared to hydrocortisone and prednisolone. Other studies have compared the relative potency of GC to inhibit the formation of granulomatous lesions, to

exert thymolytic actions (8), and to inhibit skin fibroblat growth rate (9).

There is a considerable variation between these previously described potencies. Additionally, just a small number of synthetic GCs were compared by the same technique, preventing direct comparison of the anti-inflammatory potencies among different glucocorticoids. Limited information is also available for comparison among recently synthesized novel glucocorticoids. Glucocorticoid receptor binding capability can be detected by radioreceptor-assay, and this characteristic has been correlated to the anti-inflammatory potency of these steroids. Using this method, anti-inflammatory potency observed for methylprednisolone, dexamethasone and betamethasone were considered higher than that established by other methods (10).

Studies evaluating GC antiproliferative effects are even more scarce. Using an MTT assay to compare prednisolone and dexamethasone on its relative antileukemic activity, a 16-fold higher potency was observed for dexamethasone (11). Another study evaluated the relative cytotoxicity of these two glucocorticoids by flow-cytometric analysis of cells from patients with acute lymphoblastic leukemia, and the authors concluded that dexamethasone had a cytotoxic activity five to six times higher than prednisolone (12). Despite the existing data evaluating and comparing the antiproliferative and apoptotic GC actions, these studies usually compare only two glucocorticoids (dexamethasone and prednisolone).

In this study, the relative potency of eight different glucocorticoids were compared regarding their antiproliferative and apoptotic activity, by examining cell survival. We described GC potencies considering the minimal glucocorticoid concentration able to start its effect on cell survival, the concentration at which the maximal reduction was obtained, the concentration at which 50% of the maximal effect was detected (EC50), and the maximal cell reduction observed after 48h of steroid therapy. This is the first report comparing multiple glucocorticoids in their effects or cell survival. As a group, hydrocortisone, methylprednisolone and dexamethasone started their anti-proliferative and apoptotic effect at "physiological" concentrations (10^{-7}M). The same effect was observed with budesonide and momethasone but at a 10-times smaller concentration. Momethasone was the GC able to induce the grater reduction on cell number and to require the smallest dose to start its effects.

We observed in this study, employing different glucocorticoids, that betamethasone, budesonide and momethasone have their EC50 at similar levels and under the 10^{-8} molar concentration, suggesting that further studies should evaluate even smaller doses of these compounds.

The discrepancy between anti-inflammatory and the cell proliferation and apoptotic potencies observed in this study are potentially related to the unique pathways involved in cell cycle control and apoptosis, different from pathways activated during inflammation. These discrepancies on relative glucocorticoid potency suggest that, if the regulation of cell number is the major target of therapy, specific dosage and type of glucocorticoid should be titrated for this specific effect. Future studies should determine the relative potency of new synthetic glucocorticoids and establish these effects for even smaller concentrations.

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