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Glucocorticosteroids and in vitro effects on chemiluminescence of isolated bovine blood granulocytes

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Abstract

The effects of glucocorticosteroids on respiratory burst of bovine granulocytes were studied in vitro by means of (1) chemiluminescence (luminol-dependent, phorbol 12-myristate 13-acetate (PMA)-stimulated), (2) a cell-free chemiluminescence assay, and (3) a myeloperoxidase assay. Significant effects on cellular chemiluminescence were only observed at the highest, not obtainable in vivo, concentration for all drugs except betamethasone. Prednisolone induced inhibition at therapeutic doses. Also, flumethasone and dexamethasone induced significant inhibition at lower concentrations. In the cell-free assay, all glucocorticosteroids, except betamethasone, inhibited chemiluminescence at high concentrations. None of the glucocorticosteroids tested affected myeloperoxidase activity. The results indicated that the drugs do not affect NADPH-oxidase activity. The adverse effects may be due to scavenging of free oxygen radicals, or to interference with the interaction between luminol and the myeloperoxidase–H_2O_2–halide system. It can be concluded that most glucocorticosteroids show no adverse effects on the respiratory burst of bovine granulocytes in vitro at therapeutical concentrations.

Keywords: Glucocorticosteroid; Polymorphonuclear leukocyte; Respiratory burst

1. Introduction

Glucocorticosteroids are very well-known anti-inflammatory agents. Because of this feature, glucocorticosteroids are widely used in the treatment of a variety of inflammatory processes such as acute E. coli mastitis in periparturient cows. Glucocorticosteroids are administered locally in order to reduce swelling and edema, and systemically to reduce symptoms of endotoxic shock (Phillips et al., 1987). However, both endogenous (e.g., cortisol) and exogenous glucocorticosteroids can suppress several functions of the immune system and affect the formation, tissue distribution and function of immune cells such as polymorphonuclear leukocytes (Cupps and Fauci, 1982; Phillips et al., 1987; Munck and Guyre, 1991). There are a number of contradictory reports on the influence of glucocorticosteroids on the respiratory burst activity of polymorphonuclear leukocytes in bovine (cows) and other species (Phillips et al., 1987). These studies were done with various techniques. One of these techniques was chemiluminescence, induced after phagocytosis of opsonized zymosan particles or bacteria, or after stimulation of the polymorphonuclear leukocytes by phorbol 12-myristate 13-acetate (PMA). Although these techniques yield different results, they are very useful to study the respiratory burst activity of polymorphonuclear leukocytes (Allen et al., 1972; Allen and Loose, 1976). Only a few papers reported on other techniques used to investigate subcellular interactions between glucocorticosteroids and polymorphonuclear leukocytes. These additional techniques, such as the cytochrome *c* reduction test, may be very helpful to elucidate the mechanisms of drug interaction with the respiratory burst cascade of the polymorphonuclear leukocytes. Because the efficacy of polymorphonuclear leukocytes against bacterial invasion is highly dependent on their reactive oxygen species-generating capacity (Heyneman et al., 1990), because highly conflicting results concerning the effect of glucocorticosteroids on chemiluminescence in different species have been reported, and because of the lack of more fundamental research concerning bovine species on

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how and at what point glucocorticosteroids interact with the respiratory burst cascade of bovine polymorphonuclear leukocytes, the purpose of this study was to evaluate the effects of several commonly used glucocorticosteroids on the respiratory burst activity of bovine blood polymorphonuclear leukocytes in vitro. For this purpose, three assays were used: PMA-induced chemiluminescence, chemiluminescence in a cell-free system, and a myeloperoxidase (EC 1.11.1.7.) assay.

2. Material and methods

2.1. Animals

Nine high-yielding Holstein cows were selected from the Dairy herd of the University of Ghent. The cows were of different ages and were completely healthy. At the time of the experiment, the animals were in early lactation (2–5 weeks post partum).

2.2. Drugs

The effect of eight glucocorticosteroids on chemiluminescence was investigated. All drugs were dissolved in dimethyl sulphoxide (DMSO). The drugs were cortisone, hydrocortisone (98%), prednisolone, 6α-methylprednisolone, dexamethasone, betamethasone-21-acetate, flumethasone, and triamcinolone-acetonide. All drugs were tested at 5 final concentrations, i.e., from 2×10^{-8} M up to 2×10^{-4} M, except betamethasone and triamcinolone. These two glucocorticosteroids were tested at concentrations from 2×10^{-8} to 2×10^{-5} M. A higher concentration (2×10^{-4} M) was not used because of solubility problems. Dilutions were made by adding Dulbecco's phosphate buffered saline solution (DPBS) (Gibco BRL, Life Technologies, Gaithersburg, MD, USA). All drugs were provided by Sigma (St. Louis, MO, USA).

2.3. Isolation of polymorphonuclear leukocytes from blood

Blood (40 ml) was aseptically collected from the external jugular vein with evacuated tubes containing lithium heparin as anticoagulant (Laboratoire EGA, F-28210 Nogent-le Roi, France). The polymorphonuclear leukocytes were isolated according to a method described previously (Hoeben et al., 1997a,b). Briefly, after removal of the plasma layer, the buffy-coat and the top layer of the packed red cells were discarded. After washing with 0.9% NaCl, the remaining red blood cells were lysed by adding double distilled water and gently mixing the suspension for one min. After restoration of isotonicity by the addition of 2.7% NaCl and washing, the final cell pellet was resuspended in 1 ml of DPBS. After isolation, the cells were counted with an electronic cell counter (Coulter Counter ZF, Coulter Electronics, Luton LU3 3RH, UK). The viability of the polymorphonuclear leukocytes immediately after isolation was determined by trypan blue dye exclusion and differential cell counts were performed on eosin–giemsa-stained smears (Hemacolor®, E. Merck, D-64271 Darmstadt, Germany). On the average, 95% of the isolated cells were polymorphonuclear leukocytes with a viability of 97%. After counting and differentiation of the cells, cell suspensions were adjusted to a concentration of 5×10^6 viable polymorphonuclear leukocytes per milliliter and stored on ice until use.

2.4. Chemiluminescence assay

Luminol-dependent cellular chemiluminescence was used to measure the respiratory burst activity of isolated polymorphonuclear leukocytes. A stock solution (1×10^{-1} M in DMSO) of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Sigma) was stored at −20°C, and immediately before use, was thawed and diluted to a concentration of 1×10^{-3} M with DPBS. The respiratory burst of the isolated bovine polymorphonuclear leukocytes was activated by phorbol 12-myristate 13-acetate (PMA) (Sigma; 99%). A stock solution was prepared by dissolving 200 μg of PMA in 1 ml of DMSO, and stored at −20°C. The chemiluminescence assay was performed at 37°C with a microtiterplate luminometer (LB96P, EG and G Berthold, D-75312 Bad Wildbad, Germany). The total volume used in the wells was 200 μl. The cell suspension, with a final concentration of 2×10^6 cells/ml, was preincubated with medium (DPBS) and 20 μl of the drugs for 30 min at 37°C. After this incubation, 0.5 mM luminol and 200 ng/ml of PMA were added and the chemiluminescence reaction was immediately recorded in duplicate for 30 min. The total DMSO concentration in the incubation mixture was approximately 0.8%. Preliminary studies showed no effect of this concentration on chemiluminescence. A chemiluminescence index was calculated by dividing the area under the curve (AUC) of the glucocorticosteroid-incubated polymorphonuclear leukocytes by the AUC of the control polymorphonuclear leukocytes and multiplying by 100. This AUC was calculated over a time period of 30 min.

2.5. Effects of glucocorticosteroids on chemiluminescence in a cell-free system

The chemiluminescence in a cell-free system was measured in quadruplicate according to the method of Briheim and Dahlgren (1987) as modified by Hoeben et al. (1997a,b). The supernatant (25 μl) of sonicated polymorphonuclear leukocytes from one cow, 0.1 mM of luminol and 0.1 mM of H_2O_2 was incubated with the different concentrations of the glucocorticosteroids or DPBS, and the chemiluminescence response was measured for 30 min at 37°C with a microtiterplate luminometer (LB 96P, EG and G Berthold, D-75312 Bad Wildbad, Germany). The

Table 1
Components of the assays used in these experiments

Cellular assay	Cell-free assay	Myeloperoxidase assay
active NADPH-oxidase	inactive NADPH-oxidase	inactive NADPH-oxidase
active myeloperoxidase	active myeloperoxidase	active myeloperoxidase
chemiluminescence	chemiluminescence	no chemiluminescence

isolated polymorphonuclear leukocytes were sonicated according to the method previously described by Hoeben et al. (1997a,b).

2.6. Effect of glucocorticosteroids on myeloperoxidase activity

The effect of the glucocorticosteroids on myeloperoxidase activity was measured in triplicate according to the method previously described by Somersalo et al. (1990) and as modified by Hoeben et al. (1997a,b) as the oxidation of *ortho*-dianisidine-di-hydrochloride by a neutrophil extract containing H_2O_2 in the presence or absence of the drugs. Sonicated cells (25 μl of supernatant after sonication) from one cow, DPBS and drugs (20 μl) were incubated at 37°C for 5 min. After incubation, 0.1 mM of H_2O_2 and 0.8 mM of *ortho*-dianisidine-di-hydrochloride were added and absorption was measured in a spectrophotometer (Multiskan Plus Type 314, Labsystems Oy, 00881 Helsinki, Finland) at 450 nm.

2.7. Experimental design

The experimental design is presented in Table 1. In chemiluminescence, three aspects can be distinguished: (1) NADPH-oxidase activity, (2) myeloperoxidase activity, and (3) the interaction between luminol and hypochlorite. Our experimental design included three assays: (1) the cellular assay with viable cells, (2) the cell-free assay with the supernatants of sonicated cells, and (3) the myeloperoxidase assay.

In the cellular assay, NADPH-oxidase and myeloperoxidase are active and luminol reacts with hypochlorite to yield chemiluminescence (Table 1). Consequently, an effect in the cellular assay can be due to an effect on one, two or three of these components or to scavenging of oxygen radicals. In the cell-free assay, NADPH-oxidase is inactive, myeloperoxidase is active and there is again an interaction of hypochlorite with luminol. Consequently, an effect in the cell-free assay can be due to an effect on myeloperoxidase activity, to an effect on the interaction between hypochlorite and luminol, or to scavenging effects on hypochlorite, but not to an effect on NADPH-oxidase. In the myeloperoxidase assay, NADPH-oxidase is absent, there is an active myeloperoxidase, but no interaction of hypochlorite with luminol. As a consequence, an effect in the myeloperoxidase assay can be due to an effect on myeloperoxidase itself or to scavenging of hypochlorite, but not to interference with the interaction between luminol and hypochlorite.

As shown in Table 2 (example 1), the combination of inhibition in the cellular assay and in the cell-free assay, but no effect in the myeloperoxidase assay, indicates inhibition of the cellular chemiluminescence which is not due to an effect on either myeloperoxidase or NADPH-oxidase, but to an effect on the interaction between luminol and hypochlorite or scavenging of hypochlorite. Inhibition in the cellular assay, no effect or a smaller effect in the cell-free assay, and no effect in the myeloperoxidase assay (example 2) indicates that inhibition in the cellular assay is due to an effect on neither the myeloperoxidase activity nor on the interaction between hypochlorite and luminol, but to an effect on NADPH-oxidase activity. Scavenging of peroxide or superoxide is also a possibility.

2.8. Statistical analyses

Statistical analysis of the data from the chemiluminescence assay was performed for each glucocorticosteroid by using a two-way analysis of variance (Snedecor and Cohran, 1967) with the concentration of the drugs as a fixed factor, the cows ($n = 9$) as a randomized factor and their interaction term as the error term. Means were compared using the method of least significant differences (Snedecor and Cohran, 1967). Logarithmic transformation of the values for the chemiluminescence data calculated from the area under the curves was used. Bartlett's test of equal variances was used to study the equality of variances of the different groups. The results of this test allowed us to apply the analysis of variance.

Table 2
Possible combinations of results observed in this experiment

Cellular assay	Cell-free assay	Myeloperoxidase assay	Explanation
(1) inhibition	inhibition	no effect	interaction with chemiluminescence assay
(2) inhibition	no effect	no effect	interaction with NADPH-oxidase

Statistical analyses of the data from the cell-free assay ($n = 4$) and the myeloperoxidase assay ($n = 3$) were performed for each glucocorticosteroid by a one-way analysis of variance. Statistical analyses were done after a logarithmic transformation of the data from the different assays. Means were compared using the method of least significant differences. Bartlett's test of equal variances was used to study the equality of variances. The Statistix program package (v. 4.0, 1992, Analytical Software, Tallahassee, FL 32317-2185, USA) was used. Significance of differences was accepted at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

3. Results

3.1. Effect of glucocorticosteroids on cellular chemiluminescence

All drugs reduced the chemiluminescence reaction at their highest concentration (Fig. 1A and B), except betamethasone. Methylprednisolone significantly ($P < 0.001$) reduced the chemiluminescence response by 18% at a concentration of 2×10^{-4} M. Cortisone ($P < 0.05$) and hydrocortisone ($P < 0.01$) significantly decreased the chemiluminescence response of the polymorphonuclear leukocytes by 7 and 13%, respectively, at the concentration of 2×10^{-4} M. Triamcinolone at its highest concentration (2×10^{-5} M) also showed significant ($P < 0.01$) inhibitory effects, about 10%, on the reaction. As mentioned before, betamethasone showed no significant effect on the chemiluminescence response of bovine blood polymorphonuclear leukocytes. Flumethasone decreased the chemiluminescence response at a concentration of 2×10^{-6} M by 8% ($P < 0.05$), at 2×10^{-5} M by 13% ($P < 0.01$) and at 2×10^{-4} M by 13 to 14% ($P < 0.001$). Prednisolone induced the most pronounced effects. It reduced the chemiluminescence response of the bovine polymorphonuclear leukocytes significantly ($P < 0.001$) at the three highest concentrations (2×10^{-6} M to 2×10^{-4} M), causing 15, 23, and 25% inhibition. Dexamethasone decreased the chemiluminescence response by 13% at a concentration of 2×10^{-5} M ($P < 0.001$) and by 9% at a concentration of 2×10^{-4} M ($P < 0.05$). No significant differences could be observed between control samples and glucocorticosteroid-incubated samples for the slopes of the chemiluminescence response and the time-to-peak values.

3.2. Effect of glucocorticosteroids on chemiluminescence of a cell-free system

Betamethasone was the only drug which had no effect on chemiluminescence of a cell-free system (Fig. 2A and B). Hydrocortisone and methylprednisolone induced significant ($P < 0.001$) inhibitory effects, 10 and 28%, respectively, at the concentration of 2×10^{-4} M. Dexametha-

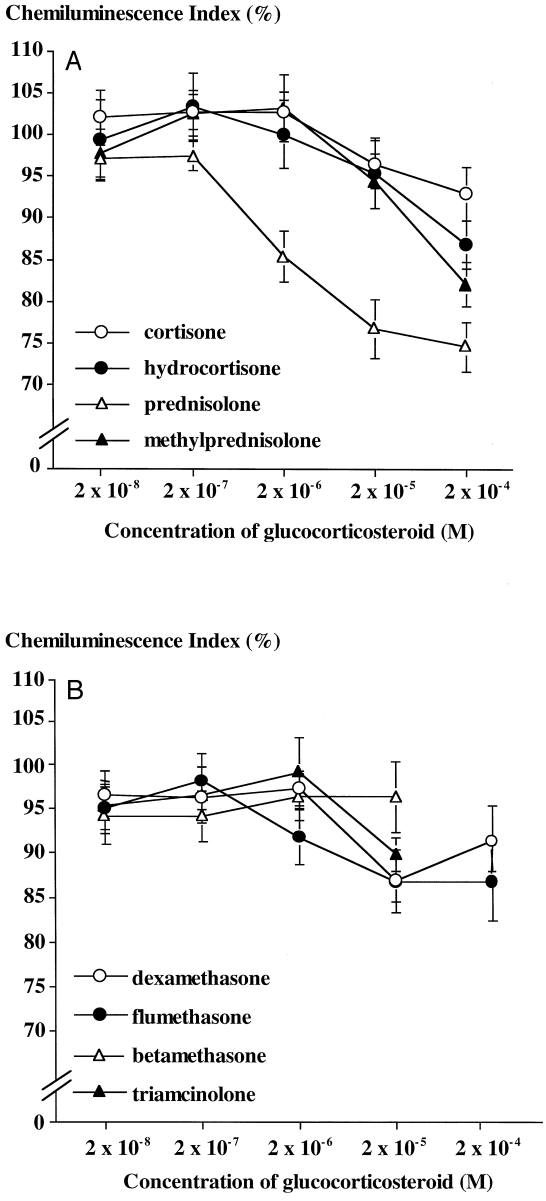

Fig. 1. (A) Effect of different concentrations of cortisone, hydrocortisone, prednisolone, and methylprednisolone on cellular chemiluminescence of bovine polymorphonuclear leukocytes. Chemiluminescence index = [(area under the curve of the drug-incubated cells)/(area under the curve of the vehicle-treated cells)] × 100. Data are means from nine cows ± S.E.M. Means of the absolute values (RLU/s) of the control samples from 9 cows ± S.E.M. were: 568 111 ± 64 962 for cortisone; 471 344 ± 67 843 for hydrocortisone; 377 433 ± 45 626 for prednisolone; and 476 611 ± 61 232 for methylprednisolone. (B) Effect of different concentrations of dexamethasone, flumethasone, betamethasone, and triamcinolone on cellular chemiluminescence of bovine polymorphonuclear leukocytes. Chemiluminescence index = [(area under the curve of the drug-incubated cells)/(area under the curve of the vehicle-treated cells)] × 100. Data are means from nine cows ± S.E.M. Means of the absolute values (RLU/s) of the control samples from nine cows ± S.E.M. were: 521 344 ± 92 699 for dexamethasone; 488 455 ± 106 720 for betamethasone; 385 511 ± 68 138 for flumethasone; and 483 467 ± 66 515 for triamcinolone. RLU, Relative Light Units per second.

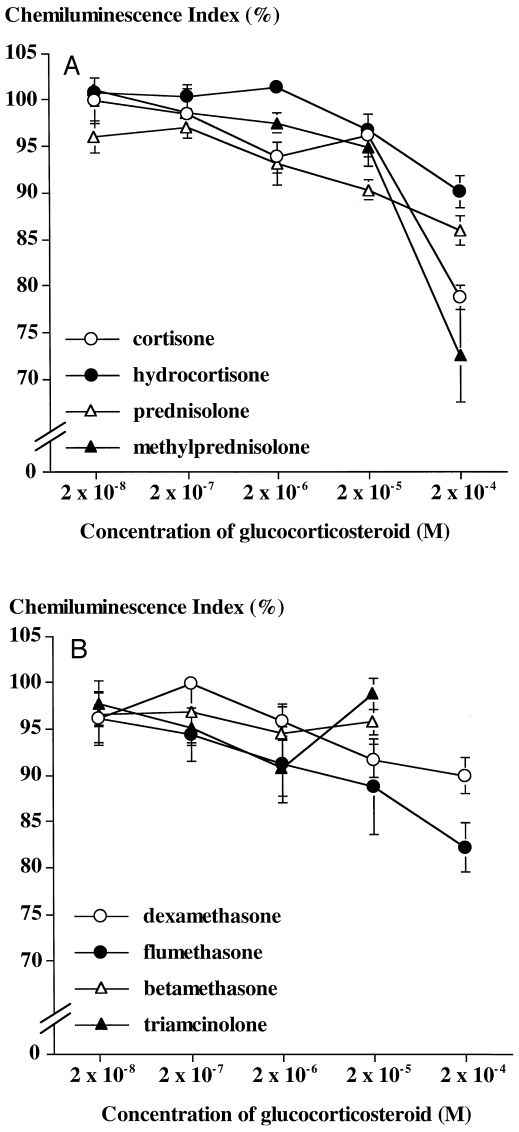

Fig. 2. (A) Effect of different concentrations of cortisone, hydrocortisone, prednisolone, and methylprednisolone on cell-free chemiluminescence of bovine polymorphonuclear leukocytes. Chemiluminescence index = [(area under the curve of the drug-incubated cells)/(area under the curve of the vehicle-treated cells)] × 100. Data are means of four measurements ± S.E.M. Means of the absolute values (RLU/s) of the four control samples ± S.E.M. were: 2 495 250 ± 299 175 for cortisone; 2 416 250 ± 257 956 for hydrocortisone; 2 442 250 ± 263 722 for prednisolone; and 2 330 250 ± 257 217 for methylprednisolone. (B) Effect of different concentrations of dexamethasone, flumethasone, betamethasone, and triamcinolone on cell-free chemiluminescence of bovine polymorphonuclear leukocytes. Chemiluminescence index = [(area under the curve of the drug-incubated cells)/(area under the curve of the vehicle-treated cells)] × 100. Data are means of four measurements ± S.E.M. Means of the absolute values (RLU/s) of the four control samples ± S.E.M. were: 2 337 500 ± 272 247 for dexamethasone; 2 130 500 ± 221 618 for betamethasone; 2 432 750 ± 183 475 for flumethasone; and 2 124 250 ± 175 464 for triamcinolone. RLU, Relative Light Units per second.

sone reduced chemiluminescence by 8% at 2×10^{-5} M ($P < 0.01$) and by 10% at a concentration of 2×10^{-4} M ($P < 0.001$). Flumethasone also had significant inhibitory effects at the same concentrations, 11% ($P < 0.05$) and 18% ($P < 0.01$), respectively. Prednisolone showed inhibitory effects at concentrations of 2×10^{-6} M ($P < 0.01$, 7%), at 2×10^{-5} M ($P < 0.001$, 10%), and at 2×10^{-4} M ($P < 0.001$, 14%). Cortisone also induced significant effects at these concentrations: 6% inhibition at concentrations of 2×10^{-6} and 2×10^{-5} M ($P < 0.05$), and 21% at a concentration of 2×10^{-4} M ($P < 0.001$). At a concentration of 2×10^{-6} M, triamcinolone significantly reduced by 9% the chemiluminescence response of the cell-free assay ($P < 0.01$).

3.3. Effect of glucocorticosteroids on myeloperoxidase-activity

None of the glucocorticosteroids tested showed a significant inhibitory or stimulatory effect in the myeloperoxidase assay. The absolute values of the data for the control samples with their S.E.M. were: 0.315 ± 0.003 for cortisone and hydrocortisone; 0.313 ± 0.004 for prednisolone and methylprednisolone; 0.326 ± 0.003 for betamethasone and triamcinolone; and 0.331 ± 0.003 for dexamethasone and flumetasone.

4. Discussion

Luminol-dependent, PMA-induced chemiluminescence of polymorphonuclear leukocytes is a good indicator of the production of reactive oxygen species such as H_2O_2 by these cells (Allen et al., 1972; Allen and Loose, 1976). The soluble stimulant, PMA, activates the cells directly by activation of the protein kinase C, without being phagocytised. This allows the study of effects of drugs on burst activity directly, without interference by phagocytosis. Chemiluminescence in a cell-free system with the supernatant of sonicated polymorphonuclear leukocytes provides information as to whether or not the drugs affect the key enzyme of the respiratory burst, i.e., NADPH-oxidase. The myeloperoxidase assay was performed to find whether drugs have an effect on this enzyme and whether they scavenge reactive oxygen species such as H_2O_2 and OCl^-.

Betamethasone was the only glucocorticosteroid that showed no significant effect on the chemiluminescence of bovine polymorphonuclear leukocytes, which was in contrast with the results of Niwa et al. (1987). As with all other drugs, no effect on myeloperoxidase activity was observed. Glucocorticosteroids showed no effect on the degranulation of myeloperoxidase (Roshol et al., 1995).

Cortisone and methylprednisolone induced more pronounced effects in the cell-free than in the cellular assay. This indicates that these drugs have no effect on NADPH-oxidase activity. Myeloperoxidase activity also was not affected. Methylprednisolone and cortisone may interfere with the interaction between luminol and the myeloperoxidase–H_2O_2–halide system, since significant effects were

only observed when luminol was present in the incubation mixture, i.e., in the cellular and the cell-free chemiluminescence assays, but not in the myeloperoxidase assay. These drugs probably have no effect on the production of H_2O_2 or O_2^- radicals. Were there an effect on the production of H_2O_2, it would not be observable in the cell-free system because this system had H_2O_2 added externally. Were there an effect on the production of O_2^-, again, no effect would be observed in the cell-free system because this system lacks NADPH-oxidase, which is responsible for O_2^- production.

Dexamethasone, hydrocortisone, flumethasone, and triamcinolone showed similar inhibitory results in both the cellular and the cell-free assays. The inhibition induced by these drugs was only slightly higher in the cellular assay. These drugs might have scavenging effects on reactive oxygen species (H_2O_2; O_2^-). According to Niwa et al. (1987), glucocorticosteroids exert their effects on reactive oxygen radicals at high concentrations, not by inhibition of cell metabolism, but by scavenging of these radicals, as was also observed by Oyanagui (1976, 1980). Glucocorticosteroids, however, have no effect on the activity of myeloperoxidase or NADPH-oxidase. However, Amezaga et al. (1992) observed a dose-dependent reduction of mRNA coding for the gp91-phox component of the NADPH-oxidase by dexamethasone. The production of H_2O_2 and O_2^- is probably not affected for the same reasons as pointed out earlier. Interference with the interaction between luminol and the myeloperoxidase–H_2O_2–halide system might also be a factor. The differences between the results for this group of glucocorticosteroids and those for cortisone and methylprednisolone might be due to differences in penetrability of the drugs into the polymorphonuclear leukocytes.

The inhibitory effects induced by prednisolone in the cellular assay were twice as great as those in the cell-free assay. This indicates that prednisolone might have a slight inhibitory effect on NADPH-oxidase. Activation of glucocorticosteroid receptors might affect NADPH-oxidase (Roshol et al., 1995). However, this is not the only reason for the decreased cellular chemiluminescence. Scavenging of reactive oxygen intermediates (H_2O_2; O_2^-) and interference with the interaction of luminol with the myeloperoxidase–H_2O_2–halide system may again play a role. In contrast with our results, it was shown that prednisolone (1×10^{-6} M) did not affect the PMA-induced chemiluminescence of human polymorphonuclear leukocytes in vitro (Humphreys et al., 1993).

Phillips et al. (1987) observed no effect of therapeutic concentrations of dexamethasone on the zymosan-induced chemiluminescence of isolated and whole blood bovine polymorphonuclear leukocytes, which is in agreement with our results. According to Mandell et al. (1970) and Olds et al. (1974), hydrocortisone may act as an inhibitor of NADPH-oxidase at high concentrations, but has no effect on granulocyte chemiluminescence at pharmacological concentrations (Van Dyke et al., 1979; Horan et al., 1982; Phillips et al., 1987). In contrast with our results, physiologically unachievable concentrations of hydrocortisone increased chemiluminescence of human polymorphonuclear leukocytes (Horan et al., 1982).

The presence of very low concentrations of DMSO cannot explain the observed effects, since preliminary studies showed that DMSO induced no significant effect on chemiluminescence at concentrations up to 2%.

The contradictory and conflicting results reported in the literature may be due to species variation, differences in sensitivity among cell types and cell classes, differences between in vivo and in vitro administration, differences in concentrations and differences in procedures and methods (Phillips et al., 1987).

It can be concluded from the present results that the glucocorticosteroids tested have no adverse effects on the chemiluminescence of bovine polymorphonuclear leukocytes in vitro at therapeutic concentrations. Glucocorticosteroids probably have no effect on the production of reactive oxygen species at normal concentrations, but the effects observed in the chemiluminescence assay are due instead to an effect on the interaction between luminol and the myeloperoxidase–H_2O_2–halide system. Except for prednisolone, significant effects were only observed at supratherapeutic concentrations, not normally achievable in vivo. However, these very high concentrations may be reached in vivo after local and repeated administration i.e., after intramammary injection. Therefore, attention has to be paid to possible immunodepressant effects of glucocorticosteroids after repeated and local administration. Excessive use of glucocorticosteroids in vivo has to be avoided and these drugs may only be administered under controlled circumstances.

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