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STEROIDS DECREASE GRANULOCYTE MEMBRANE FLUIDITY, WHILE PHORBOL ESTER INCREASES MEMBRANE FLUIDITY Studies Using Electron Paramagnetic Resonance

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Abstract—High concentrations of corticosteroids inhibit granulocyte responses and disrupt agonist receptor function. Dose–response and time-course considerations make it unlikely that these effects are mediated via the glucocorticoid receptor, a concept further supported by the ability of sex steroids to work similar effects. We postulated that steroids nonspecifically altered granulocyte membrane fluidity, which we measured directly by electron paramagnetic resonance. As predicted, methylprednisolone caused a dose-dependent increase in order parameter (decrease in fluidity) calculated on the basis of EPR spectra, using 5-doxylstearic acid (5-DS) as a probe of resting PMN membranes. This trend was highly significant (P < 0.001; P at 0.5 mg/ml < 0.01). Qualitatively similar results (but with different dose–response features) were obtained with conjugated estrogen. Granulocyte agonists (such as PMA) showed an opposite effect, which was not oxidatively mediated and which was steroid-inhibitable. 16-DS showed less prominent effects, suggesting that the membrane leaflets were more strongly affected than was the deep region of the membrane. Ibuprofen, which has similar effects to those of methylprednisolone on PMN aggregation and receptor function, caused a fluidizing rather than a stiffening of the membrane; this surprising result may indicate that there is a critical range of membrane fluidity for normal function, outside of which—in either direction—agonist receptor dysfunction occurs. We conclude that the immediate effects of very high doses of steroids are probably not mediated by corticoid receptors; instead, they may be due to changes in membrane fluidity.

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INTRODUCTION

High concentrations of corticosteroids have been found to blunt a variety of granulocyte functions, an effect suggested to account for some of the pharmacologic actions of extremely high doses of these drugs in vivo (1–3). A difficulty in understanding and interpreting these observations pharmacologically is that the doses and/or concentrations of steroid required to blunt granulocyte function acutely are far in excess of those required to saturate the cytoplasmic glucocorticoid receptors in mammalian cells. Furthermore, also arguing against a critical role for glucocorticoid receptors in these drug effects, the functional inhibition worked by high-dose corticoids is virtually instantaneous (2–4), while receptor-mediated effects of steroids require the time-consuming induction of protein synthesis and elaboration of a mediator (5, 6).

We therefore postulated that the unique effects of very high doses of glucocorticoids were not corticoid–receptor-mediated and were therefore probably not highly specific; rather, they seemed likely to be a nonspecific physicochemical effect of sterols as a class. In support of this hypothesis, we recently reported that conjugated estrogens and two highly water-soluble synthetic sex steroids each inhibited granulocyte aggregation, chemotaxis, and chemiluminescence at doses similar to those observed for methylprednisolone (7); furthermore, conjugated estrogens (available in greater supply and therefore tested more extensively) were found to induce dysfunction of the granulocyte surface receptor for N-f-Met-Leu-Phe in manner analogous to that of methylprednisolone (3, 4, 7).

From these observations, we postulated that steroids might work their effect upon granulocyte function by intercalating within the plasma membrane or by layering over the plasma membrane, altering the membrane fluidity and secondarily altering the function of receptors associated with the membrane. We then assessed this question specifically, using electron paramagnetic resonance (EPR) as a measure of membrane fluidity.

MATERIALS AND METHODS

Preservative- and filler-free methylprednisolone sodium succinate and sodium ibuprofen were provided by the Upjohn Company (Kalamazoo, Michigan) and were dissolved and serially diluted in isotonic phosphate-buffered saline (PBS; pH 7.4). Conjugated equine estrogens were obtained as a clinical preparation (Premarin, Ayerst Laboratories, New York, New York); studies utilizing this agent employed the drug’s simethicone-containing vehicle as a blank, because the drug was not available in a preservative- and filler-free form. Phorbol myristate acetate (PMA) was obtained from Sigma Fine Chemicals (St. Louis, Missouri); it was initially dissolved in a small volume of dimethyl sulfoxide, then serially diluted in PBS.
Granulocytes were obtained from the heparinized venous blood of normal human volunteers by a minor modification of the method of Hjørth et al. (8); they were separated by centrifugation on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient after hetastarch (2%) pre-sedimentation of the erythrocytes for 45 min at 1 g. The cells were resuspended in isotonic HEPES-buffered (pH 7.4) saline to a cell count of $5 \times 10^7$ PMN/ml. Preincubation of cells was carried out by mixing 1 ml of the cell suspension with 250 µl of the appropriate drug dilution and allowing the suspension to stand at room temperature for 10 min. When the granulocyte agonist PMA was being tested in the same incubation with a drug, the dilutions were chosen such that 125 µl of the drug solution and an equal volume of PMA were employed; in this case, the incubation was carried out for 30 min at 37°C.

The spin labels employed were 5-doxylstearic acid (5-DS) and 16-doxylstearic acid (16-DS), which were dissolved at 20 mM in dimethylformamide. Two microliters of the 20 mM spin-label solution were added to 1 ml of the cell suspension ($5 \times 10^7$ PMN/ml) after incubation with drug and/or agonist, and 2 min at room temperature (or 5 min at 0°C for samples exposed to PMA) were allowed for incorporation of the label into the cells. This spin-label dose was low enough that increasing or decreasing it by a factor of two had no effect on the resulting EPR spectrum, i.e., it was without significant effect of its own on the measurements being made. The cells were then centrifuged at 400 g for 40 sec; the supernatant fluid was decanted, and the remaining cells were aspirated into the capillary lumen of a 50-µl micropipet. The end of the pipet was then sealed with inert clay, and the tube was centrifuged at 400 g for 10 sec to sediment the granulocytes into the bottom of the sealed capillary tube.

Electron paramagnetic resonance spectra were then obtained using a Varian E-4 EPR spectrometer (Varian Instruments, Palo Alto, California), equipped with a temperature controller capable of maintaining the temperature within ±0.2°C of the selected value. The operating settings employed were: operating frequency, 9.1 GHz; field modulation, 100 kHz, with an amplitude of 2.0 G; field set, 3300 G; field sweep, 200 G. First-derivative spectra were obtained at gain settings and scan times that were chosen to maximize the signal-to-noise ratio at the spectral peaks.

For mathematical interpretation of the spectra and for statistical assessment, an order parameter, $S$, was calculated for samples labeled with 5-DS (9), while an empirical order parameter, $R$, was calculated according to the method of Haak et al. (10) for the samples employing 16-DS. Each parameter is related to membrane fluidity in that an increase in the value indicates a decrease in spin label mobility (decreased fluidity), while a decrease of the value shows that the spin label is in a more fluid environment.

Statistical significance was assessed using Student’s $t$ test for paired data (11-13), using ANOVA-derived variances to correct, where appropriate, for the availability of multiple paired comparisons (14, 15).

**RESULTS**

Methylprednisolone caused a dose-dependent decrease in membrane fluidity of resting PMNs (Figure 1). This decrease in membrane fluidity was manifest by an increase in order parameter, which achieved statistical significance at 0.5 mg steroid/ml when 5-DS was used as the probe.

Because earlier studies had indicated that noncorticoid steroids influenced granulocyte function in manner similar to glucocorticoids, we then tested whether conjugated estrogen would effect a similar increase in granulocyte
membrane stiffness. This was the case, although the dose–response characteristics were somewhat different. As seen in Figure 2, the amount of estrogen required to effect detectable membrane fluidity changes was greater than the amount of methylprednisolone required. Lower doses of estrogen caused a slight fluidizing of the membrane (which did not achieve statistical significance with an n of 12). However, at concentrations of estrogen sufficient to increase the order parameter (decrease membrane fluidity), the observed effect was greater than that seen with the same concentration of methylprednisolone. The simethicone-containing vehicle was without effect on order parameter when added to granulocytes at the corresponding concentrations.

Having found that high-dose steroids led to a decreased fluidity in the granulocyte membrane, we postulated that a granulocyte agonist might have the opposite effect (as certainly suggested by the morphologic concomitants of activation). We therefore incubated granulocytes with 10 ng of PMA/ml and observed an increase in membrane fluidity (i.e., a decrease in order parameter) (Figure 3). This increase in membrane fluidity was blocked if methylprednisolone (1 mg/ml) had been added to the cells before the agonist was applied.

The same series of experiments was then carried out using 16-DS, a probe with the radical situated deeper in the membrane; this was done in an attempt to demonstrate whether the rigidifying effect of steroids involves the entire membrane. Under each experimental condition, the effect was less with 16-DS;

![Fig. 1. Methyprednisolone decreases granulocyte membrane fluidity. In a dose-dependent fashion, the incubation of granulocytes with methylprednisolone (MP) leads to a decrease in membrane fluidity, reflected in an increase in the order parameter calculated from the electron paramagnetic resonance spectrum of pelleted granulocytes containing the stable free-radical probe, 5-doxystearic acid. The trend shown was statistically significant (product–moment correlation) at P < 0.001; asterisks indicate P < 0.01 by ANOVA-corrected t test.](image-url)
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Fig. 2. Noncorticoid steroids (conjugated estrogen shown) decrease PMN membrane fluidity. Data are displayed in a manner analogous to those in Figure 1. The trend shown is again highly statistically significant, but the dose–response characteristics are different from those for methylprednisolone. The threshold for demonstration of an effect is higher, but the effect itself is greater at high concentrations of drug.

Fig. 3. Phorbol myristate acetate (PMA) increases PMN membrane fluidity. Because of different incubation conditions, the order parameter is slightly higher in the blank incubation than is the case in Figures 1 and 2. The presence of 10 ng PMA/ml leads to fluidizing of the membrane, reflected in a decrement in order parameter (second bar). If 1 mg methylprednisolone (MP) per milliliter was also present (third bar), the membrane fluidity remained indistinguishable from the baseline value. Truly enormous steroid concentrations (right-hand bar) were able to produce net stiffening of the membrane, even in the presence of PMA.
Fig. 4. Ibuprofen (IBU) fluidizes the PMN membrane. At concentrations that inhibit granulocyte activation and disrupt FMLP receptor function, we had expected that IBU, like MP, would stiffen the PMN membrane. The opposite was observed, suggesting that there may be a critical range of membrane fluidity for proper receptor function.

thus, the leaflets (or at least the outer leaflet) are more strongly affected than is the central portion of the membrane.

Finally, we were aware from previous studies (15) that the nonsteroidal antiinflammatory drug ibuprofen also inhibited granulocyte function at high doses and also had an effect on granulocyte agonist receptor function. We wondered whether this agent might have similar effects upon membrane fluidity, or if in fact its effect was worked by some other mechanism. In resting PMNs tested in a manner analogous to those treated with steroids, we found that concentrations of ibuprofen that inhibit granulocyte aggregation enhance rather than impair the fluidity of the granulocyte membrane (Figure 4). Thus, the latter hypothesis seems more likely.

**DISCUSSION**

A number of authors have advocated the use of corticosteroids in shock or the shock lung syndrome (16–19), because they are protective against death and pulmonary dysfunction in a variety of experimental models and because some
clinical studies have suggested that they may be useful in patients as well. Both animal and patient studies show benefit only if the drugs are introduced very early in the disease process, something which is hard to achieve in the clinic [as recently underscored by two negative prospective studies of steroids in septic shock (20, 21)]. This suggests that the mechanism of beneficial action of steroids is probably related to blunting of inciting events, rather than because of an effect upon the disease process once it is well underway.

It is unclear which of the many effects of high-dose corticosteroids is (are) responsible for their efficacy. It has long been our view that at least part of this effect derives from the ability of high-dose steroids transiently to blunt granulocyte responses to a variety of agonists (22), and thus to blunt the contribution of granulocytes to the pathophysiologic process. Because the concentrations and doses required for these effects are much higher than those required to saturate glucocorticoid receptors, we have favored the hypothesis that steroids work their influence upon granulocytes by a nonspecific physicochemical effect at the cell surface, an hypothesis strengthened by the observation that noncorticoid steroids have similar effects and have in fact been reported by one group to be protective in endotoxin shock (23).

One simple physicochemical effect that could account for many previous observations and that was liable to measurement was a change in granulocyte membrane fluidity. This was especially attractive to study, because it had been reported by Schieren et al. (24) that the incorporation of hydrocortisone into β-doxylcholestone-labeled lecithin–cholesterol liposomes blunted the effects of aggregated IgG upon the mobility of the probe and upon the rate of chromate efflux. In the present study, we have shown that methylprednisolone and a noncorticoid steroid both do rigidify the membrane of resting granulocytes, an effect that seems greater when probed near the surface of the membrane (using 5-DS) than when probed near the center of the bilayer (with 16-DS). In contrast, a variety of granulocyte agonists caused increases in membrane fluidity; with partially purified C5a or FMLP, this effect was short-lived and of highly variable degree, making experiments technically difficult to conduct and to analyze statistically. We therefore collected most of our data using PMA, which yielded more consistent and prolonged changes in membrane fluidity. The changes in response to agonists were prevented by the prior addition of methylprednisolone.

While these observations are in good agreement with our working hypothesis, there are some aspects of our findings that suggest rigidification of the membrane is not the only mechanism whereby steroids work their effect. First, conjugated estrogens had different dose–response characteristics from those of methylprednisolone. Even though estrogen has a lower 50%-attenuative concentration for granulocyte aggregation (7), it required more estrogen to effect a change in membrane fluidity. Interestingly, although the threshold was different
in a direction that we would not have predicted, the magnitude of the effect with estrogen was greater in the concentration range where an effect was observed.

The technique of assessing membrane fluidity by EPR is, unfortunately, one which requires the pelleting of the cells in order to get a dense enough signal for the reliable measurement of a spectrum. It is therefore not possible to measure membrane fluidity in the same cells both before and after their experimental perturbation. This, combined with the large number of cells needed to conduct the assay and its time–labor intensity, has made it difficult to dissect further the mechanisms of the effects we have observed. However, it does not appear that the effect of PMA is a consequence of the production of oxidants by the respiratory burst of the granulocytes: the same effects were observed if the cells were incubated in the presence of superoxide dismutase and catalase, and the deliberate exposure of the spin-labels to a superoxide-generating system (xanthine–xanthine oxidase) did not change the spacing of the spectral lines when the label was subsequently used (the height of the peaks was attenuated somewhat, but it is their spacing rather than their height that enters into the order parameter calculations).

Finally, we were a bit surprised that ibuprofen did not rigidify the cell membrane in a manner analogous to steroids, knowing that ibuprofen and methylprednisolone had very similar effects on granulocyte aggregation, respiratory burst, and FMLP receptor function. On the other hand, we had previously shown synergy between the two agents (25), strongly suggesting that at some level the mechanism of action was likely to be different between them. Clearly, the current observations imply that membrane stiffening is not the only way to interfere with agonist receptor function. The findings with ibuprofen could indicate that there is a critical range of membrane fluidity for agonist receptor function, with deviations in either direction causing dysfunction. Alternatively, they could mean that altering membrane fluidity is only one of many possible ways to disrupt receptor function.

We conclude that a nonspecific effect of sterols upon granulocyte membrane fluidity is likely to be a part of the mechanism whereby these agents influence granulocyte function, and perhaps whereby they work clinical benefit. Although the further observations outlined above suggest that this may not be the whole story, we suggest that noncorticoid steroid drugs might be devised for clinical use in certain applications, such as shock, that achieve the desired effect upon effector cell function without the undesired and undesirable side effects of glucocorticoids.

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REFERENCES


