Age-associated increase of skin fibroblast-derived prostaglandin E$_2$ contributes to reduced collagen levels in elderly human skin

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Abstract

Production of type I collagen declines during aging, leading to skin thinning and impaired function. Prostaglandin E$_2$ (PGE$_2$) is a pleiotropic lipid mediator that is synthesized from arachidonic acid by the sequential actions of cyclooxygenases (COX) and PGE synthases (PTGES). PGE$_2$ inhibits collagen production by fibroblasts *in vitro*. We report that *PTGES1* and *COX2* progressively increase with aging in sun-protected human skin. *PTGES1* and *COX2* mRNA was increased 3.4-fold and 2.7-fold, respectively, in the dermis of elderly (>80 years) versus young (21-30 years) individuals. Fibroblasts were the major cell source of both enzymes. PGE$_2$ levels were increased 70% in elderly skin. Fibroblasts in aged skin display reduced spreading due to collagen fibril fragmentation. To investigate the relationship between spreading and PGE$_2$ synthesis, fibroblasts were cultured on micropost arrays or hydrogels of varying mechanical compliance. Reduced spreading/mechanical force resulted in increased expression of both *PTGES1* and *COX2* and elevated levels of PGE$_2$. Inhibition of PGE$_2$ synthesis by diclofenac enhanced collagen production in skin organ cultures. These data suggest that reduced spreading/mechanical force of fibroblasts in aged skin elevates PGE$_2$ production, contributing to reduced collagen production. Inhibition of PGE$_2$ production may be therapeutically beneficial for combating age-associated collagen deficit in human skin.

Keywords

prostaglandin E$_2$; skin; aging; collagen; extracellular matrix; cellular mechanical force

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Introduction

Elderly skin is typically thin and fragile, with increased susceptibility to bruising and impaired wound healing (Fisher *et al.*, 2008). These alterations largely reflect fragmentation and reduction of type I collagen fibrils, which comprise the bulk of the dermal extracellular matrix (ECM) (Varani *et al.*, 2006). Dermal fibroblasts secrete type I procollagen, which is converted into collagen via proteolytic modifications. Mature collagen self-assembles into collagen fibrils, which are stabilized by inter- and intra-fibrillar crosslinking (Canty and Kadler, 2005). Although mature cross-linked collagen fibrils are very stable, with a half-life of approximately 15 years (Verzijl *et al.*, 2000), gradual cleavage, mediated by matrix metalloproteases (MMPs), occurs during aging (Fisher *et al.*, 2009). This cleavage results in accumulation of cross-linked fragments, due to resistance of cross-links to proteolysis. Thus during aging, the dermal ECM becomes progressively degraded and disorganized, which deleteriously alters the function of resident fibroblasts (Fisher *et al.*, 2008).

Fibroblasts attach to surrounding collagen fibrils to form adhesion complexes, which act through the cytoskeleton to exert contractile forces. Resistance to this contraction generates mechanical forces within fibroblasts, which largely determine morphology, cytoskeletal organization, signal transduction, and gene expression (Hegedus *et al.*, 2008; Mammoto *et al.*, 2013; Tarutani *et al.*, 2003). Collagen fragmentation causes loss of attachment sites, resulting in reduced fibroblast spreading, which is observed in aged human skin. This contracted state, with concomitant reduced mechanical force is associated with down-regulation of collagen fibril production, in human skin (Fisher *et al.*, 2008; Varani *et al.*, 2006; Xia *et al.*, 2013).

PGE_2 is a pleiotropic lipid signaling molecule produced by multiple cell types (Humphrey *et al.*, 2014). PGE_2 is synthesized from arachidonic acid by the sequential actions of cyclooxygenases (COX1 and COX2) and prostaglandin E synthases (PTGES1, 2, and 3) (Iskratsch *et al.*, 2014; Liu *et al.*, 1995). Elevated COX2 often coincides with PTGES1 induction in a wide variety of tumor lesions, and in response to inflammatory stimuli (Pickup *et al.*, 2013; Quan and Fisher, 2015; Verrecchia *et al.*, 2001). COX1, PTGES2, and PTGES3 are not typically inducible or involved in excess PGE_2 production seen in abnormal conditions. PGE_2 is a major prostaglandin in human skin and is normally synthesized at low levels, but is markedly increased in skin squamous cell carcinoma and inflammatory conditions, such as sunburn (Muller-Decker, 2011). Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of commonly used drugs including aspirin, ibuprofen, indomethacin and diclofenac, which inhibit the activities of both COX1 and COX2, thereby inhibiting PGE_2 production (Qin *et al.*, 2014). COX2 and PTGES1 induction in response to acute inflammatory stimuli has been extensively investigated (Pickup *et al.*, 2013; Quan and Fisher, 2015; Samuelsson *et al.*, 2007; Verrecchia *et al.*, 2001), however, the potential effects of cellular mechanical forces on COX2 or PTGES1 expression are unknown.

In cell cultures, PGE_2 inhibits fibroblast collagen production, in part through impeding actions of TGF-β (Sandulache *et al.*, 2007). The inhibitory effect of PGE_2 on collagen synthesis has been shown to play a protective role against lung fibrosis, where impaired

PGE$_2$ production and action are implicated in excessive fibroblast collagen deposition (Huang *et al.*, 2007).

This study reports that *PTGES1* mRNA expression progressively increases during aging, and investigates the role of PGE$_2$ in the age-related decline of type I collagen production in human skin. Taken together, the data support the concept that the dermal microenvironment raises PTGES1 and PGE$_2$ levels, which contributes to reduced collagen in aged skin. Given that PGE$_2$ synthesis can be effectively inhibited by a wide range of agents (Qin *et al.*, 2014), targeting PGE$_2$ is an appealing strategy to combat age-associated skin collagen deficiency.

Results

PTGES1 mRNA expression progressively increases during aging in human skin *in vivo*

In order to investigate age-related alterations in gene expression, total RNA was extracted from full-thickness skin samples obtained from sun-protected buttock skin of persons between the ages of 18-75 (N=62) (Swindell *et al.*, 2012). Global gene expression analysis was conducted using an *in situ* oligonucleotide array platform (Affymetrix Human Genome U133 Plus 2.0 array). Out of 19,851 human genes, 268 exhibited statistically significant age-associated changes in expression levels (FDR <0.10). *PTGES1* expression positively correlated with age, and this correlation was the most statistically significant. *PTGES1* expression was assessed by two probes targeting two different regions of the *PTGES1* transcript and yielded similar correlation coefficients and yearly rates of increase, as calculated by linear regression. The results obtained from one probe, 210367_s_at, are shown in **Fig. 1a**. The linear correlation between increased *PTGES1* expression and increased age was highly statistically significant (p=2.6×10^{-7}, n=62) and the degree of linearity, expressed as Pearson's correlation coefficient was r=0.6 (r can vary from zero to one, with zero representing no correlation and one representing perfect linear correlation).When compared to young skin (18 years of age), elderly skin (75 years of age) had a 1.6-fold overall increase in *PTGES1* expression.

In order to substantiate the age-associated increase of *PTGES1*, we acquired buttock skin samples from an additional 40 subjects aged 21-94. Consistent with our microarray data, *PTGES1* expression progressively increased with age as determined by qPCR, and increased 2.92-fold overall in 94-year versus 21-year old skin (N=40, p=1.38×10^{-7}, r=0.73) **(Fig. 1b)**. Taken together, these data demonstrate an intriguing correlation between aging and skin *PTGES1* gene expression.

Dermal fibroblasts are the primary source of increased expression for both *PTGES1* and *COX2* mRNA in aged human skin

Elevation of COX2 expression often occurs concomitantly with *PTGES1* induction. However, *COX2* mRNA levels in whole skin preparations did not significantly differ between young and aged individuals (data not shown). We hypothesized that dermal expression of *PTGES1* and *COX2* mRNA may account for age-dependent differences. In order to determine dermal and epidermal *PTGES1* and *COX2* expression, we used laser capture microdissection (LCM) of skin sections, followed by qPCR. Consistent with our

hypothesis, dermal, but not epidermal, *PTGES1* and *COX2* gene expression were significantly different in young vs. aged individuals. *PTGES1* and *COX2* levels were significantly increased 3.4-fold (p<0.05) and 2.7-fold, respectively (p<0.05) in aged dermis (**Fig. 2a & 2b**). This finding suggests that aging dermal, but not epidermal, cells express more *PTGES1* and *COX2*.

In order to determine whether fibroblasts are responsible for elevated dermal *PTGES1* gene expression, we separated fibroblasts from other dermal cells in skin samples using anti-fibroblast antibody-coated magnetic microbeads. Fibroblast enrichment was validated by mRNA quantification of several cell markers (Supplemental Table S1). Isolated cells were directly analyzed without culturing. *PTGES1* mRNA levels were approximately 10-fold (p<0.05) higher in fibroblast-enriched cells than in fibroblast-depleted dermal cells (**Fig. 2c**). These data indicate that dermal fibroblasts are responsible for the majority of dermal *PTGES1* expression.

In order to determine whether dermal fibroblasts are responsible for the changes to *PTGES1* expression in aging, we isolated fibroblasts from young (21-30 years) and aged (>80 years) skin. Consistent with our data, *PTGES1* mRNA expression was 2.2-fold higher in fibroblasts from aged skin (p<0.05) (**Fig. 2d**). We also quantified *COX2* mRNA expression in isolated fibroblasts, however, *COX2* mRNA levels in freshly isolated fibroblasts were markedly elevated by the isolation process. Taken together, the data suggest that dermal fibroblasts are the primary cell source of elevated *PTGES1* and *COX2* mRNA expression in aged skin.

PTGES1 protein expression is elevated in skin fibroblasts of elderly (>80 years), versus young (21-30 years) individuals

In order to determine whether PTGES1 protein levels were increased in aged dermal fibroblasts, we performed immunohistochemistry on both young and aged skin samples. Positive staining that overlapped with or was immediately adjacent to nuclei was most prominently seen in dermal stromal cells in both young and aged skin. In addition, PTGES1-positive dermal cells displayed morphology characteristic of fibroblasts, including: being embedded in collagenous ECM without direct contact with other cells or structures, elongated cell bodies, and oval shaped nuclei (**Fig. 3**). Statistical analysis showed that the percentage of positively-stained dermal stromal cells was increased 2.1-fold (75% vs. 35%, p<0.01) in aged skin. These data suggest that PTGES1 protein is preferentially expressed by fibroblasts, and fibroblast-derived PTGES1 protein expression is increased in the aged dermis, consistent with increased *PTGES1* mRNA expression (**Fig. 2**).

COX2 protein expression was also examined by immunohistochemistry but did not yield specific staining, consistent with previous studies showing that COX2 protein expression in normal human skin is below the limit of immunohistochemistry detection (Hoot *et al.*, 2010).

PGE$_2$ levels are higher in elderly versus young skin

Age-associated increases of *COX2* and *PTGES1* likely result in enhanced PGE$_2$ production. To examine this possibility, we incubated fresh samples of buttock skin from young (21-30

years) and aged (>80 years) persons in culture medium for five minutes, and quantified PGE_2 levels in the conditioned media. PGE_2 levels were 1.7-fold higher (p<0.05) in skin specimens from elderly individuals (**Fig. 4**). Taken together, these data indicate that increased *COX2* and *PTGES1* expression in aged dermal fibroblasts is associated with increased PGE_2 production in human skin *in vivo*.

Enhanced ECM compliance elevates *COX2* and *PTGES1* expression and PGE$_2$ synthesis in primary adult human dermal fibroblasts

Reduced fibroblast spreading/mechanical force is considered a driving force of fibroblast dysfunction in elderly skin (Fisher *et al.*, 2008). We therefore examined whether *PTGES1* and *COX2* expression were regulated by changes to ECM compliance. For these studies, we utilized micropost arrays and hydrogel assays. Fibroblasts were cultured on a micropost array with two different compliances, low compliance (15.3kPa) and high compliance (1.3kPa).

Electron micrographs (**Fig. 5a**) of fibroblasts adhered to type I collagen coated micropost arrays revealed bending of the microposts through cytoskeleton contractile forces. More compliant microposts (1.3kPa) were more deformed, resulting in reduced fibroblast spreading, resembling fibroblasts seen in fragmented collagenous ECM of aged dermis *in vivo* (Fisher *et al.*, 2008).

Less compliant microposts (15.3kPa) resisted deformation and fibroblasts had a spread morphology, resembling the stretched fibroblasts seen in the intact collagenous ECM of young dermis *in vivo* (**Fig. 5a**). On average, fibroblasts cultured on 1.3kPa microposts, displayed a 7.9-fold lower traction force than those cultured on 15.3-kPa microposts (**Fig. 5b**), as measured by calculating the number of posts deformed by a cell as well as the degree of deformation. These data confirm that fibroblasts grown on a less compliant ECM experience more mechanical force. To determine the effect of mechanical force on PGE_2 production, *PTGES1* and *COX2* mRNA expression were measured in fibroblasts cultured on 15.3kPa and 1.3kPa microposts. *PTGES1* and *COX2* expression increased by 1.7-fold and 3.0-fold, respectively, when cultured on more compliant (1.3kPa), compared to less compliant (15.3kPa) microposts (**Fig. 5c**). In addition, PGE_2 levels in the conditioned media increased by 1.9-fold (**Fig. 5d**), indicating that increased ECM compliance results in increased PGE_2 production.

To further confirm the stimulatory effects of high ECM compliance on PGE_2 synthesis, we analyzed fibroblasts cultured on type I collagen-coated polyacrylamide hydrogels. Fibroblasts cultured on a more compliant hydrogel (7kPa) exhibited increased *PTGES1* (2.0-fold) and *COX-2* (3.8-fold) mRNA expression and enhanced PGE_2 levels (4.5-fold) compared to fibroblasts cultured on a less compliant 30kPa hydrogel (**Fig. 5e & 5f**). In concert with changes of PGE_2, but in an opposite direction, procollagen mRNA and protein expression were increased by 2.2 and 2.8-fold, respectively, in 30kPa hydrogels compared with 7kPa hydrogels **(Supplemental Fig.1).** Taken together, these data indicate that increased ECM compliance results in reduced fibroblast spreading/mechanical force, which leads to enhanced PGE_2 production and reduction of procollagen expression.

PGE$_2$ inhibits procollagen production in human dermal fibroblasts

We next assessed the effects of PGE$_2$ on collagen production of fibroblasts from young (21-30 years) skin. Fibroblasts were cultured in serum-free medium in the presence of PGE$_2$ or vehicle (DMSO) for 24 hours. Type I procollagen protein was examined by Western blot. PGE$_2$ treatment reduced procollagen protein levels by approximately 66% (p<0.05) (**Fig. 6a**).

In order to determine if inhibition of PGE$_2$ production can enhance collagen production, we examined the effect of the general COX inhibitor, diclofenac, and the COX2-specific inhibitor, celecoxib, in organ culture of skin samples obtained from buttock skin. Fresh skin biopsies were cultured in the presence of inhibitor or vehicle (DMSO) for 16 hours. Type I procollagen protein in conditioned medium was quantified by EIA. Biopsies also underwent RNA extraction for quantification of type I procollagen expression. Diclofenac significantly reduced PGE$_2$ levels by 92% $\pm$ 7% (N=3, p<0.05, data not shown), and enhanced procollagen mRNA and protein expression by 2.3-fold and 1.9-fold, respectively (p<0.05) (**Fig. 6b).** Celecoxib reduced PGE$_2$ levels by approximately 80%, and enhanced procollagen mRNA expression by 1.9-fold (**Supplemental Fig. 2**), suggesting that PGE$_2$ derived from COX2 is largely responsible for inhibition of procollagen expression in human skin organ cultures. The results are consistent with the notion that increased COX-2 expression in aged dermis can lead to increased PGE$_2$ production and consequently reduced collagen expression.

Discussion

Chronological aging is a progressive process driven by the accumulation and synergy of subtle alterations, resulting in declining organ structure and function. Because of its accessibility, skin is an ideal organ for investigating the cumulative nature of aging and its underlying causalities in humans (Fisher *et al.*, 2008). Age-related increase of PTGES1 mRNA and protein expression was consistently revealed by examining whole skin, isolated dermis, and freshly isolated fibroblasts, using different methods and different groups of donors. Fibroblasts in aged human skin displayed comparable increases of mRNA (2.2-fold) and protein (2.1-fold) levels, although direct quantitative comparisons between results obtained from two different techniques should be interpreted with caution.

Although we found that epidermal COX2 and PTGES1 mRNA expression do not differ between young and aged, the results do not exclude the possibility that aged epidermal cells may also contribute to enhanced PGE$_2$ levels observed in aged skin. The relative contribution of dermal and epidermal cells to enhanced PGE$_2$ levels observed in whole skin specimens remains to be determined by future investigations.

We have proposed that age-associated molecular and cellular alterations in the dermis create a self-perpetuating cycle that drives the progression of ECM degeneration (Fisher *et al.*, 2014). Age-associated fragmentation of collagen fibrils impairs interactions of fibroblasts with the collagenous ECM, leading to suppression of collagen production and enhanced production of MMPs, which collectively cause further deterioration of the ECM. Impaired ECM causes further fibroblast dysfunction in a self-sustaining loop (Fisher *et al.*, 2008;

Varani *et al.*, 2006; Xia *et al.*, 2013). The present study implicates PGE_2 in this self-perpetuating cycle. Impaired ECM causes reduced mechanical force within fibroblasts, which in turn elevates *COX2* and *PTGES1* gene expression, likely through activations of transcription factors such as NF-kB, which are known to regulate expression of these two genes (Bage *et al.*, 2010).

Several age-associated alterations found in skin fibroblasts *in vivo* may conspire to cause reduced collagen production in the context of collagen fibril fragmentation. In addition to PGE_2, previous studies have found reduced expression of CTGF/CCN2 (connective tissue growth factor) and enhanced expression of CCN1 (cysteine-rich protein 61) in aged human skin fibroblasts. These alterations inhibit collagen production, at least in part, through blockade of TGF-β signaling (He *et al.*, 2014; Quan *et al.*, 2006; Quan *et al.*, 2010). Therapeutics targeting these pathways may retard or reverse the decline of skin collagen production during aging, thereby improving skin health. Elevated PGE_2 is an appealing target for intervention, as PGE_2 production in human skin can be effectively reduced by topical NSAIDs (Qin *et al.*, 2014). The present study provides rationale for investigation into the potential beneficial effects of NSAIDs for collagen deficiency in elderly skin. Mice models that display elevated skin PGE_2 levels and resultant collagen reduction would provide opportunity for both further mechanistic studies and evaluation of agents that could retard age-related collagen deficiency via the inhibition of PGE_2 synthesis and actions.

In addition to a possible contribution to collagen deficiency, fibroblast-derived PGE_2 may be a risk factor for other prevalent age-associated skin disorders. PGE_2 may impair functions of adjacent immune cells and epidermal keratinocytes via a paracrine mechanism. Given the critical role of PGE_2 in promoting cutaneous squamous cell carcinoma and inhibiting functions of antigen-presenting cells required for allergenic contact dermatitis, elevated fibroblast-derived PGE_2 may contribute to the observed increase of skin cancer and reduced skin immunity in the elderly (Muller-Decker, 2011; Scott *et al.*, 2014). In addition to local effects, skin-derived PGE_2 has been demonstrated to have a systemic impact. This effect is mediated by transit of PGE_2 and PGE_2-induced cytokines into the circulation (Scott *et al.*, 2014; Soontrapa *et al.*, 2011; Ullrich and Byrne, 2012). Whether constitutively elevated PGE_2 in chronologically aged, sun-protected, skin impacts other organ systems is an intriguing question for future investigations.

Materials and Methods

Human tissue procurement

Full thickness skin biopsies (2, 4, and 6mm in diameter) were taken from buttocks of healthy human subjects as previously described (Li *et al.*, 2013). All procedures involving human subjects were approved by the University of Michigan Institutional Review Board. Informed written consent was obtained from all human subjects.

cDNA microarray analysis

Affymetrix Human Genome U133 Plus 2.0 arrays (54,675 probe-sets of 19,851 genes) were used for expression profiling. Normalized expression values were calculated using the

robust multichip average (RMA) algorithm (Irizarry *et al.*, 2003). One probe-set was analyzed for each gene. The representative probe-set was chosen by the highest average RMA expression intensity among all 62 samples. Raw RMA intensities were adjusted to remove gender and batch variation. Simple linear regression was then used to identify genes significantly changed with age. Raw p-values were adjusted using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). FDR-adjusted p-value threshold of 0.10 was used.

Laser-capture microdissection (LCM)

LCM was performed as previously described (Li *et al.*, 2013). Briefly, 30 tissue sections were prepared from each skin biopsy embedded in OCT. Appendage-free dermis and interfollicular-epidermis were separately collected using LCM (Leica ASLMD System; Leica Microsystems, Wetzlar, Germany).

Fibroblast isolation

Skin biopsies were digested with collagenase (5 mg/ml in DMEM) for 16 hours at 37ºC as described previously (Li *et al.*, 2013). Fibroblasts that were released from the dermis were separated from other cell types using anti-fibroblast Microbeads (Miltenyl Biotec, Germany) according to manufacturer's instruction.

Cell-culture

Fibroblasts were cultured from punch biopsies (6-mm) from buttock skin (aged 21-30 years), and maintained as previously described (Fisher *et al.*, 2009). Fibroblasts between passages 3-8 were used in this study.

RNA extraction and qPCR

RNA was extracted using an RNA extraction kit (Qiagen, Chatsworth, CA). qPCR reagents were from Applied Biosystems (Foster City, CA). Ribosomal protein 36B4 was used to normalize gene expression (Li *et al.*, 2013).

Micropost arrays and hydrogels

Polydimethylsiloxane micropost arrays were fabricated, analyzed by scanning electron microscopy, and used for fibroblast traction force measurements, as previously described (Bonnans *et al.*, 2014; Fu *et al.*, 2010). Hydrogels were fabricated on glass coverslips as previously described (Caulin *et al.*, 2007).

Protein extraction and Western blot analysis

Protein extraction from primary fibroblasts and Western analysis were performed as previously described (Li *et al.*, 2013).

Immunohistochemistry

Immunohistochemistry was performed with anti-PTGES1 mouse monoclonal antibody (Santa Cruz Biotech, Santa Cruz, CA) on frozen skin sections as previously described (Rittie et al., 2009). Fifteen microscopic fields of each section were used for quantitation of cell staining.

PGE$_2$ Enzyme immunoassay (EIA)

After excision, skin buttock biopsies (2mm) were immediately incubated in 200μl α-MEM medium for five minutes. PGE$_2$ levels in the conditioned media were quantified using EIA assay (Cayman, Ann Arbor, MI). DNA was extracted from skin biopsies using the Micro-DNA Extraction Kit (Qiagen) and quantified by ultraviolet spectrometry. PGE$_2$ levels were normalized to DNA content in corresponding biopsies. This method was validated by quantifying increased PGE$_2$ levels in skin biopsies from acute ultraviolet-irradiated skin, compared to non-irradiated skin.

Type I procollagen EIA from organ cultures

Fresh skin biopsies were cultured in the presence of diclofenac or vehicle (DMSO) for 16 hours. Type I procollagen in organ culture conditioned medium was determined by an EIA kit (Takara) and normalized to total protein based on the manufacture's instruction.

Statistical Analysis

The correlations between *PTGES1* levels and age were analyzed using linear regression. Differences between two groups of samples were analyzed using the two-tailed t-test. Differences were considered significant when $p<0.05$. Data are presented as mean±SEM. N numbers represent sample size of each group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

List of Abbreviations used

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PGE$_2$	Prostaglandin E$_2$
PTGES	Prostaglandin E Synthase
COX2	Cyclooxygenase 2
ECM	extracellular matrix
MMP	matrix metalloprotease
NSAID	non-steroidal anti-inflammatory drug
LCM	laser capture microdissection
CTGF	connective tissue growth factor
RMA	robust mulitichip average
EIA	enzyme immunoassay

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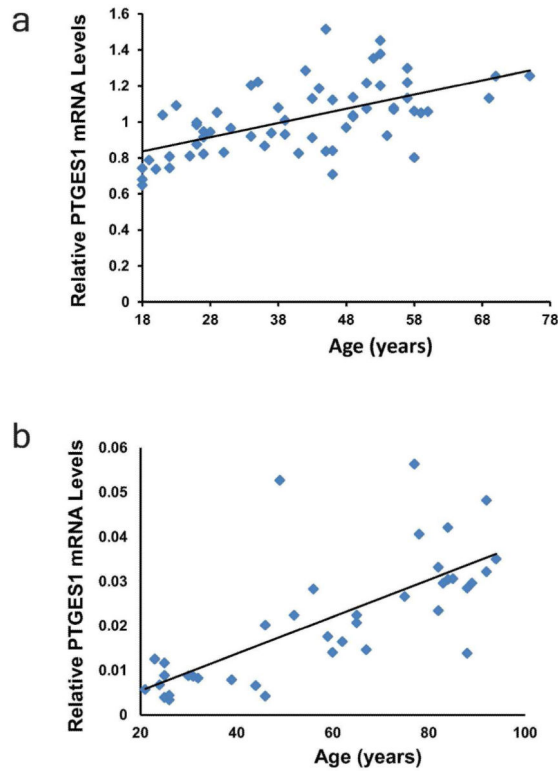
Figure 1. *PTGES1* mRNA expression progressively increases in human skin during chronological aging

(a) Relative *PTGES1* mRNA expression in human buttock skin was determined by cDNA microarray analysis. Results show positive correlation of *PTGES1* mRNA level and age of donors (N=62, p=2.6×10^{-7}, r=0.60). (b) *PTGES1* mRNA expression in buttock skin was determined by qPCR. (N=40, p=1.38 10^{-7}, r=0.73). The subjects used in microarray analysis were different from the subjects used in qPCR analysis.
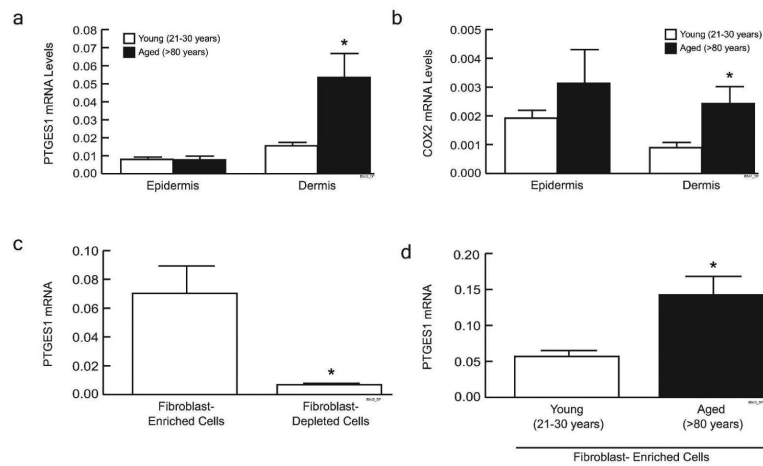
Figure 2. Dermal fibroblasts are a major cell source for increased expression of *PTGES1* and *COX2* in aged (>80 years) versus young (21-30 years) skin

Dermis and epidermis of frozen skin sections were separated and collected using laser capture microscopy. Relative (a) *PTGES1* and (b) *COX2* mRNA levels in the epidermis and dermis of young and aged individuals were determined by qPCR normalized to 36B4 mRNA. N=6, *p<0.05. (c-d) Cells were released from fresh sun-protected skin samples by collagenase digestion. Released cells were fractionated by immuno-affinity magnetic beads to separate fibroblasts for other cell types. Total RNA was extracted from isolated cells. (c) *PTGES1* mRNA levels were determined in fibroblast-enriched and fibroblast-depleted cells (N=4, *p<0.05). (d) *PTGES1* mRNA levels were determined in fibroblast-enriched cells isolated from skin samples from young and aged individuals. (N=5-7, *p<0.05).
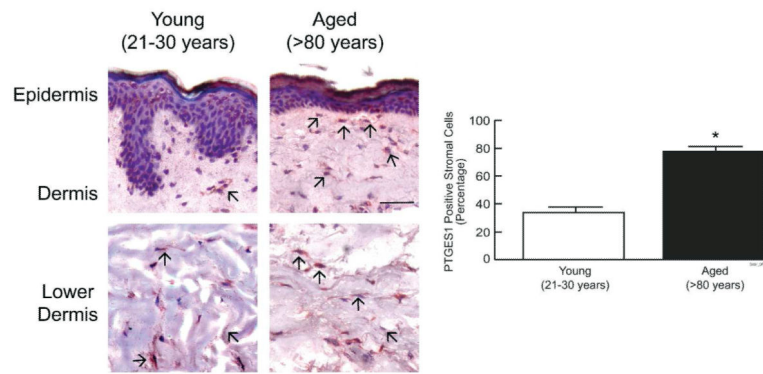
Figure 3. PTGES1 protein expression is increased in fibroblasts in aged human skin
Immunohistochemistry of PTGES1 protein in young (21-30 years old) and aged (>80 years old) sun-protected buttocks human skin. Left panels: representative immunostaining in upper and lower dermis. PTGES1 protein staining is brownish, and nucleus counterstaining with hematoxylin is blue. Arrows point to PTGES1 positively stained cells. Right panel: Percentage of total dermal stromal cells that were PTGES1 positive. (N=6, *p<0.01) Scale=50μm
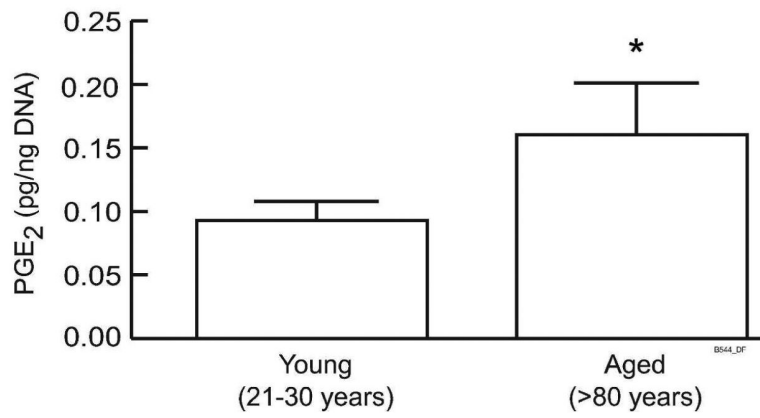
Figure 4. PGE$_2$ levels are increased in the skin of elderly compared to young individuals
Skin biopsies (2mm) were obtained from young (21-30 years) and elderly (>80 years) sun-protected buttock skin. PGE$_2$ levels were quantified by substrate enzyme immunoassay and normalized to DNA content of corresponding skin samples. (N=10, *p<0.05).
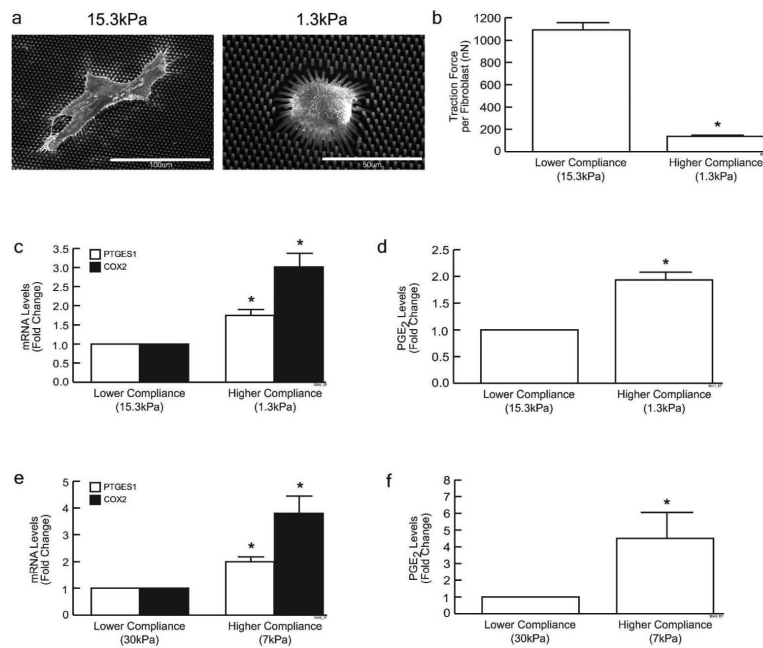
Figure 5. Reduced spreading/mechanical force elevates *COX2* and *PTGES1* expression and PGE$_2$ levels in human skin fibroblasts

(a-d) Fibroblasts obtained from young (21-30 years) individuals seeded on micropost arrays. (a) Scanning electron microscopy of fibroblasts. Micrographs are representative of three experiments examining >60 fibroblasts. (b) Quantification of traction force (nanonewton, nN) for 10 fibroblasts/condition (N=3, *p<0.001). (c) *COX2* and *PTGES1* mRNA levels was determined by qPCR (N=3, *p<0.05). (d) PGE$_2$ levels in conditioned-medium were quantified by EIA, and normalized to cell number (N=3, *p<0.05). (e-f) Fibroblasts cultured from young skin were seeded on type I collagen-coated hydrogels with low or high compliance. (e) *PTGES1* and *COX2* mRNA levels were quantified by qPCR. (N=3, *p<0.05). (f) PGE$_2$ levels in conditioned media quantified by EIA. (N=3, *p<0.05).
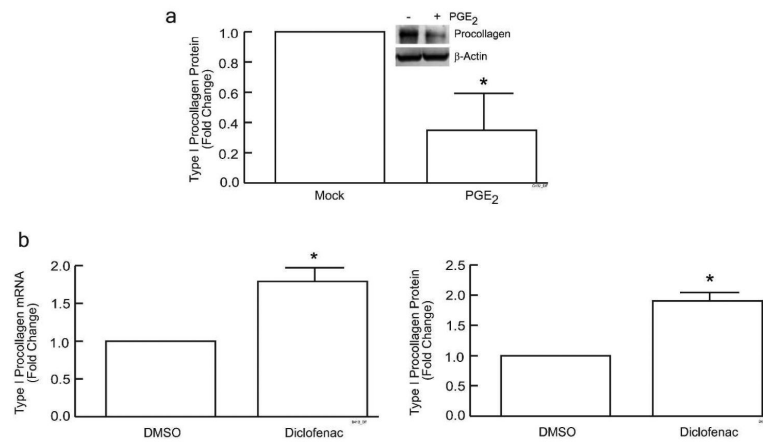
Figure 6. PGE$_2$ inhibits procollagen production in adult human skin fibroblasts
(a) Fibroblasts were cultured with the addition of PGE$_2$ (10nM) or vehicle (DMSO) for 24 hours. Type I procollagen protein levels in cell lysates were determined by Western blot, normalized to β-actin. Inset shows representative Western blots. (N=5, *p<0.05). (b) Skin samples were incubated in serum-free α-MEM media with the addition of diclofenac (10 μM) or vehicle (DMSO) for 16 hours. Type I collagen mRNA levels were quantified by qPCR and normalized to 36B4 mRNA levels (N=6). Type I procollagen protein levels in conditioned media were quantified by EIA. (N=3, *p<0.05).