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DHEA has strong antifibrotic effects and is decreased in idiopathic pulmonary fibrosis

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Running title: DHEA in IPF

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Abstract

Idiopathic pulmonary fibrosis (IPF) is an aging-related lung disorder characterized by expansion of the myofibroblast population and aberrant lung remodeling. Dehydroepiandrosterone (DHEA), a steroid pro-hormone, decreases with age but an exaggerated decline has been associated with chronic-degenerative diseases.

We quantified the plasma levels of DHEA and its sulfated form (DHEA-S) in 137 IPF patients and 58 controls and examined the effects of DHEA on human lung fibroblasts.

Plasmatic DHEA/DHEA-S were significantly decreased in male IPF patients, (DHEA, median (max-min): 4.4 (0.2-29.2) versus 6.7 (2.1-15.2) ng/ml; $p<0.01$; DHEA-S, median: 47 (15.0-211) versus 85.2 (37.6-247.0) μg/dl; $p<0.001$), while in women only DHEA-S was significantly decreased (median: 32.6 (15.0-303.0) versus 68.3 (16.4-171); $p<0.001$). DHEA caused a decrease of fibroblast proliferation and a ~2-fold increase of fibroblast apoptosis, likely through the intrinsic pathway with activation of caspase-9. This effect was accompanied by upregulation of several pro-apoptotic proteins (Bax and cyclin-dependent kinase-inhibitor CDNK1A) and downregulation of anti-apoptotic proteins such as c-IAP1/c-IAP2. DHEA also caused a significant decrease of TGF-β1-induced collagen production and fibroblast to myofibroblast differentiation, and inhibited PDGF-induced fibroblast migration.

These findings demonstrate a disproportionate decrease of DHEA/DHEA-S in IPF patients and indicate that this molecule has multiple antifibrotic properties.

Key words: pulmonary fibrosis, fibroblasts, dehydroepiandrosterone, DHEA.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease of unknown etiology that causes progressive and irreversible destruction of the lung architecture (1, 2). The disease has a dismal prognosis, with a median survival of approximately 2-3 years after diagnosis (3). Patients with IPF are usually between 50-70 years old and its frequency increases markedly with age (4). Some studies indicate that at least in part, the aging association can be related to an accelerated shortening of telomeres (5). However, the mechanisms linking aging with IPF are largely unknown (1, 6).

Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEA-S) are the most abundant adrenal steroids in humans (7). Blood levels of DHEA/DHEA-S reach a peak between the ages of 25 and 30 years and thereafter decline gradually, so that by the age of 60, the concentrations are only 10-20% of corresponding values in young adults (7).

Abnormally decreased DHEA has been linked with immunosenescence, a decline of the immune system that occurs with aging (8), as well as with several chronic diseases also associated with elderly, such as insulin resistance, atherosclerosis, systemic arterial hypertension, and Sjögren syndrome (9-11). Accordingly, DHEA has been proposed as aging-protective molecule.

In this study, we analyzed the plasma and bronchoalveolar lavage levels of DHEA and DHEA-S in patients with IPF and age-matched controls. We further determined whether DHEA could affect the proliferation, migration and apoptosis of human lung fibroblasts, as well as the differentiation of fibroblasts to myofibroblasts and collagen synthesis. Our results showed that DHEA/DHEA-S is abnormally decreased in IPF patients and that this adrenal steroid has strong antifibrotic effects in vitro.

Materials and Methods

Study Population

Plasma samples were obtained from 137 consecutive IPF patients, 87 males and 50 females (64.1±9.9 years) diagnosed at the Institute, and from 58 age- and sex-matched controls. The healthy donors, 30 males and 28 females (56.5±4.6 years) were enrolled from the personnel of our Institute and from our Smoking Cessation Program, according to their willingness to participate in the study. The Bioethics Committee of the National Institute of Respiratory Diseases approved the protocol, and signed consent was obtained from patients and controls.

Bronchoalveolar lavage (BAL) was carried out as previously described (12) in 3 control donors, (58±7.7 years) and 12 IPF patients, all males (62.8±4.6 years).

Control subjects underwent a brief clinical history to exclude the presence of other disorders such as diabetes, cardiovascular disease, endocrine disorders or cancer. Diagnosis of IPF was confirmed according to ATS/ERS guidelines (2).

DHEA/DHEA-S analysis

Plasma and BAL levels of DHEA were measured by using a solid-phase ^{125}I radioimmunoassay (Siemens Medical Solutions Diagnostics, Los Angeles, CA; DHEA RIA DSL 9000) and DHEA-S by chemiluminescent assay (Siemens Medical Solutions Diagnostics, CA; DHEA SC chemiluminescent LKDS-1).

Cell culture

Human normal lung fibroblasts CCD25-Lu were acquired from the ATCC. Fibroblast primary cell lines from normal and IPF lungs were obtained as described (13). CCD25-Lu was cultured in DMEM while primary cell lines were cultured in Ham's F-12 medium all of them supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml

of streptomycin. Cell cultures were maintained at 37ºC in a humidified atmosphere of 5% CO2. Cell viability was assessed by trypan blue exclusion.

Growth rate assay

Cell number was assessed by tetrazolium colorimetric WST1 assay (Roche, Germany), as described (13). Fibroblasts were plated into 96-well microplates (10,000 cells/cm^2) and cultured with or without DHEA at concentrations of 50, 100 and 200 µM for 24, 48 and 72 hours. At the end of the experiment, medium was replaced by fresh medium containing WST1 solution and absorbance was analyzed on an ELISA plate reader (Sinergy HT, BioTek VT). Effect of DHEA on growth rate was expressed as a percent of control. These DHEA concentrations have been used elsewhere (14-16).

Bromodeoxyuridine Incorporation

Cells were seeded at a density of 10,000/cm^2 in 96-well microplates, allowed to attach overnight and treated with or without DHEA (50, 100 and 200 µM) for 24 and 48 hours. Fibroblast proliferation was evaluated by analyzing 5′-Bromo-2′-Deoxy-uridine (BrdU) incorporation into newly synthesized DNA using a cell proliferation enzyme linked immunosorbent assay (Cell proliferation ELISA, Roche) (17). Optical density was measured using an ELISA plate reader (Sinergy HT, BioTek VT) at 450/595 nm. Proliferation was expressed as a percent of control.

Apoptosis assay

Apoptosis was examined by flow cytometry using a dual staining with annexin V-FITC and propidium iodine (Annexin staining kit, BD Biosciences, CA) according to manufacturer's instructions as previously described (18). Briefly, 1x10^6 cells in 100 µl annexin buffer were stained with propidium iodide staining solution and FITC annexin V staining solution. Cells were incubated at room temperature in the dark for 15 minutes and acquired in a FACSAria flow cytometer (Becton-Dickinson, CA). Data

were analyzed using FlowJo 8.7 software (Stanford University).

Caspase activity

Fibroblasts were lysed in caspase buffer (10 mM Tris-HCl, 10 mM NaH2PO4, 130 mM NaCl, 1% (v/v) Triton X-100). Protein concentration was assessed by Bradford method, and caspase activity was assayed by a fluorometric method as previously reported (19) in a luminescence spectrometer (Sinergy HT, BioTek VT, USA) at 380/430-460 nm. Acetyl tetrapetides coupled to 4-methycoumaryl-7-amide were used as substrates: Ac-DEVD-AMC (caspase-3/7/8) and Ac-LEHD-AMC (caspase-9) (PeptaNova GmdH Sandhausen, Germany). Results are expressed as the change in UF/min/mg protein produced after substrate cleavage.

Human apoptosis antibody array

The expression profile of 35 apoptosis-related proteins was measured using a human apoptosis array kit (Proteome Profiler Array ARY009, R&D Systems) following the manufacturer´s instructions as described (20). Mean spot pixels were quantified by densitometry. Data were expressed as fold-change and arbitrarily considered as biologically important a change > 30%.

Migration assay

Fibroblast migration was assayed using 24-well collagen-coated Boyden chambers (Chemicon, Millipore, Bedford, MA, USA) with an 8-µm pore size as described (21). Cells ($2x10^5$) were seeded in the upper chamber and DHEA (100 µM), PDGF 20 ng/ml (R&D Systems, Minneapolis, MN) or both were added in the lower chamber containing 0.5 ml of medium with 5% BSA. After incubation for 8 h the migrating cells were quantified according to manufacturer's instructions.

Western blotting

Fibroblasts were lysed with RIPA buffer containing multiple protease inhibitors (Sigma Aldrich). Sample proteins (40 μg) were separated by 10 % SDS-PAGE electrophoresis, transferred onto nitrocellulose membranes (Bio Rad, Hercules CA) and blocked following standard protocols. Membranes were incubated overnight at 4°C with primary antibodies: α-SMA (1:500; Sigma Aldrich.), caspase-9 (1:500; Abcam, MA), and β-Tubulin (1:500; Santa Cruz Biotechnology Inc.), followed by secondary antibodies (anti-mouse and anti-rabbit IgG, Invitrogen Life Technologies, CA) coupled to horseradish peroxidase for 1 h at room temperature and visualized by Super Signal West Pico Enhanced Chemiluminescence detection system (Thermo Scientific, Rockford, IL. Signals were normalized against β-tubulin.

Immunofluorescence

Fibroblasts were plated on coverslips and incubated with serum-free medium alone or containing DHEA 100 μM, TGF-β1 (5 ng/ml), or both for 48 h. Cells were fixed with acetone/methanol at 4°C, permeabilized with Triton 0.5% and incubated with human monoclonal α-SMA antibody (Sigma Aldrich) at 4°C overnight in a humidified chamber. Then, cells were treated with an affinity-purified goat anti-mouse IgG DyLigtht 549 (Biocare Medical, Concord CA) during 1 h in a humidified chamber at room temperature. Slides were counterstained with DAPI mounting medium (Santa Cruz Biotechnology Inc., CA).

Collagen Assay

Fibroblasts were seeded into 25 cm^2 culture flasks and cultured until 70% confluence and stimulated in serum-free medium with DHEA (100 μM), TGF-β1 (5ng/ml) or both for 48 hours. Supernatants were dialyzed, lyophilized and resuspended to a volume of 100 μl. Collagen concentration was determined by colorimetric Sircol soluble collagen assay (Biocolor Ltd., UK) according to manufacturer's instructions (22). Optical density

was measured at 555 nm using an ELISA plate reader (Sinergy HT, BioTek VT). Collagen concentration was expressed as relative to control.

Statistical analysis

Plasma levels results were not normally distributed and were logarithmically transformed for statistical analysis. Since the study groups did not follow the normal distribution, we use a non-parametric approach for the descriptive statistics (median and range) and the statistical analysis (Mann-Whitney U test). In vitro data are given as mean ± SD. Differences were assessed by one way ANOVA or Wilcoxon test. $P<0.05$ was considered significant. Data were analyzed using the statistical program SPSS for Windows, release 14.0 (SPSS Inc., Chicago, IL, USA).

Results

DHEA and DHEA-S are decreased in patients with IPF

Plasma levels of DHEA and DHEA-S were measured by radioimmunoassay and chemiluminescent assays, respectively, in 137 IPF patients and 58 controls. As illustrated in **Figures 1A and 1B**, both molecules were significantly decreased in male IPF patients, (DHEA, median (max-min): 4.4 (0.2-29.2) versus 6.7 (2.1-15.2) ng/ml; $p<0.01$; DHEA-S median: 47 (15.0-211) versus 85.2 (37.6-247.0) μg/dl; $p<0.001$), while in women only DHEA-S was significantly decreased (median: 32.6 (15.0-303.0) versus 68.3 (16.4-171); $p<0.001$). DHEA in women showed a non-significant tendency to decrease (median: 4.3 (0.9-28.9) versus 5.7 (0.98-18.3), $p=0.2$).

DHEAS concentration was also significantly decreased in BAL samples from IPF patients (2.31 ± 0.61 versus 1.37 ± 0.14 μg/dl; $p<0.01$). No differences were observed in DHEA levels.

No correlations were found between clinical and functional parameters (e.g., forced vital capacity) and the DHEA or DHEA-S plasma concentrations (data not shown).

DHEA decreases lung fibroblast proliferation

Cell growth was quantified by the WST-1 proliferation assay in the CCD25Lu normal cell line as well as in three primary cell lines, one obtained from normal lungs, and two from IPF lungs. No differences in growth rate were observed under basal conditions between IPF and control fibroblasts (152 ± 10 versus 157 ± 7 at 48 h and 214.0 ± 2.99 versus 200.4 ± 10.1 at 72 h). As shown in **Figure 2A**, DHEA treatment caused a dose and time dependent decrease of cell growth. In general, this effect was significant with DHEA concentrations ≥ 50 μM and was stronger after 48 hours. The inhibitory effect of DHEA on human lung fibroblast proliferation was corroborated measuring BrdU

incorporation into DNA (**Figure 2B**). In normal and IPF fibroblasts, DHEA caused a significant decrease in cell proliferation from concentrations of 50 μM.

DHEA induces apoptosis in lung fibroblasts

The effect of DHEA on cell death was examined using 100 and 200 μM. To detect early apoptosis, DHEA-treated and control fibroblasts were assayed for the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane using annexin V binding assay. As shown in **Figure 3A**, at 24 and 48 hours of culture both DHEA concentrations provoked a significant increase in fibroblast apoptosis. To examine whether the apoptosis induced by DHEA was mediated by the extrinsic or intrinsic pathway we evaluated caspase-8 and caspase-9 by immunoblotting. Stimulation with 100 μM DHEA induced in lung fibroblasts at 24 hours an increase of the active caspase-9 (**Figure 3B**). Activation of pro-caspase-9 was corroborated by fluorometry (**Figure 3C**). No changes were observed with pro-caspase-8 (data not shown). Then, we examined whether DHEA induced apoptosis in IPF fibroblasts and if it was influenced by the inhibition of caspase 9. We found that basal apoptosis (2.8 ± 1.3) increased to 6.1 ± 0.6 when cells were exposed to DHEA 100 μM ($p<0.05$). Caspase-9 inhibitor attenuated DHEA-induced fibroblast apoptosis (3.6 ± 0.3; $p<0.05$).

To further explore the apoptotic/antiapototic proteins likely involved in DHEA-induced cell death we used a human apoptosis antibody array (**Figure 4**). Our results showed that DHEA increased some apoptosis receptors, such as tumor necrosis factor receptor 1 (36.5±5.2) and TRAILR2 (43.9±8.7). An increase of the pro-apoptotic marker Bax (49.7±6) and of the cyclin-dependent kinase inhibitor CDNK1A (p21/Cip1/CDNK1A) which inhibits cell cycle progression (23) was also detected. In addition, DHEA induced a decrease in the levels of several anti-apoptotic proteins such as the cellular inhibitor of

apoptosis c-IAP1 (34±1.5), c-IAP2 (44.4±3.7), survivin (60.5±11), claspin (70±2.2) and livin (41.6±7).

DHEA inhibits PDGF-induced migration of lung fibroblasts

The effect of DHEA on cell migration was examined using collagen-coated Boyden chambers in two normal (**Figures 5A and 5B**) and two IPF (**5C and 5D**) fibroblast cell lines. The number of cells that migrated in the presence of medium plus 5% BSA alone was used as control, while PDGF, a potent fibroblast chemoattractant, was used as a positive control. As shown in **Figure 5**, PDGF-induced fibroblasts migration was significantly reduced by DHEA in both normal human lung and IPF fibroblasts. Around 30-70% PDGF-BB-induced fibroblast migration was inhibited by 100 µm DHEA.

DHEA inhibits TGFβ-induced fibroblast to myofibroblast differentiation

The effect of DHEA on the expression of α-SMA, the hallmark of myofibroblasts, was examined by Western blot using two different approaches. In the first of them, fibroblasts were cultured simultaneously during 48 hours with TGF-β1 and DHEA. As exemplified in **Figure 6A**, 100 µM DHEA concentrations induced a strong reduction of TGF-β1-induced α-SMA in both normal **(N1 and N2)** and IPF **(IPF1 and IPF2)** fibroblasts. In the other approach, fibroblasts were first stimulated with TGF-β1 for 48 h, and then treated with DHEA for additional 48 h, without removing the growth factor. As shown in **Figure 6B**, an inhibitory effect was observed with 100 µM DHEA in normal human lung fibroblasts. The attenuation of fibroblast to myofibroblast differentiation by DHEA was corroborated by immunofluorescence, both in normal lung fibroblasts (**Figure 7 a-h**) and IPF fibroblasts (**Figure 7 i-p**).

DHEA reduces TGFβ-induced collagen production

To investigate whether DHEA has an effect on collagen synthesis, human lung fibroblasts were stimulated with TGF-β1 and collagen protein was measured in cell

culture supernatants. As shown in **Figure 8**, collagen production increased 2-3-fold upon stimulation with TGF-β1 as assessed by the Sircol assay. This effect was significantly reduced by DHEA at 100 μM in both normal human lung fibroblasts (**Figures 8A and 8B;** $p<0.01$), and IPF derived fibroblasts (**Figures 8C and 8D;** $p\leq 0.05$).

Discussion

IPF is a progressive and usually lethal lung disease of unknown etiology occurring primarily in elderly adults (1, 4, 6). However, the mechanisms that link aging with IPF remain elusive, although accelerated shortening of telomeres that may affect alveolar epithelial cell regeneration, and epigenetic processes that seem to compromise both epithelial cells and fibroblasts have been proposed (1, 5, 6, 24).

Dehydroepiandrosterone and DHEA-sulfate are anabolic prohormones secreted by the human adrenal cortex. DHEA-S is the hydrophilic storage form that circulates in the blood while evidence indicates that lipophilic DHEA is the one converted intracellularly to androgens and estrogens. Circulating concentrations of DHEA and DHEA-S decline progressively throughout adult life so that after 60-70 years of age, the levels are only 10-20% of those detected in young adults.

Lower circulating levels have been correlated with the incidence of inflammatory and malignant processes (25, 26). Thus, blood DHEA-S level has been found independently and inversely associated with immunosenescence, autoimmune diseases, non-alcoholic steatohepatitis, insulin resistance, atherosclerosis, and systemic arterial hypertension (8-11, 27). Likewise, evidence indicates that low levels of DHEA-S predict death from all causes, cardiovascular disease and ischemic heart disease in older men (28). Moreover, it has been recently shown that long-term DHEA replacement may reduce insulin resistance and improved glucose tolerance, in elderly, overweight or obese women and men with abnormal glucose tolerance (29).

In our study we found, for the first time to our knowledge, that IPF patients had a disproportionate decrease of the circulating levels of DHEA-S compared with age-adjusted controls. Studies in other fibrotic disorders are scanty, although more advanced

nonalcoholic fatty liver disease, as indicated by the presence of severe fibrosis, is also strongly associated with low circulating DHEA-S (26, 30).

However, the underlying mechanisms associated with the putative protective, antifibrotic, role of DHEA are unknown.

In this context, it is important to emphasize that studies dealing with the mechanisms by which DHEA exerts it effects, primarily in fibroblasts, are scanty and have given contradictory results. For example, regarding collagen synthesis, it has been reported that DHEA induced in skin fibroblasts a modest increase in collagen gene expression, but without any effect in collagen synthesis (31). By contrast, and similar to our results, treatment with DHEA resulted in a significant decrease in procollagen type I mRNA and in procollagen type I protein synthesis in cardiac fibroblasts (32).

Regarding apoptosis, results in the literature are also contradictory. Thus, while a protective role of DHEA against apoptosis was reported in immortalized human keratinocytes and neurons (33, 34), in other cell types such as human hepatocellular carcinoma cells, and pulmonary artery smooth muscle cells DHEA induced apoptosis (15, 35). Therefore, it is likely that the cell response to DHEA is related with the cell type, and/or cell tissue origin.

In the present study, we evaluate the effects of DHEA on some important lung fibrogenic processes, including migration, proliferation, apoptosis and collagen synthesis by human lung fibroblasts as well as on fibroblast to myofibroblast differentiation. DHEA was used because DHEA-S is the hydrophilic storage form that circulates in the blood, while DHEA is the functional form and the principle form used in steroid hormone synthesis. For this reason, despite that DHEA-S is the form usually found decreased in blood studies in different chronic diseases associated to aging, it is DHEA which is used for in vitro or in vivo experiments (36).

Fibroblast migration to the injured areas in response to chemoattractant signaling molecules, and their subsequent expansion in the lesion are two important profibrotic mechanisms. In this context, our results showed that DHEA is a potent inhibitor of PDGF-induced migration of fibroblasts through a type 1 collagen matrix as well as on fibroblast proliferation, although the specific pathways that promote these activities remain to be determined.

In the fibroblastic foci of IPF lungs, fibroblasts undergo a phenotypic change to myofibroblasts with the subsequent upregulation of extracellular matrix macromolecules (1). This process plays a key fibrogenic role in this disease where myofibroblasts persist and contribute to pathological scar formation. In this regard, we found that TGF-β1-induced fibroblast to myofibroblast differentiation, evaluated through the expression of α-SMA, was significantly reduced in a dose-dependent manner by DHEA. Attenuation of myofibroblasts differentiation was also observed by immunofluorescence in IPF derived fibroblasts.

Fibrotic disorders such IPF are characterized by an increased deposition of fibrillar collagens, and we assumed that DHEA would suppress collagen synthesis by lung fibroblasts. Paralleling the inhibitory effect on myofibroblasts differentiation, we observed that DHEA repressed TGF-β1-induced collagen synthesis.

The reasons why fibroblasts and myofibroblasts persist in the IPF fibroblastic foci and appear to be resistant to apoptosis are unclear. TGF-β1 plays an important role, promoting two myofibroblast prosurvival signaling pathways, focal adhesion kinase and protein kinase B (37, 38). Therefore, we next examined the effect of DHEA on fibroblast cell death. Using FACS analysis to quantify the level of apoptosis, we observed that DHEA promotes fibroblast cell death, in a time dependent manner. Importantly, our findings also showed that treatment with DHEA resulted in activation

of caspase-9 indicating that DHEA activates the intrinsic pathway of apoptosis. Progression through the intrinsic pathway leads to mitochondrial release of cyt C into the cytosol inducing apoptosome formation with the activation of procaspase-9, which in turn cleaves and thereby activates downstream effector caspases (39-41). To identify other molecules likely be involved in the DHEA-induced changes of the finely balance of pro-apoptotic and anti-apoptotic factors we employed a human apoptosis antibody array. This array allows the semi-quantitative analysis of the changes occurring in critical apoptosis and checkpoint pathway proteins. Important antiapoptotic proteins (c-IAP1, c-IAP2, livin, survivin, claspin) where down regulated further supporting that intrinsic apoptotic pathway is activated while the increase of death receptors (TNFR1 and TRAILR2) suggests a crosstalk between intrinsic and extrinsic apoptotic pathways.

All these findings on normal and IPF fibroblasts provide strong evidence that DHEA could act as an anti-fibrotic compound against fibroproliferative diseases, including, but not limited to, pulmonary fibrosis, although the intracellular mechanisms involved in these different biological effects need to be explored.

In this context, it is important to emphasize that the cell receptors and intracellular signaling pathways by which DHEA exerts its effects are presently unclear. Moreover, since DHEA is a multifunctional steroid with a broad range of biological effects, molecular mechanisms may be varied. Although DHEA binding to membrane-associated proteins has been demonstrated, definitive evidence for a DHEA-specific membrane receptor including isolation and sequencing of the gene for the purported receptor is lacking (42). It has been hypothesized that DHEA may exert its effects through the estrogen/androgen receptors. However, different studies have shown that the effects of DHEA on proliferation and apoptosis are independent of both receptors (43, 44) whereas some studies have suggested the implication of one receptor

potentially coupled to a G-protein (42, 43). In the last 10 years, a series of orphan receptors, namely, peroxisome proliferators activated receptor, pregnane X receptor, and constitutive androstanol receptor, have been reported and there is evidence that DHEA (or some of its metabolites) either bind to or activate these newly characterized receptors (36).

Regarding signaling pathways, it has demonstrated that DHEA affects the cell cycle by blocking the PI3K pathway (15). There is also evidence that DHEA negatively regulates the MAPK pathway in humans via a novel MAPK phosphatase, suppressing the p38-MAPK cascade (45). Additionally, in an experimental model of pulmonary hypertension, DHEA prevented the activation of RhoA–ROCK signaling pathway although the cell target was not identified (46).

In summary, our findings demonstrate that the circulating levels of DHEA/DHEA-S is decreased in IPF and indicate that DHEA display a strong antifibrotic effect on fibroblasts affecting migration, proliferation, differentiation to myofibroblasts, collagen synthesis and survival. These findings may open new therapeutic options for this destructive lung disease.

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Legend for Figures

Figure 1: Sulfated DHEA is decreased in patients with IPF. Plasma levels of DHEA (**A**) and DHEA-S (**B**) were examined by radioimmunoassay and chemiluminescent assay in male and female healthy donors and IPF patients. Values represent median (min-max). $^{\&}p<0.001$ and $^{\&\&}p<0.01$.

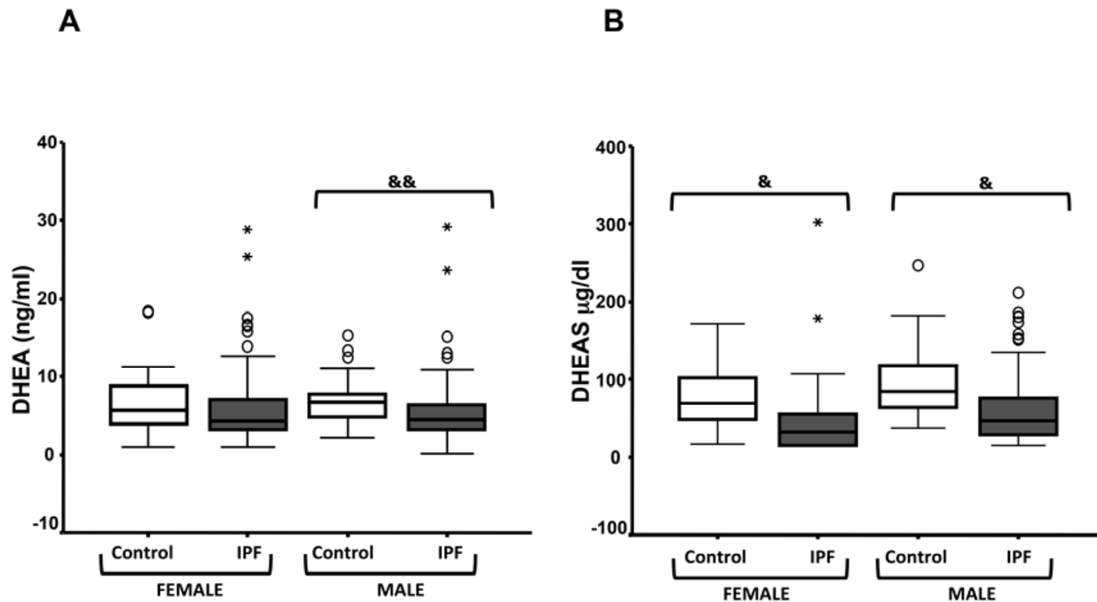

Figure 1

Figure 2: DHEA decreases growth rate and proliferation in normal and IPF human lung fibroblasts. (A) Growth rate was measured by WST-1 assay in two normal **(N1** and **N2)** and two IPF **(IPF1** and **IPF2)** human lung fibroblast cell-lines at 24, 48 and 72 hours in the absence or presence of increasing concentrations of DHEA (50, 100 and 200 μM). Data are expressed as mean ± SD from 3 independent experiments; *$p<0.01$; **$p<0.05$. **(B)** Cell proliferation was measured by BrdU incorporation into DNA at 24 hours in a normal **(N)** and an **IPF** fibroblast cell-line in

the absence or presence of increasing concentrations of DHEA (50 and 100 μM). Data are expressed as mean ± SD from 3 independent experiments. *p<0.01.

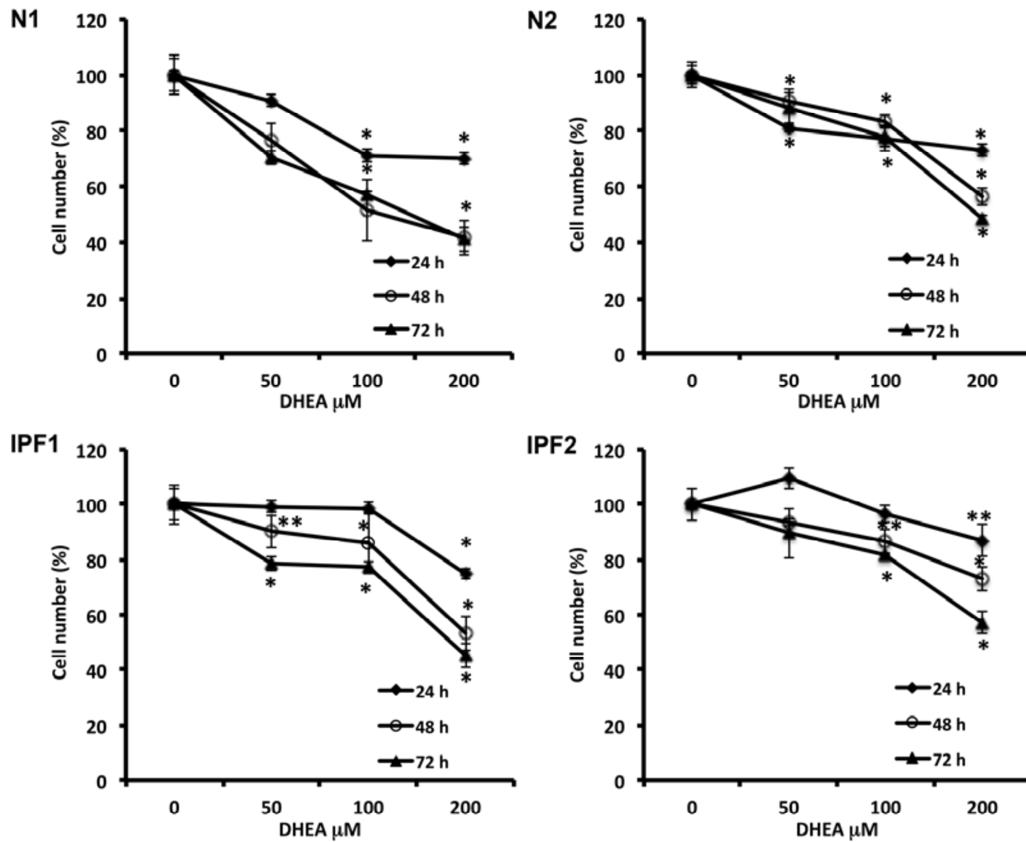

Figure 2A

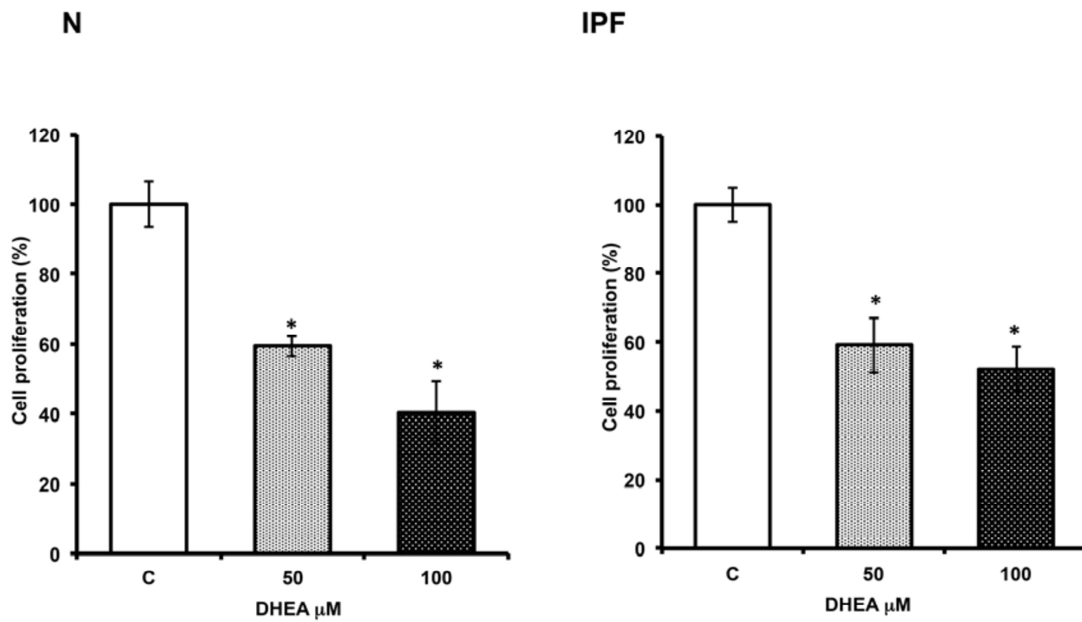

Figure 2B

Figure 3: DHEA induces apoptosis of human lung fibroblasts. A: Normal fibroblast cell-line CCD25Lu was incubated for 12, 24 and 48 hours in the absence or presence of increasing concentrations of DHEA (100 and 200 μM) in FBS supplemented medium. Apoptosis and necrosis were quantified by FACS using Annexin V-FITC and PI respectively. Levels of apoptosis are expressed as % in the total number of acquired events in at least three independent experiments. Data are expressed as mean ± SD; *$p<0.01$. **B:** Representative western blot analysis of pro-caspase-9 and active caspase-9 expression in total lysates obtained from the normal human lung fibroblast cell-line CCD25Lu. Fibroblasts were exposed to DHEA 100 μM for 0, 4, 12 and 24 h, and β-tubulin was used as loading control to normalize by densitometric analysis (bottom). **(C)** Caspase-9 activity was also assayed by a fluorometric method using LEDH-AMC as substrate in lysates of normal cells exposed during 12 and 24 h with or without 100 μM DHEA. Data are expressed as mean ± SD from 3 independent experiments; *$p<0.01$.

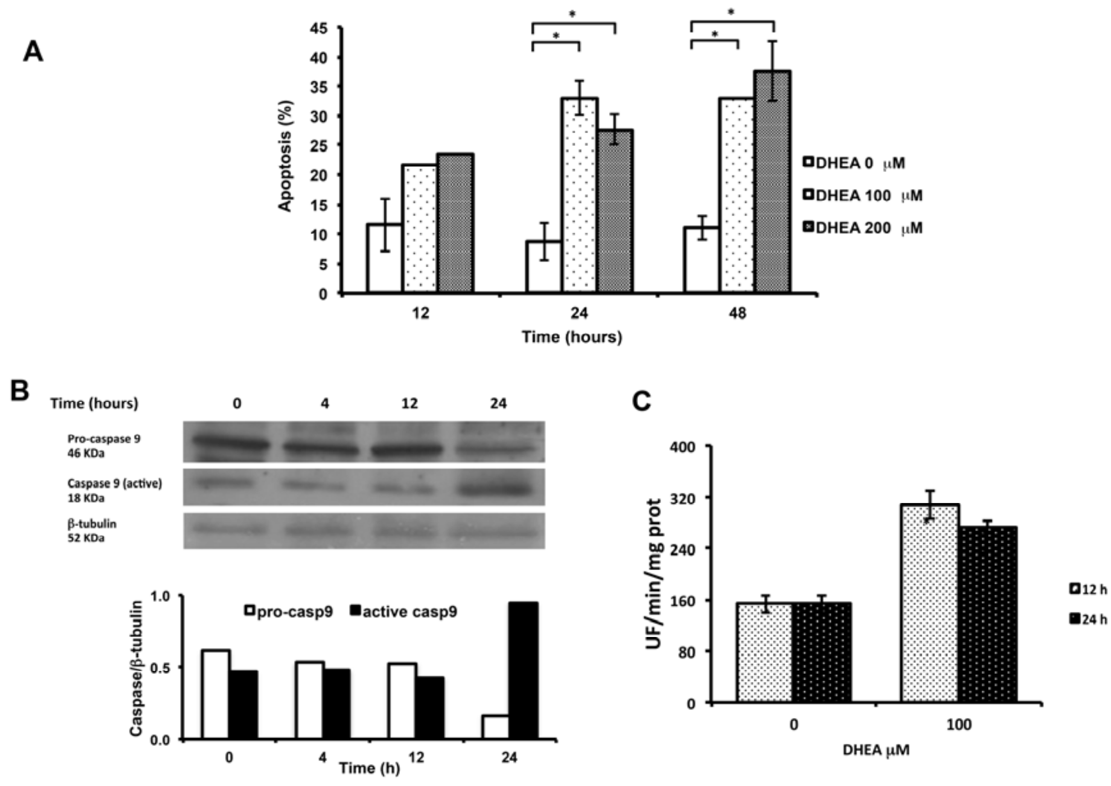

Figure 4: Effect of DHEA on proteins involved in apoptosis. The expression profile of 35 apoptosis-related proteins was measured using a human apoptosis array kit as described in Methods. Normal fibroblasts CCD25Lu were treated with or without DHEA 100 μM for 24 hours and cell lysates were used for antibody array. Data are expressed as fold-change ± SD related to the non-treated control.

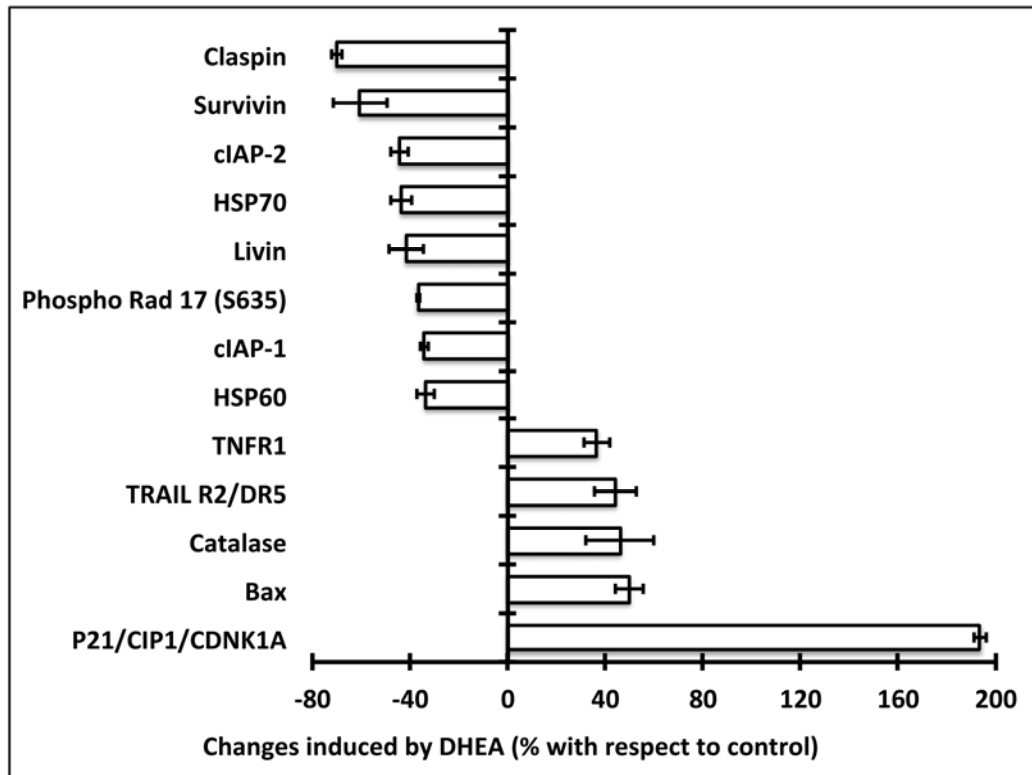

Figure 4

Figure 5: DHEA inhibits PDGF-induced migration of lung fibroblasts. Cell migration assay was performed in normal (A and B) and IPF (C and D) lung fibroblasts in the absence (control) or presence of DHEA 100 μM and/or PDGF-BB 25 ng/ml during 8 hours in collagen covered Boyden chambers as described in methods. Data are expressed as mean ± SD from 3 independent experiments. *$p<0.01$ and **$p<0.05$.

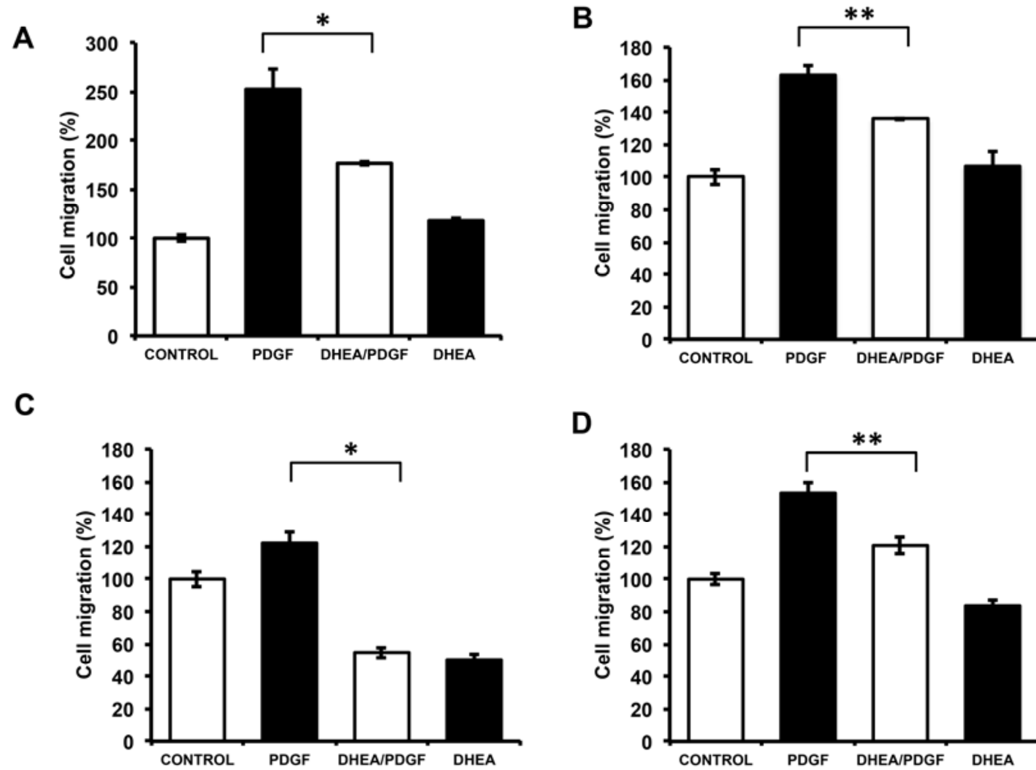

Figure 5

Figure 6: DHEA inhibits fibroblast to myofibroblast differentiation induced by TGFβ. Representative Western blots of α-SMA in total lysates of fibroblasts that were exposed to 5 ng/ml of TGF-β1 and/or to DHEA in FBS supplemented medium for 48 h. **(A)** Normal (N1 and N2) and IPF fibroblasts (IPF1 and IPF2) were stimulated simultaneously with TGF-β1 and DHEA 100 μM. Densitometric analysis of each experiment is included to the right. DHEA caused a 2-4 fold decrease of TGF-β induced aSMA expression ($p<0.05$). **(B)** Normal fibroblasts were stimulated with TGF−β1 for 48 hours and subsequently with increasing concentrations of DHEA (25, 50 and 100 μM) for an additional period of 48 hours without removing TGF−β1. β-tubulin content was used as loading control for normalization.

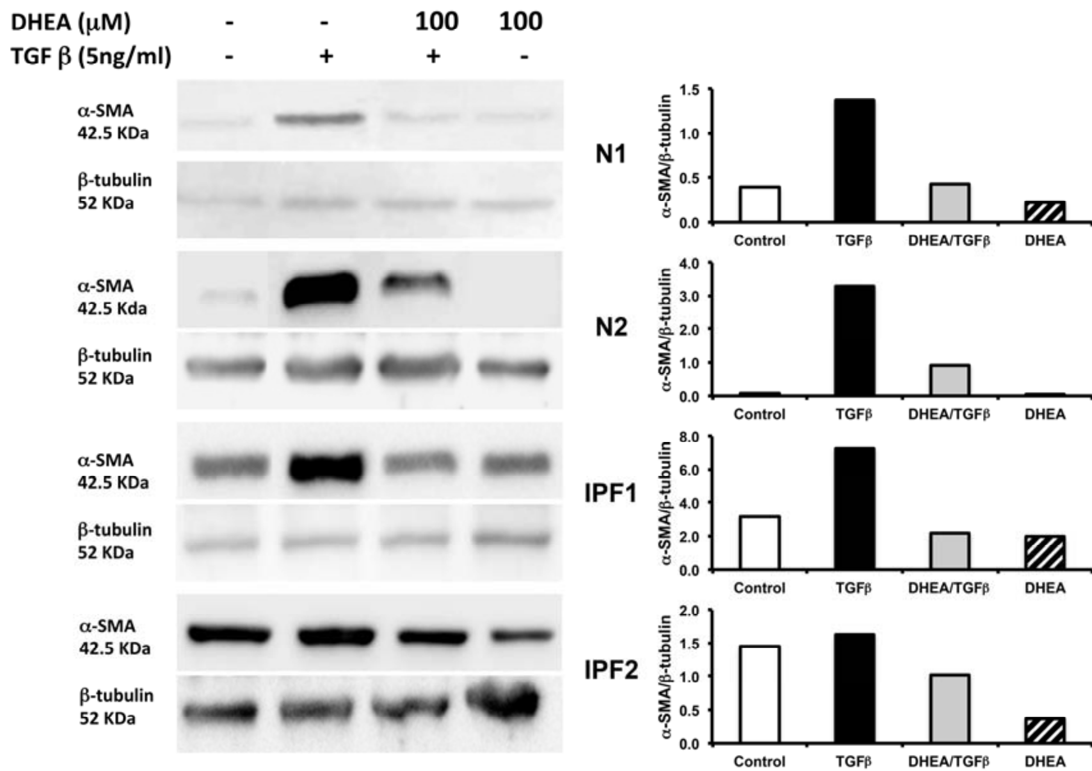

Figure 6A

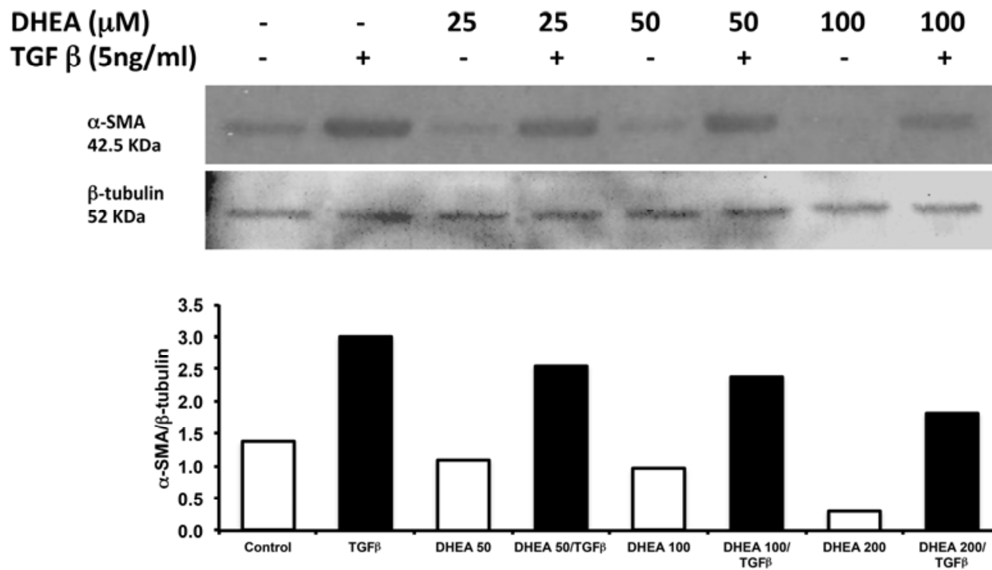

Figure 6B

Figure 7: DHEA inhibits α-SMA expression induced by TGF-β in normal and IPF lung fibroblasts. Representative immunofluorescence staining for α-SMA from human normal and IPF fibroblasts stimulated with or without TGF-β1 (5 ng/ml) and/or 100 μM

DHEA for 48 h. Simultaneous staining of nuclei with DAPI is also shown in blue. Original magnification: X20.

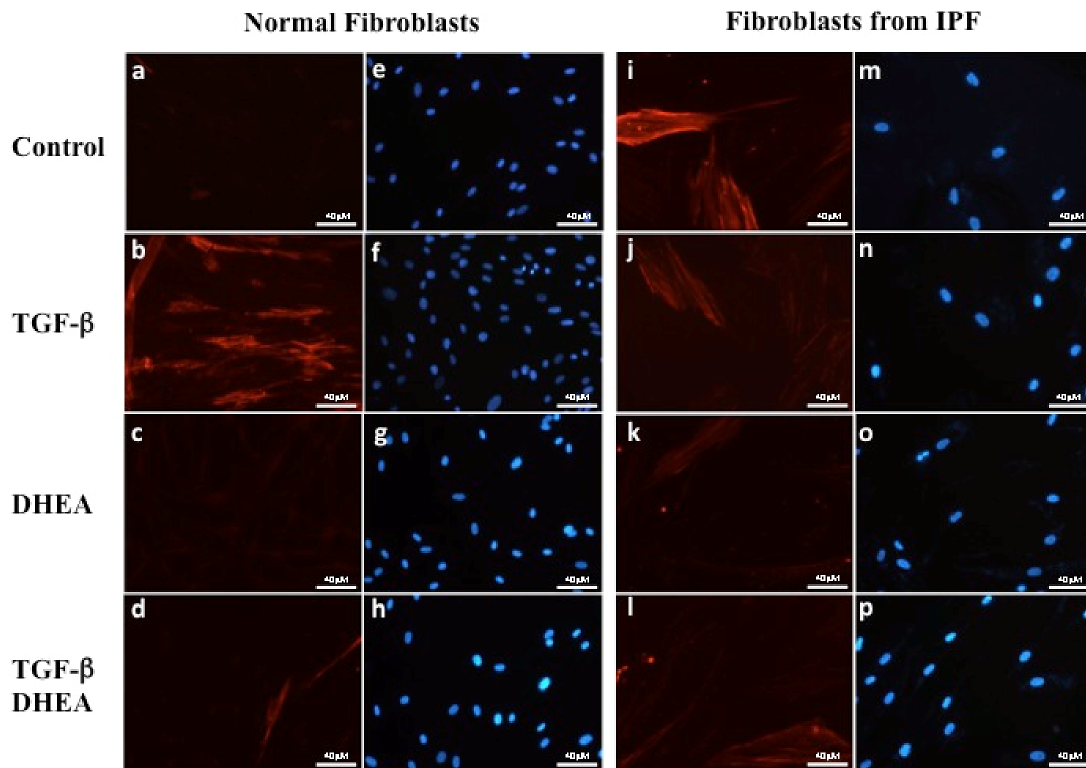

Figure 8: DHEA reduces TGF-β-induced collagen production. Normal (A and B) and IPF (C and D) lung fibroblasts were treated with or without TGF-β1 (5 ng/ml) and/or DHEA 100 μM for 48 h. Collagen concentration in conditioned media was quantified by Sircol collagen soluble assay. Data are expressed as mean ± SD from 3 independent experiments; *$p<0.01$, **$p\leq 0.05$.

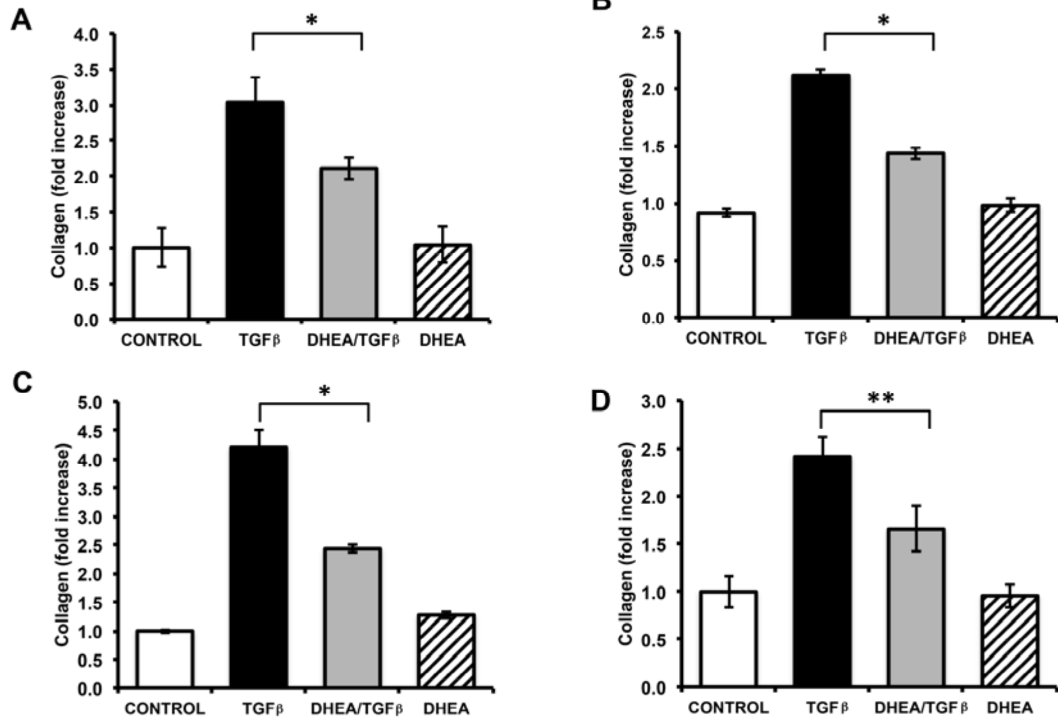

Figure 8