DHEA-S inhibits human neutrophil and human airway smooth muscle migration

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ABSTRACT

Airway diseases such as asthma, emphysema, and chronic bronchitis are, in part, characterized by reversible airflow obstruction and inflammation. In severe disease, marked decreases in lung function are associated with airway smooth muscle proliferation and airway neutrophilia. Inhaled glucocorticoids attenuate increased airflow obstruction and airway inflammation that occur, in part, due to increased smooth muscle migration and proliferation, as well as the airway neutrophilia. Glucocorticoids, however, have adverse side effects and, in some patients, are ineffective despite high doses. Recent research has explored the effects of non-traditional steroids on attenuation of inflammation associated with airway diseases. These non-traditional steroids have improved side effect profiles in comparison to glucocorticoid therapy. Our studies assessed effects of dehydroepiandrosterone-3-sulfate (DHEA-S) on migration of both human peripheral blood neutrophils (PMN) and human airway smooth muscle cells (HASM). DHEA-S dose-dependently inhibited chemotaxis of PMN and HASM while having no effect on the phosphorylation levels of Akt, ERK1/2, p38 MAPK or PKC, canonical positive regulators of cell migration. These studies demonstrate direct effects of DHEA-S on cell migration, thereby suggesting that DHEA-S may attenuate airway inflammation and cell migration.

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1. Introduction

Asthma and other airway diseases are marked by inflammation of the airways, increased migration of airway smooth muscle cells, and inflammatory cell influx that contribute to airway remodeling. Current therapeutics that modulate these responses traditionally comprise glucocorticoids and β_2 adrenergic receptor (β_2AR) agonists. Unfortunately, some patients exhibit steroid insensitivity despite high doses of glucocorticoids.

Dehydroepiandrosterone-3-sulfate (DHEA-S) and its metabolite dehydroepiandrosterone (DHEA) are metabolites of cholesterol that can be further metabolized into testosterone and estradiol. Evidence suggests that levels of DHEA and DHEA-S in patients with asthma are decreased, both in the stable phase of the disease as well as during exacerbations [1]. Research in animal models and in clinical trials has demonstrated that DHEA-S and DHEA are effective in attenuating Th$_2$ cytokines associated with allergic disease, cytokine elaboration and by inhibiting influx of inflammatory cells into the airways [2–4]. Therefore, supplementation of these naturally occurring steroids may be therapeutically beneficial and offer a steroid-based therapy without the adverse side effects observed with traditional glucocorticoid treatments. Although evidence supports the existence of both cell surface and nuclear receptors that bind DHEA [5–7], the signaling pathways by which DHEA or DHEA-S mediates its effects remain unclear.

We sought to determine the effects of DHEA-S on cells known to be involved in the pathogenesis of asthma and COPD, human airway smooth muscle cells (HASM) and primary human neutrophils (PMN). We found that treatment with DHEA-S inhibited chemokinesis and chemotaxis of PMN, and chemokinesis of HASM. To identify a mechanism by which DHEA-S inhibits cell migration, phosphorylation of Akt, ERK1/2, p38 MAPK and PKC were examined.

2. Methods

2.1. Neutrophil chemokinesis/chemotaxis

Peripheral blood (20–30 ml) was drawn from healthy donors, in accordance with the Declaration of Helsinki and approved by the

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Committee on Studies Involving Human Beings at the University of Pennsylvania, layered over PMN isolation medium (Matrix) and spun at room temperature for 35 min at 1500 rpm. The plasma and mononuclear layers as well as half of the clear dextrose layer were aspirated off, and an equal volume of 0.45% NaCl was added, filling the remaining volume with 0.9% NaCl. The resulting solution was centrifuged for 15 min at 1500 rpm at room temperature. Supernatant was removed, and the pellet was resuspended in 0.2% NaCl, and an equal volume of 1.6% NaCl was added to ensure red blood cell lysis. The solution was centrifuged at 1500 rpm for 15 min, supernatant removed, and PMNs resuspended in media. Total number of cells was counted, and 100,000 cells were incubated with DHEA-S (Epi-12323, Epigenesis Pharmaceuticals, Cranbury, NJ) (0.01–10 μM) for 4 h prior to loading into 12-well transwell plates with or without 10 nM fMLP in the bottom chamber. Chemokinesis and chemotaxis were measured by counting the number of cells present in the bottom chamber 2 h after their addition onto the transwell insert. Cell viability was assessed by trypan blue exclusion and caspase activation. A single concentration of DHEA-S (10 μM) was chosen as the concentration to use for subsequent migration and signaling experiments, which was based on the dose responses performed in the neutrophils but also as a result of this concentration being used in clinical trials [3].

2.2. Human airway smooth muscle cell isolation and migration analysis

HASM cells were dissociated from human tracheas obtained from human lung transplant donors, in accordance with the Declaration of Helsinki and approved by the Committee on Studies Involving Human Beings at the University of Pennsylvania, as described previously [8]. Cells derived from a minimum of two healthy donors were cultured in Ham's F12 media (Life Technologies, Grand Island, NY), supplemented with 10% FBS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Before the experiments, HASM cells were maintained for 48 h in serum-free Ham's F12 media, supplemented with 0.1% BSA. Cell migration was examined using a Boyden chamber apparatus as we described previously [9]. Briefly, HASM cells, pre-incubated with 10 μM DHEA-S or diluent for 18 h, were briefly trypsinized by 0.05% trypsin/0.53 mM EDTA, centrifuged at 900 rpm for 10 min and resuspended in serum-free media supplemented with BSA. Cells were then placed into the upper wells of the Boyden chamber (5×10^4 cells/well) fitted with an 8-μm pore membrane coated with Vitrogen (100 μg/ml). Vehicle or 10 ng/ml PDGF-BB in serum-free media supplemented with BSA was added to the lower chambers. Cells in the Boyden chamber were incubated for 4 h at 37 °C in a 5% CO_2 incubator. Non-migrated cells were scraped off, the membrane was fixed with methanol, stained with Hemacolor stain set (EM Industries, Inc., Gibbstown, NJ) and scanned. Cell migration was analyzed using the Gel-Pro analyzer program (Media Cybernetics, Silver Spring, MD). Cell viability was measured by trypan blue exclusion and caspase activation.

2.3. Cell lysate preparation and immunoblot analysis

HASM cells were cultured in Ham's F12 media, supplemented with 10% FBS, and primocin™ (Invivogen, San Diego, CA). Before the experiments, HASM cells were maintained for 48 h in serum-free Ham's F12 media, supplemented with 0.1% BSA. Cells were incubated for 18 h with 10 μM DHEA-S, 1 μM dexamethasone (Sigma Aldrich, St. Louis, MO), or for 40 min with 30 μM LY294002 (Cayman Chemicals, Ann Arbor, MI), treated with 10 ng/ml PDGF-BB or diluent for 10 min, and cell lysates were prepared as we described previously [10]. Cells were also stimulated with PDGF (1 ng/ml) for 2, 5 or 30 min in the presence and absence of 10 μM DHEA-S. Immunoblot analysis with anti-phospho-Akt, anti-phospho ERK1/2, anti-phospho p38 MAPK,

anti-phospho PKC and anti-COX-4 (Cell Signaling Technology, Danvers, MA) was performed similarly as described [11].

2.4. Data analysis

Data points from individual assays represent mean values ± standard error. Statistically significant differences among groups were assessed with the analysis of variance (ANOVA) (with the Bonferroni–Dunn correction), with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses. All experiments were designed with matched control conditions within each experiment to enable statistical comparison as paired samples and to obtain statistically significant data.

3. Results

3.1. DHEA-S inhibits PDGF-induced migration of human airway smooth muscle cells (HASM)

To determine whether DHEA-S modulates migration of peripheral blood neutrophils (PMN), we evaluated chemokinesis and fMLP-induced chemotaxis of human primary PMN. Cells were incubated for 4 h with 0.01–10 μM DHEA-S, 0.01–10 μM roflumilast (a selective phosphodiesterase 4 inhibitor), or 1 μM dexamethasone, and then PMN migration was assessed in the presence or absence of 10 nM fMLP. As seen in Fig. 1, DHEA-S, roflumilast and dexamethasone significantly inhibited PMN chemokinesis. Stimulation with fMLP induced a ~13-fold increase in PMN migration compared to diluent-treated cells (Fig. 2A). Chemotaxis to fMLP was significantly attenuated by pre-incubation with 0.1, 1, and 10 μM DHEA-S, 1 and 10 μM roflumilast, and 1 μM dexamethasone (Fig. 2B). Treatment with DHEA-S did not affect PMN viability (data not shown). Taken together, these data suggest that DHEA-S is effective at attenuating both chemokinesis and fMLP-induced chemotaxis.

3.2. DHEA-S inhibits PDGF-induced migration of human airway smooth muscle cells (HASM)

To evaluate effects of DHEA-S on HASM cell migration, cells were pre-incubated for 18 h with DHEA-S, and then migration of HASM in the presence or absence of PDGF was examined. We found that DHEA-S had little effect on HASM cell chemokinesis (Fig. 3). PDGF increased migration of HASM three-old over basal, and pretreatment with DHEA-S significantly reduced PDGF-induced HASM migration

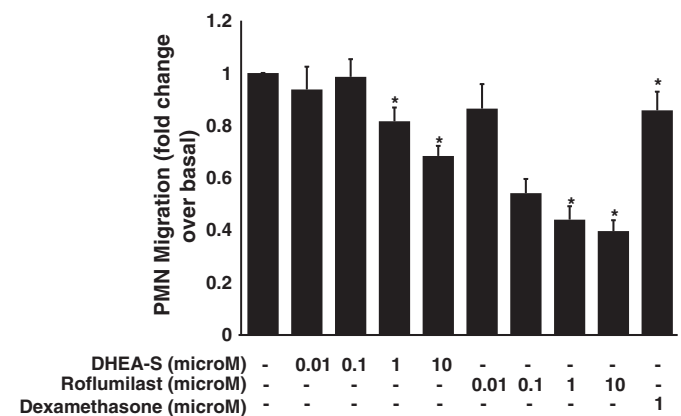| DHEA-S (microM) | - | 0.01 | 0.1 | 1 | 10 | - | - | - | - | - |
| Roflumilast (microM) | - | - | - | - | - | 0.01 | 0.1 | 1 | 10 | - |
| Dexamethasone (microM) | - | - | - | - | - | - | - | - | - | 1 |

Fig. 1. DHEA-S inhibits neutrophil chemokinesis. Human peripheral blood neutrophils were incubated with 0.01–10 μM DHEA-S, 0.01 μM roflumilast or 1 μM dexamethasone for 4 h, transferred to transwell inserts and allowed to migrate in the absence of chemoattractant. Data is represenatative of 3 separate experiments with triplicate measurement of each condition. (* p < 0.0001 compared to unstimulated control).
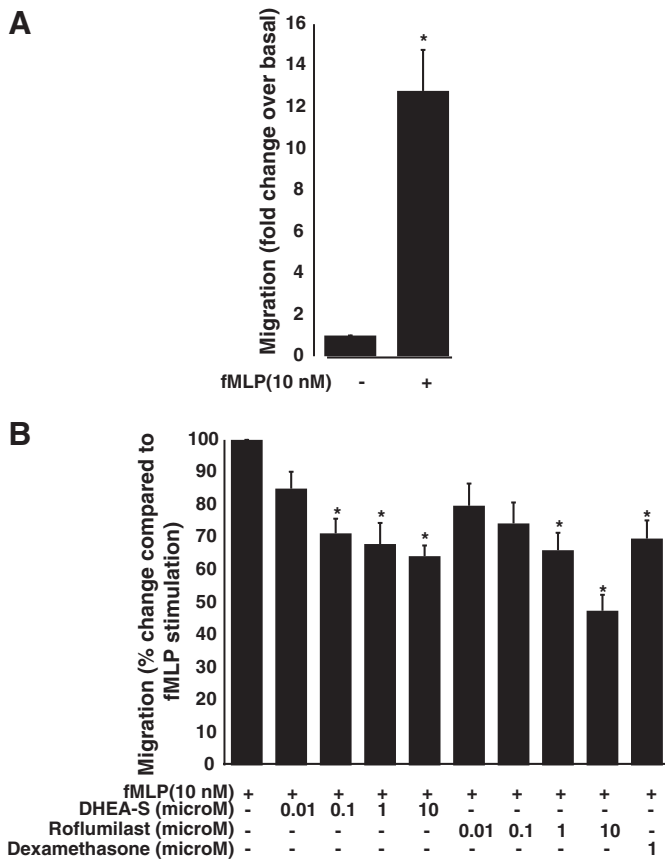
A

B

Fig. 2. DHEA-S inhibits fMLP-induced neutrophil migration. Human peripheral blood neutrophils were incubated with 0.01–10 μM DHEA-S, 0.01 μM roflumilast or 1 μM dexamethasone for 4 h, transferred to transwell inserts and allowed to migrate in the presence of 10 nM fMLP for 2 h (A) and (B). Inhibition by treatment with compounds (B) was expressed as a percentage of fMLP stimulated alone (set at 100%). Data is representative of three to seven measurements per condition tested (*$p < 0.0001$).

(Fig. 3). These data show that DHEA-S inhibits PDGF-induced chemotaxis, but not chemokinesis of HASM cells.

3.3. DHEA-S has no effect on PDGF-induced Akt, ERK1/2, p38 MAPK and PKC phosphorylation

To delineate potential mechanisms whereby DHEA-S attenuates migration of HASM following PDGF stimulation, we first assessed phosphorylation of Akt as a downstream substrate for PI3 kinase activation. Previously, we reported that PI3 kinase (PI3K) activity is required for PDGF-induced HASM migration by utilizing the pharmacological inhibitor LY294002 to inhibit PI3K [11]. We reasoned that DHEA-S alters PI3K-Akt signaling pathways to decrease PDGF-induced migration of HASM. We found that pretreatment of HASM with 10 μM DHEA-S or 1 μM dexamethasone, which inhibited PDGF-induced HASM migration (Fig. 3 and [9]), has little effect on PDGF-induced phosphorylation of Akt. Pretreatment with PI3K inhibitor LY294002 under similar experimental conditions suppressed Akt phosphorylation (Fig. 4A and B). The phosphorylation state of other signaling molecules known to positively modulate cellular migration, including ERK1/2, p38 MAPK and PKCα were examined to assess a potential role for these in DHEA-S-mediated attenuation of PDGF-induced migration (Fig. 4A–D). The phosphorylation of all proteins tested was unaffected by pretreatment with DHEA-S and subsequent stimulation with PDGF. These data suggest that DHEA-S inhibits PDGF-mediated HASM migration independently of canonical pathways known to regulate migration.
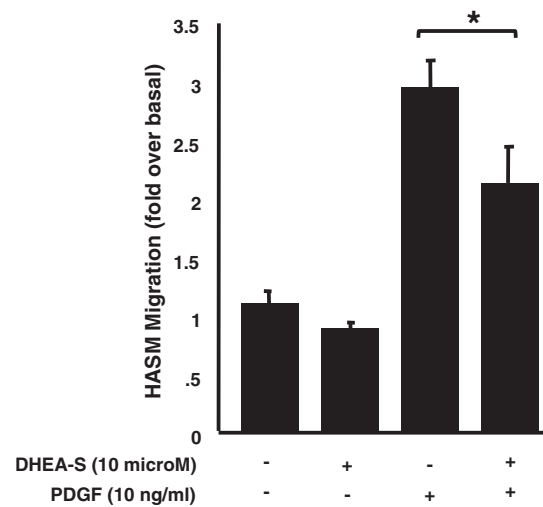

Fig. 3. DHEA-S inhibits PDGF-mediated airway smooth muscle migration. HASM cells were incubated with 10 μM DHEA-S overnight (18 h), loaded into a Boyden chamber and allowed to migrate to 10 ng/ml PDGF for 4 h. Data is representative of triplicate measurements from two different cell lines (*$p < 0.005$).

4. Discussion

In this study, we sought to examine the effects of DHEA-S on migration of human neutrophils (PMNs) and airway smooth muscle cells (HASM). We found that DHEA-S dose-dependently decreased chemokinesis and fMLP-induced chemotaxis of PMNs, as well as PDGF-mediated chemotaxis of HASM. Two types of commonly used asthma and COPD therapeutics were used as comparison since dexamethasone, a glucocorticoid, and roflumilast, a phosphodiesterase 4 inhibitor, inhibit PDGF-induced airway smooth muscle migration [9]. We show that at 10 μM, but not at lower concentrations, roflumilast is more effective than DHEA-S at inhibiting fMLP-induced chemotaxis. Given these dose responses, we show that roflumilast is more potent and effective at similar concentrations of DHEA-S. In an effort to elucidate potential mechanisms for DHEA-S-mediated attenuation of migration, we assessed Akt phosphorylation, a surrogate for PI3K activation, as well as ERK1/2, p38 MAPK and PKCα phosphorylation — signaling molecules known to modulate cell migration. We found that treatment with DHEA-S had little effect on PDGF-mediated Akt, ERK1/2, p38 MAPK and PKCα phosphorylation. Interestingly, a study of vascular smooth muscle demonstrated that DHEA inhibited phosphorylation of Akt at a concentration ten-fold higher than we utilized to inhibit cell migration [12], a finding that contrasts with effect of DHEA-S that we show. Our data suggests that other mechanisms governing cytoskeletal rearrangements necessary for coordinated cell movement are affected and attenuated by DHEA-S treatment, rather than canonical pathways governing cell migration.

Glucocorticoids, a mainstay in the treatment of lung disorders, decrease airway inflammation in asthma and COPD. Although effective at reducing inflammation, steroids have undesirable effects, and some patients are insensitive to glucocorticoids. Use of anti-inflammatory alternatives exhibiting fewer adverse effects may serve as alternative strategies. DHEA and DHEA-S offer potential options to traditional glucocorticoid therapy. In mice, OVA-induced allergic airway inflammation was markedly attenuated by DHEA-S, and this treatment decreased eosinophilic infiltration of the lungs as well as inhibited increases in eotaxin-1 and eotaxin-2, IL-5, IL-13, IL-6 and TNFα expression. Interestingly, DHEA treatment also decreased levels of PGE$_2$ [2]. Collectively, these data suggest that DHEA-S can attenuate cytokine-induced hyper-responsiveness of airway smooth muscle, but may increase the agonist-mediated bronchoconstriction by decreasing endogenous levels of PGE$_2$. Others showed, using *Der f*, a house dust mite allergen, eosinophil influx, and IL-4 and IL-5 levels into the lungs were
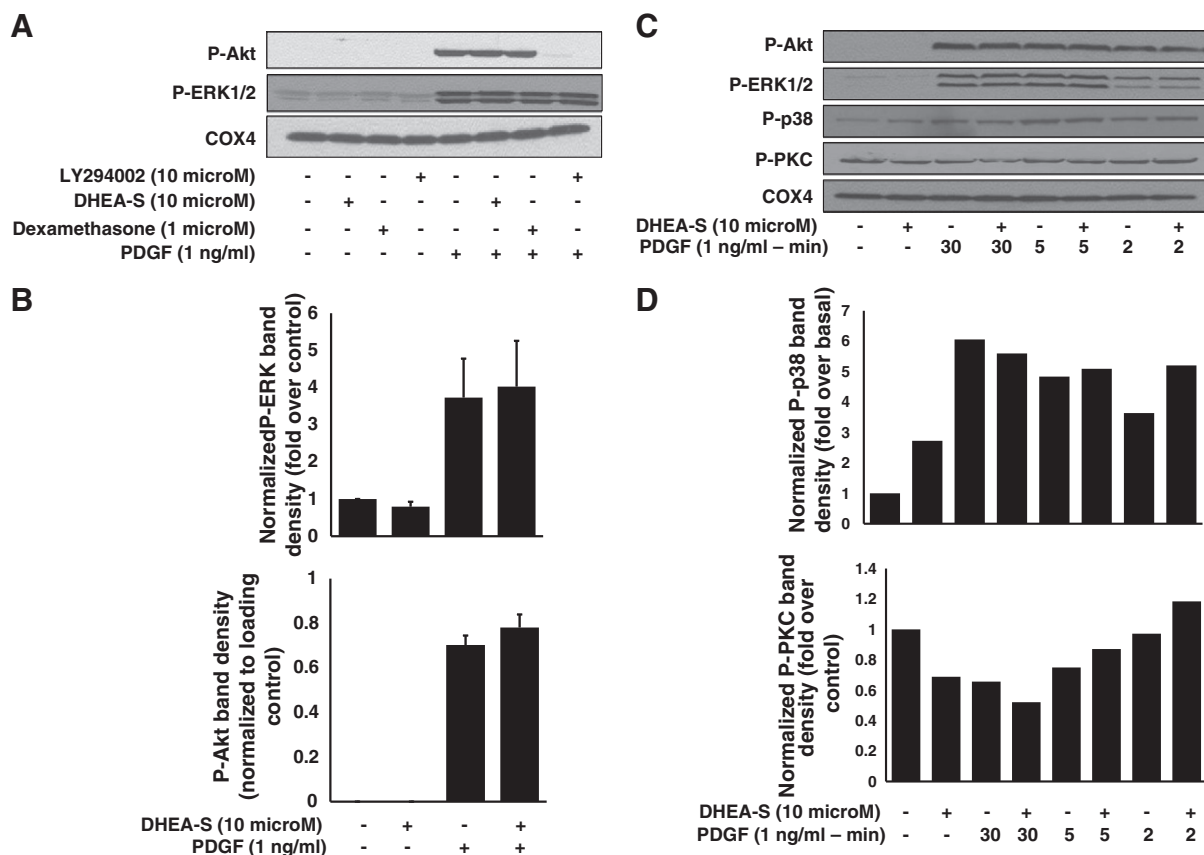
Fig. 4. DHEA-S does not inhibit PDGF-dependent phosphorylation of Akt, ERK1/2, p38 MAPK or PKC. (A–B) HASM cells were incubated with 10 μM DHEA-S (18 h), 1 μM dexamethasone (18 h) or 30 μM LY294002 (40 min) prior to stimulation with 1 ng/ml PDGF (10 min). Phosphorylation of Akt and ERK1/2 was assessed by immunoblotting. Total COX-4 was used as a loading control. (C–D) HASM cells were incubated with 10 μM DHEA-S (18 h) prior to stimulation with 1 ng/ml PDGF (2, 5 and 30 min). Phosphorylation of Akt, ERK1/2, p38 MAPK, and PKC was assessed, using total COX-4 as a loading control. Data is representative of three independent experiments in three different cell lines.

decreased following administration of DHEA [4]. Collectively, these data suggest that DHEA and DHEA-S may modulate inflammatory responses associated with allergen-induced airway diseases. In human studies, nebulized DHEA and DHEA-S attenuated allergen-induced late phase response in mild asthma subjects [3]. Using human PBMCs, DHEA also decreased IL-10, IL-5 and IFNγ production, and this decreased cytokine production was more pronounced in individuals exhibiting airway hyperresponsiveness [13].

Radford et al. demonstrated that treatment of human neutrophils with DHEA-S increased superoxide formation, in both the presence and absence of priming with GM-CSF [14]. Others, utilizing neutrophils and endothelial cells, showed that DHEA treatment increased L-selectin expression, decreased LPS-induced CD11b expression, and had little effect on CD18 expression in neutrophils. On endothelial cells, there was also little effect on LPS-induced levels of VCAM or ICAM-1 expression. Levels of E-selectin, however, were decreased following DHEA alone or in combination with LPS stimulation [15]. In rat tracheal smooth muscle, DHEA treatment decreased proliferation of the smooth muscle following stimulation with FBS or PDGF [16]. Utilizing a rabbit vascular smooth muscle cell line, Furutama et al. demonstrated that DHEA-S administration inhibited PDGF-induced cell migration, proliferation and adhesion to fibronectin [5]. This same study noted using Scatchard analysis that a majority of DHEA-S was found to be bound in the nucleus, suggesting that it is indeed binding a nuclear receptor. Given current evidence, we hypothesized that DHEA-S may have direct effects on neutrophils and HASM that modulate inflammation observed in asthma. We found that treatment of human neutrophils with DHEA-S inhibited both chemotaxis and chemokinesis of the cells. In animal studies, DHEA-S inhibited immune cell recruitment to the lungs following allergen challenge. Our data also suggest that the effects of DHEA-S may not be exclusively a function of decreasing levels of inflammatory mediators necessary to recruit cells, but may also modulate cytoskeletal arrangements within cells. We demonstrated that migration of HASM to the growth factor PDGF was also inhibited by DHEA-S, suggesting a generalized effect of DHEA-S on cell migration. We also showed that a dose of DHEA-S that inhibited migration had little effect on PDGF-induced phosphorylation of Akt, which is a downstream substrate of PI3K that is required for PDGF-mediated HASM migration [11]. The effect of DHEA-S on phosphorylation of ERK1/2, p38 MAPK and PKCα was examined as other potential signaling molecules modulating cell migration. A study examining migration of neutrophils to fMLP noted that inhibition of myristoylated alanine-rich c-kinase substrate (MARCKS), a target of PKCα in addition to ERK1/2 and p38 MAPK, abolished chemokine-dependent migration [17]. We examined phosphorylation of PKCα in our study and found that there was no modulation of phosphorylation of these molecules. Further studies are necessary to define the probable mechanism by which DHEA-S attenuates cell migration; we speculate that DHEA-S directly modulates cytoskeletal rearrangements. Collectively, DHEA-S may have benefit in the treatment of airway disorders such as asthma and COPD, not only by inhibiting inflammatory mediator secretion but also by inhibiting myocyte and immune cell migration.

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