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# Glucocorticoid-mediated alterations in fluidity of rabbit cardiac muscle microvessel endothelial cell membranes: influences on eicosanoid release

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**Experiments were conducted to determine effects of the synthetic glucocorticoid, dexamethasone, on the lipid fluidity of cultured rabbit cardiac muscle microvessel endothelial cells and the possible role(s) for altered fluidity in the steroid inhibition of cellular eicosanoid production. Following a sixteen hour exposure to $10^{-7}$ M dexamethasone, membranes prepared from treated cells exhibited a decreased fluidity compared to their control counterparts, as assessed by steady-state fluorescence polarization techniques using 1,6-diphenyl-1,3,5-hexatriene (DPH). Examination of the effects of temperature on the anisotropy values of DPH using Arrhenius plots revealed consistent differences in the steroid treated cells over the entire temperature range (40–5°C). These dexamethasone-dependent fluidity changes were associated with increases in the cholesterol/phospholipid ratio of membrane lipids. Restoration of membrane fluidity to control values with the fluidizing agent, 2-(2-methoxyethoxy)ethyl-8-(*cis*-2-*n*-octylcyclopropyl)octanoate (A2C), partially reversed dexamethasone induced inhibition of A23187-stimulated eicosanoid release. These observations suggest that at least part of dexamethasone's inhibitory actions on eicosanoid generation in microvessel endothelial cells are mediated by alterations in membrane composition and fluidity.**

## Introduction

Glucocorticoids influence the structure and/or function of many cell types through alterations in ion transport and membrane bound enzyme activity [1–3]. Steroid inhibition of prostaglandin release from endothelial [4–7] and other cell types [8–10] is glucocorticoid specific, dose-dependent, and requires new RNA and protein synthesis. One proposed mechanism for this anti-inflammatory action of glucocorticoids involves inhibition of arachidonic acid release, possibly via induction of phospholipase inhibitory protein (lipocortin) activity [8,11–13]. However, recent data challenge this hypothesis, as several laboratories have been unable to demonstrate induction of lipocortins in various glucocorticoid sensitive cells [14–16]. Davidson et al. [17] demonstrated that the ability of lipocortin to inhibit phospholipase activity was the result of substrate sequestration; and, further, they proposed that this mechanism was unlikely to account for the observed inhibitory actions of glucocorticoids. Recently our laboratory [18] reported that glucocorticoid treatment of microvessel endothelial cells did not inhibit the release of arachidonic acid (when the mass of endogenous AA was determined). However, significant alterations in the fatty acid composition of membrane phospholipids by dexamethasone were observed [18] which led to the present study of glucocorticoid actions of the lipid fluidity of membranes prepared from microvessel endothelial cells.

The results of the present study demonstrate that membranes prepared from dexamethasone treated microvessel endothelial cells exhibit relative reductions in lipid fluidity, compared with corresponding controls, as assessed by steady-state fluorescence polarization studies using the fluorophore, 1,6-diphenyl-1,3,5-hexatriene. Based on the results from this, and an earlier study [18], steroid reduction of membrane fluidity may be mediated by an increase in the cholesterol : phospholipid ratio and increased membrane fatty acid saturation. To de-

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termine if these alterations in fluidity might play a role in dexamethasone-induced inhibition of eicosanoid synthesis, we evaluated the actions of the membrane fluidizing agent 2-(2-methoxyethoxy)ethyl-8-(*cis*2-*n*-octylcyclopropyl)octanoate (A2C). 'Restoration' of anisotropy (reciprocal of fluidity) to control levels with A2C partially reversed the inhibitory effects of dexamethasone on prostaglandin synthesis in intact microvessel endothelial cells.

## Materials and Methods

*Materials.* Dexamethasone, prostaglandin $E_2$ ($PGE_2$), 6-ketoprostaglandin $F_{1\alpha}$ (6-keto$PGF_{1\alpha}$), A23187, and A2C were obtained from Sigma (St. Louis, MO). DPH was obtained from Aldrich Chemical Co. (Milwaukee, WI). Prostaglandin antisera were from Advanced Magnetics (Cambridge, MA). Arachidonic acid was obtained from Nuchek Prep (Elysian, MN). [$^3$H]$PGE_2$ and 6-keto[$^3$H]$PGF_{1\alpha}$ were from Amersham (Clearbrook, IL). Scintillation fluid (Liquiscint) was from National Diagnostics (Manville, NJ). All cell culture materials were from GIBCO (Grand Island, NY). 1,1'-Dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Di-I-Ac-LDL) was from Biomedical Technologies Inc. (Staughton, MA).

*Cell culture.* Cells used in this study were from two established cell lines of microvessel endothelial cells isolated and identified as detailed in earlier publications [19,20]. Using the criteria of Di-I-Ac-LDL uptake and staining for Factor VIII related antigen, the endothelial cell content of the cell lines was greater than 95%. Cells were routinely grown in Dulbecco's Modified Eagle's Medium (DME) supplemented with 20% fetal bovine serum (FBS). Steroid treatment was performed as follows. Cells were grown to confluence in Corning T-150 flasks and fed 24 h prior to the experiment with fresh culture media. Cells were incubated 16 h in DME containing 1% dialyzed FBS with or without dexamethasone ($10^{-7}$ M). The cells were washed twice with 50 ml of warm Hanks-25 mM Hepes buffer (pH 7.4), removed from the plates using a Corning cell scraper and pelleted by centrifugation.

*Preparation of plasma membranes.* Three to five T-150 flasks were pooled for each membrane preparation in order to have sufficient material for fluidity and lipid composition analyses. One ml of 0.1 M sucrose in 10 mM Tris (pH 7.4 at 4°C) was added to cell pellets which were incubated on ice 6 min. The pellet was then lysed by three freeze-thaw cycles, homogenized in a Dounce 1 ml homogenizer ('B' pestle) and transferred to conical centrifuge tubes. The homogenizer was washed with 1 ml cold 0.4 M sucrose in 10 mM Tris and combined with the original homogenate. Following centrifugation at $1000 \times g$ for 10 min, the supernatant was transferred to a Corex tube and the remaining pellet rehomogenized in 1 ml 0.4 M sucrose and the supernatant obtained as above. The rehomogenizing step was repeated an additional two times and all supernatants combined and centrifuged at $9000 \times g$ for 10 min. The pellet was discarded, the supernatant centrifuged at $40\,000 \times g$ for 30 min and the resulting membrane-rich pellet suspended in Dulbecco's phosphate-buffered saline (PBS) by sonication. The protein content was determined using a Pierce BCA kit with bovine serum albumin as the standard. Similar yields of protein were obtained for matched preparations of steroid and control treated microvessel endothelial cells. Table I summarizes the marker characterization of the membrane preparation. The activity of 5'-nucleotidase, determined using a Sigma diagnostic kit, was enriched 4–6-fold over the initial crude homogenate. Mitochondrial and lysosomal contamination [21] of the membranes was minor and similar in both control and dexamethasone-treated microvessel endothelial cell preparations. Membrane phospholipid and cholesterol contents were determined as described previously [18,24].

TABLE I

*Marker enzyme characterization of membranes prepared from control and dexamethasone (Dex)-treated rabbit cardiac muscle microvessel endothelial cells*

Values shown are the means of duplicate determinations, range ±5% of the values given. n.d., not detected.

| | Treatment | Homogenate | Membranes | Enrichment |
|---|---|---|---|---|
| 5'-Nucleotidase | control | 0.007 | 0.030 | 4.28 |
| (mg P/mg protein per h) | Dex | 0.006 | 0.032 | 5.33 |
| Succinate dehydrogenase [a] | control | 0.40 | 0.07 | 0.18 |
| ($\mu$mol/mg protein per h) | Dex | 0.50 | 0.11 | 0.22 |
| $\beta$-Glucuronidase [b] | control | 0.92 | n.d. | – |
| (nmol/mg protein per h) | Dex | 1.05 | n.d. | – |

[a] Determined by the method of Shelton and Rice [22].
[b] Determined by the method of Bosman and Bernacki [23].

*Prostaglandin release.* Rabbit cardiac muscle microvessel endothelial cells were grown to confluence in Falcon 24-well culture dishes. Twenty four hours prior to experiments the cells were fed with fresh culture media. Cells were incubated 16 h in DME containing 1% dialyzed FBS with or without dexamethasone as above. The cells were washed three times with PBS containing $Ca^{2+}$ and $Mg^{2+}$ (PBS/Ca), then preincubated 15 min in PBS/Ca. The preincubation buffer was removed and incubation buffer (PBS/Ca) with or without agonist (e.g. 5 $\mu$g/ml A23187) added and the cells incubated an additional 15 min. All washes, preincubations and incubations were carried out at 37°C. Each 24-well plate contained control and steroid-treated groups, as well as basal (PBS/Ca alone) and stimulated (A23187) groups. Within each experiment, each group contained 4 or 6 wells, and each experimental protocol

was repeated at least twice. The incubation buffer was removed and stored at −20°C for later radioimmunoassay. Immunoreactive levels of $PGE_2$ and 6-ketoPGF$_{1\alpha}$, the chemically stable endproduct of $PGI_2$ hydrolysis, were determined as detailed in earlier studies [4,24] and doses of agonists, steroids and time points used in this study were based on earlier publications by this laboratory [4,24].

*Membrane fluidity studies.* For these experiments the lipid-soluble fluorescence probe, DPH, was prepared according to established techniques [25]. All studies were carried out within 2 weeks of membrane isolation utilizing membranes stored at −20°C. To obtain sufficient material for assay, 3–5 T-150 flasks of endothelial cells were pooled for each membrane preparation. Estimates of relative membrane fluidity were calculated after fluorescence polarization measurements using a Shimadzu RF-540 spectrofluorophotometer fitted with a thermoregulated sample chamber and automatic rotating polarizers (C.N. Wood, Newtown, PA). In these experiments the term 'fluidity' is used to describe the motional freedom of a lipid-soluble molecular probe (DPH) within a membrane bilayer. Determination of absolute fluidity is limited in an anisotropic membrane suspension (as opposed to a homogeneous, isotropic medium) because of the inability to accurately reproduce the three dimensional structure of the hydrophobic bilayer. Therefore, the steady-state fluorescence anisotropy ($r$, the reciprocal of fluidity) is employed to estimate relative degrees of fluidity after probe incorporation into the bilayer. Values for $r$ were calculated from fluorescence polarization measurements using the equation:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ equal fluorescence intensities parallel and perpendicular, respectively, to the excitation plane (excitation wavelength 360 nm, emission wavelength 430 nm). Scattered light plus ambient medium fluorescence contributed < 5% to the total fluorescence intensity throughout the temperature range utilized in all studies [24]. Fluorescence anisotropy in cell membranes or liposomes may be further resolved according to the Perrin relationship [25] a modified form of which may be written

$$r = r_{\infty} + (r_0 - r_{\infty})[t_c/(t_c + t_f)]$$

where $r_0$ represents the maximal limiting anisotropy in the frozen state (0.365 for DPH), $t_c$ is the correlation time and $t_f$ is the mean lifetime of the excited state. The term $r_{\infty}$ represents the static component of fluidity [26] which is related to both bilayer molecular order and the degree of hindrance to probe rotation by packing of bilayer lipids (factors that cannot be distinguished under the present experimental conditions). Here, $r_{\infty}$ was calculated according the equation proposed by Van Blitterswijk et al. [26], where $r_{\infty} = (4r/3) - 0.1$. Specific determination of $r_{\infty}$ from time resolved studies (dynamic depolarization experiments utilizing DPH) [27] was not carried out in this protocol. Nevertheless, within a defined anisotropy range (0.130–0.280) calculations of $r_{\infty}$ employed herein represent a reasonable approximation of the limited hindered anisotropy [26]. Phase and modulation values for $t_f$ were measured using an SLM 4800 (SLM-Aminco, Champagne, IL) spectrofluorophotometer according to established techniques [28].

*Statistical analyses.* All results are expressed as the mean ± S.E. When multiple comparisons against a control were made, data were first analyzed by a one-way analysis of variance. If differences were noted, data were compared using Bonferroni's modified $t$-test. For other experiments, paired '$t$'-tests were used. The null hypothesis was rejected at $P < 0.05$.

## Results

*Lipid composition of endothelial cells*

In control cells, total cholesterol content was 0.075 ± 0.006 μg/μg protein, and total phospholipid content was 0.316 ± 0.008 μg/μg protein, with a cholesterol/phospholipid ratio of 0.24 (μg/μg) ($n = 4$). The dexamethasone-treated cell membranes had a total cholesterol content of 0.089 ± 0.012 μg/μg protein and the total phospholipid content was 0.266 ± 0.008 μg/μg protein, yielding a cholesterol/phospholipid ratio of 0.33. While there was no difference in cholesterol, the phospholipid content of dexamethasone-treated cells

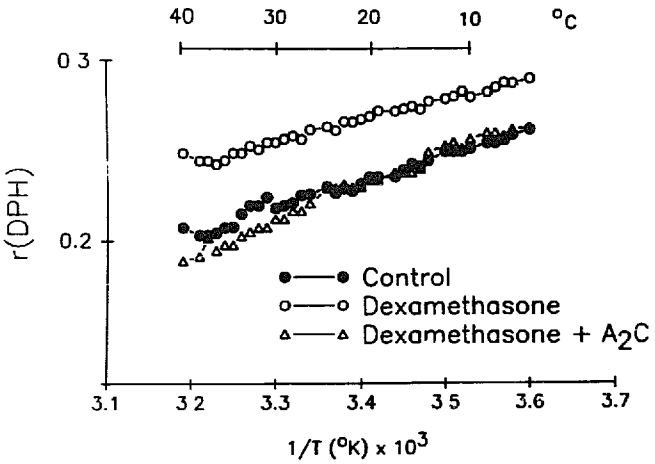


Fig. 1. Representative Arrhenius plots of the fluorescence anistropy of DPH in control (●) and dexamethasone (○)-treated rabbit cardiac muscle microvessel endothelial cell membranes. Also shown is the representative Arrhenius plot obtained from dexamethasone-treated rabbit cardiac muscle microvessel endothelial cells membranes treated with 10 μM A2C (△).

was significantly lower than that of control cells ($P < 0.005$).

*Membrane fluidity in endothelial cells*

To determine the effects of dexamethasone on relative membrane fluidity, fluorescence polarization studies were performed using DPH as the lipid-soluble fluorophore. Determination of fluorescence lifetime values (phase and modulation) demonstrated no significant differences, between control and dexamethasone-treated membranes (data not shown). As shown in Table II, mean fluorescence anisotropy (reciprocal of fluidity), and $r_\infty$ of endothelial cells membranes treated with dexamethasone was significantly higher than control membranes ($P < 0.05$, $n = 4$). Representative Arrhenius plots for $r$ (DPH) in control and dexamethasone-treated membranes are shown in Fig. 1. Throughout the temperature range examined (40–5°C) anisotropies for the dexamethasone-treated membranes were significantly higher than the corresponding control values. In both groups a distinct thermotropic transition (breakpoint) could not be identified utilizing this methodology.

*Prostaglandin release*

Dexamethasone treatment of microvessel endothelial cells reduced basal and A23187-stimulated prostaglandin release (Fig. 2). To determine the influence of altered membrane fluidity on prostaglandin release, microvessel endothelial cells were incubated with the membrane fluidizing agent, A2C (10 μM). As shown in Table II and Fig. 1, a 10-min incubation of dexamethasone-treated microvessel endothelial cell membranes with A2C restored fluorescence anisotropy ($r$) values to control levels. Similar treatment of control membranes yielded fluorescence anisotropy identical to control values given in Table II. Pretreatment of intact microvessel endothelial cells with 10 μM A2C did not alter the A23187-stimulated release of prostaglandins from untreated cells, but partially restored A23187-stimulated prostaglandin release toward control levels in dexamethasone-treated cells (Fig. 2).

## Discussion

This data illustrate four major findings concerning dexamethasone effects on rabbit cardiac muscle microvessel endothelial cell membrane lipid composition and fluidity measurements. (1) Dexamethasone treatment decreases membrane total phospholipid content resulting in a significant increase in the cholesterol/phospholipid ratio; (2) consistent with these alterations in lipid composition, glucocorticoid-treated microvessel endothelial cell membranes exhibit significant decreases in membrane fluidity; (3) dexamethasone treatment inhibits A23187-stimulated prostaglandin release from microvessel endothelial cells, in agreement with our earlier report [4]; and (4) the membrane fluidizing agent, A2C, reversed the dexamethasone-induced alterations in membrane fluidity and partially restored prostaglandin release to control values.

These observations suggest the intriguing possibility that at least part of the inhibitory actions of dexamethasone on microvessel endothelial cell prostaglandin synthesis result from steroid-induced alterations in membrane composition and fluidity. The concept that steroids could exert antiphlogistic actions by stabilizing membranes was first suggested by Eyring and Dougherty [29]; however, there is a paucity of experimental data either supporting or refuting this contention.

There have been a number of studies describing both glucocorticoid induced increases [30–33] and decreases [34] in membrane fluidity. These disparate results are likely secondary to differences among the tissues studied, the ages of the animal used, and the duration and dose of steroid treatment. Earlier studies have not attempted to relate the effects of glucocorticoids to their prostaglandin inhibitory actions.

Two factors thought to play significant roles in the determination of membrane fluidity are cholesterol content (particularly in relationship to phospholipids) and the fatty acid saturation index. Studies by Brasitus et al. [33] which examined intestinal brush border membranes showed an increase in phospholipid content, no change

TABLE II

*Fluorescence polarization studies of rabbit cardiac muscle microvessel endothelial cell membranes following dexamethasone treatment and addition of A2C (10 μM)*

Values are the means ± S.E. of four separate membrane preparations of each group. Determinations from individual membrane preparations were performed in duplicate and the average values used in the group data. Dex, dexamethasone.

| Preparation | 37°C | | 25°C | |
|---|---|---|---|---|
| | $r$ | $r_\infty$ | $r$ | $r_\infty$ |
| Control | 0.183 ± 0.011 | 0.144 ± 0.015 | 0.207 ± 0.008 | 0.176 ± 0.010 |
| Dex | 0.224 ± 0.013 * | 0.199 ± 0.018 * | 0.246 ± 0.012 * | 0.228 ± 0.016 * |
| A2C | 0.187 ± 0.012 | 0.149 ± 0.016 | 0.210 ± 0.017 | 0.179 ± 0.022 |

* Significantly different from corresponding control value, $P < 0.05$.

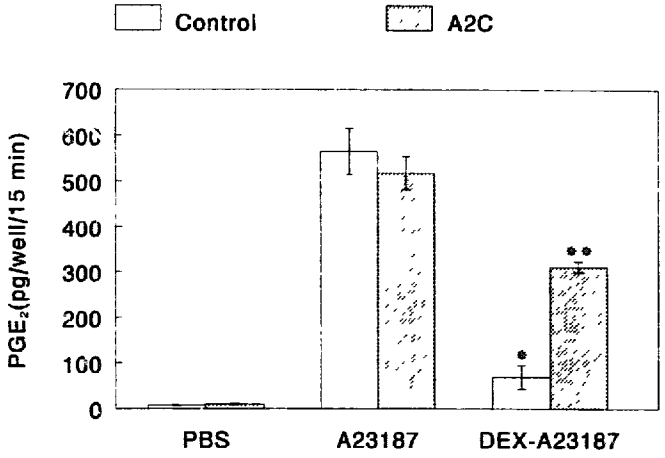


Fig. 2. A2C (10 $\mu$M) pretreatment (3 min) of rabbit cardiac muscle microvessel endothelial cells partially reverses the inhibition of A23187 (5 $\mu$g/ml) stimulated $PGE_2$ release observed in dexamethasone (DEX-A23187)-treated cells. Data are expressed as the means $\pm$ S.E., $n = 4$. * Significantly different from control rabbit cardiac muscle microvessel endothelial cells stimulated with A23187. ** Significantly different from group without fluidizer.

in cholesterol and a corresponding increase in the phospholipid/cholesterol ratio following dexamethasone treatment. These membrane compositional changes were associated with an increase in relative lipid fluidity. In the present study, we report that dexamethasone treatment of microvessel endothelial cells had the opposite effect on the phospholipid/cholesterol ratio, which resulted in a decrease of bilayer fluidity. Increased membrane saturated fatty acid content is also associated with decreased motional freedom of membrane probes [35], although the relationship between lipid saturation and fluidity is not direct [36]. Nevertheless, our observations that dexamethasone treatment of microvessel endothelial cells resulted in an increased saturation index [18] suggest that alterations in fatty acid composition contribute in part to the reduction of membrane fluidity.

A2C is a molecule expressly developed for use as a membrane fluidizing/membrane mobility agent [36]. The hydrophilic (polar) portion of this molecule bridges the region between water and the hydrocarbon domains of the phospholipid bilayer, while the hydrophobic (nonpolar) portion carriers a cyclopropane ring which promotes disorder within the hydrocarbon region of the bilayer. Addition of 10 $\mu$M A2C to microvessel endothelial cell membranes restored the membrane anisotropy to control values. The same dose of A2C, added to intact microvessel endothelial cells, did not alter basal or A23187-stimulated PG release in control cells, but partially reversed the inhibitory effects of dexamethasone. The orrelation of these two observations makes the implicit assumption that since A2C fluidizes the membrane preparations in vitro, addition of this molecule to a whole cell results in membrane fluidization. Support for this assumption is provided by the observations of others [38], which have shown fluidization of mouse brain microsacs and synaptosomes that were isolated following in vivo administration of A2C. Of some interest is the inability of A2C to 'fluidize' the plasma membranes of control rabbit cardiac muscle microvessel endothelial cells. Although the reasons for this phenomenon are not clear, previous work suggests that, under physiological conditions. cell membrane fluidity is maintained within a narrow anisotropy range, a concept referred to as homeoviscous adaptation [36]. Furthermore, available data indicate that the actions of A2C on bilayer function may only be demonstrated under specific conditions of cell/membrane perturbation [39].

In summary, the observations described herein suggest that alterations in membrane fluidity may be an important element of dexamethasone inhibition of eicosanoid synthesis. The possibility that other glucocorticoid mediated anti-inflammatory effects are related to changes in plasma membrane physicochemical properties is intriguing and warrants further investigation.

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