Sex-based differences in conjunctival goblet cell responses to pro-inflammatory and pro-resolving mediators

Menglu Yang1, Haakon K. Fjærvoll1,2, Ketil A. Fjærvoll1,2, Nicholas H. Wang1, Tor P. Utheim3,4, Charles N. Serhan5 & Darlene A. Dartt1

Many conjunctival inflammatory diseases differ between the sexes and altered conjunctival goblet cells (CGCs) response is often involved. Inflammation is initiated by the release of pro-inflammatory mediators and terminated by the biosynthesis of specialized pro-resolution mediators (SPMs). Herein, we determined the sex-based difference in the responses of CGCs to inflammatory stimuli or pro-resolving lipid SPMs and their interaction with sex hormones. CGCs were transferred 24 h before the start of experiments to phenol red-free and FBS-free media to minimize exogenous hormones. RT-PCR, immunofluorescence microscopy (IF), and Western Blot (WB) were performed to determine the presence of sex hormone receptors. Cellular response to pro-inflammatory stimuli or SPMs was studied by measuring the increase in intracellular [Ca^{2+}] ([Ca^{2+}]_i) using fura 2/AM microscopy. Use of RT-PCR demonstrated estrogen receptor (ER) α in 4/5 males and 3/3 females; ERβ in 2/4 males and 2/3 females; and androgen receptors (AR) in 3/3 male and 3/3 female CGCs. Positive immunoreactivity by IF and protein expression by WB was detected using antibodies for the ERα and ERβ in 3/3 males and 3/3 females, while AR were only present in males. Significantly different Ca^{2+} responses between sexes were found with carbachol only at 10^{-3} M, but not with histamine or leukotriene (LT) B₄ at any concentration used. Incubation with dihydrotestosterone (DHT), estrone (E1), or estradiol (E2) at 10^{-7} M for 30 min significantly inhibited the LTB₄-stimulated [Ca^{2+}]_i increase in male and female CGCs. Incubation with DHT, E1, and E2 overnight significantly inhibited the LTB₄ response in females, while DHT and E2 significantly inhibited the LTB₄ response in males. The SPM lipoxin A₄ (LXA₄) (10^{-9}–10^{-8} M), but not the resolvins D1 or D2, induced an [Ca^{2+}]_i increase that was significantly higher in males compared to females. We conclude that male and female CGCs showed differences in the expression of sex hormone receptors. Treatment with sex hormones altered pro-inflammatory mediator LTB₄-induced response. Males compared to females have a higher response to the ω-6-fatty acid derived SPM LXA₄, indicating males may terminate inflammation in conjunctival goblet cells faster than females.

The prevalence of many ocular surface diseases differs between men and women. In the US, women have an age-adjusted 70% increase compared to men in the risk of dry eye disease1,2, and more antihistamine medicines were prescribed to women6. The differences in biological characteristics are one of the major probable causes of this phenomenon1. Sex affects health at multiple biological levels, from chromosomes to sex hormones. In leukocytes, X and Y chromosomes have different profiles of immune system-related genes7; while sex hormones also moderate immunity. For example, estrogens at the level of the ovulatory phase or pregnancy suppress cytotoxicity of natural killer (NK) cells, interferon-γ production, and T-cell subpopulation and their effect on the innate immune response2. Several autoimmune diseases such as systemic lupus erythematosus (SLE), Sjogren’s disease (SJD), Grave’s disease (GD), rosacea, and rheumatoid arthritis (RA) have a higher prevalence in women.

1Department of Ophthalmology, Harvard Medical School, Massachusetts Eye and Ear, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114, USA. 2Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway. 3Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway. 4Department of Ophthalmology, Oslo University Hospital, Oslo, Norway. 5Department of Anesthesia, Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA, USA. 6email: Darlene_dartt@meei.harvard.edu
LXA₄. We hypothesize that conjunctival goblet cells respond differently to pro-inflammatory and pro-resolving levels such as menopause or pregnancy. Furthermore, androgens stimulate meibomian gland secretion by pattern in both the cytoplasm and nucleus females (Fig. 1D). was present as a fine punctate pattern concentrated in the cytoplasm in males (Fig. 1C) and the same punctate male samples (Fig. 2B). ERβ immunoreactivity was detected as a punctate pattern throughout the cytoplasm and nuclear area in both males (Fig. 2C) and females (Fig. 2D).

AR immunoreactivity appeared as a punctate pattern that was more concentrated in the cytoplasm than the nucleus in males (Fig. 3C), but no AR immunoreactivity was detected in females (Fig. 3D). Incubation with the isotype control antibody in IF showed no apparent immunoreactivity for any of the three receptors (Supplemental Fig. 1) indicating that the antibodies used were specific for each receptor.

In summary, the sex hormone receptors are differentially present in human conjunctival GCs from males and females. Although little difference was found in the expression of mRNA for all three receptors, the pattern of expression was very different at the protein level. The ERβ protein was more abundant in females than males. In contrast, AR protein was only found in males. ERα showed the most consistency between the sexes, but a sex-based difference can still be detected in the subcellular localization.

**Results**

**Differential expression of sex hormone receptors in goblet cells cultured from male compared to female human conjunctiva.** To determine the expression of the estrogen and androgen receptors in conjunctival goblet cells, RT-PCR and WB were performed using cultured human goblet cells. IF was used to determine the cellular location of the sex hormone receptors on conjunctival goblet cells grown on coverslips. 

**ERα mRNA** was expressed in 4 out of 5 males and 3 out of 3 females at the expected size of 100 bp (Fig. 1A). ERα protein was present in 3 out of 3 males and 3 out of 3 females by WB analysis (Fig. 1B). Helix pomatia lectin (HPA), a marker of goblet cell secretory product, was present in both male and female goblet cells as a rough granular pattern indicating secretory granules, consistent with previous publications. ERα immunoreactivity was present as a fine punctate pattern concentrated in the cytoplasm in males (Fig. 1C) and the same punctate pattern in both the cytoplasm and nucleus females (Fig. 1D).

**ERβ** was found in 2 out of 4 males and 2 out of 3 females at the expected size of 143 bp by RT-PCR (Fig. 2A). In WB, ERβ presented as a double band in 3 out of 3 females, and a trace of ERβ band was found in 3 out of 3 male samples (Fig. 2B). ERβ immunoreactivity was detected as a punctate pattern throughout the cytoplasm and the nuclear area in both males (Fig. 2C) and females (Fig. 2D).

**AR mRNA** was identified in 3 out of 3 males and 3 out of 3 females at the expected size of 106 bp (Fig. 3A). AR was detected by WB in 2 out of 2 male samples, however, no protein was detected in 3 out of 3 females (Fig. 3B). AR immunoreactivity appeared as a punctate pattern that was more concentrated in the cytoplasm than the nucleus in males (Fig. 3C), but no AR immunoreactivity was detected in females (Fig. 3D). Incubation with the isotype control antibody in IF showed no apparent immunoreactivity for any of the three receptors (Supplemental Fig. 1) indicating that the antibodies used were specific for each receptor.

In summary, the sex hormone receptors are differentially present in human conjunctival GCs from males and females. Although little difference was found in the expression of mRNA for all three receptors, the pattern of expression was very different at the protein level. The ERβ protein was more abundant in females than males. In contrast, AR protein was only found in males. ERα showed the most consistency between the sexes, but a sex-based difference can still be detected in the subcellular localization.

**Differential response of goblet cells cultured from male and female conjunctiva to cholinergic agonists but not allergic mediators.** To determine the rapid, short-term activation of conjunctival goblet cells, we used three different types of known goblet cell stimuli: (1) the cholinergic agonist Cch (10⁻³–10⁻⁵ M)
to mimic physiological stimulation by the parasympathomimetic neurotransmitter acetylcholine, (2) LTB₄
(10⁻¹¹–10⁻⁸ M) an eicosanoid mediator of allergic conjunctivitis as well as of other types of inflammation
and (3) histamine (10⁻⁸–10⁻⁵ M) a major mediator of allergic conjunctivitis. The stimuli were applied to
male and female goblet cells, and the change in [Ca²⁺]i was measured.

Cch (10⁻⁵–10⁻³ M) compared to basal significantly increased [Ca²⁺], in both male and female cells (Fig. 4A,B).
Only at a carbachol concentration of 10⁻³ M was the peak [Ca²⁺], response significantly different between males
and females at 112.2 ± 40.54 nM in males and 577.0 ± 213.9 nM in females (p < 0.05) (Fig. 4B).

LTB₄ used at 10⁻¹¹ to 10⁻⁸ M caused an increase in [Ca²⁺], in both male and female goblet cells with a
maximum response to LTB₄ at 10⁻⁸ M with the [Ca²⁺], significantly increased to 230.8 ± 80.44 nM in males
and 521.6 ± 161.6 nM in females (Fig. 4C,D). No significant difference in peak [Ca²⁺] response was found at any concentrat-
on of LTB₄ when comparing the response of male and female cells (Fig. 4C,D).

When the goblet cells were stimulated by histamine, histamine caused an increase in peak [Ca²⁺], in male
but not female goblet cells (Fig. 4E,F). The maximum increase in [Ca²⁺], of 593.3 ± 104.5 nM occurred at 10⁻⁶ M
histamine in males. In contrast, the maximum increase in [Ca²⁺], of 583.3 ± 130.1 nM was detected at 10⁻⁵ M in
females (Fig. 4E). No significant difference in peak [Ca²⁺], was found between the two groups at any histamine
concentration (Fig. 4F).

In all, the cellular response of goblet cells to different stimuli that mimic physiological nerve stimulation,
inflammation, and allergy was not different between males and females except for the highest concentration of
the cholinergic agonist, in which females demonstrated a greater response than males.
Sex hormones inhibit conjunctival goblet cell response to the eicosanoid inflammatory mediator LTB₄, but not cholinergic agonist carbachol nor the autacoid histamine. To study the action of sex hormones on cellular responses to inflammatory mediators, we treated goblet cells from male and female conjunctiva with a sex hormone alone or for varying times followed by the addition of LTB₄. The sex hormones used were dihydrotestosterone (DHT), estrone (E1), and 17-β-estradiol (E2). DHT is a highly potent form of testosterone, estrone a low potency estrogen synthesized in peripheral tissues, and estradiol a high potency estrogen mainly synthesized in the ovary. The addition of 10⁻⁷ M DHT, E1, or E2 alone did not significantly increase the [Ca²⁺]i compared to basal in conjunctival goblet cells of a mixed series of both sexes (Supplemental Data 2). Histamine at 10⁻⁵ M, the positive control significantly increases the [Ca²⁺]i from basal.

To determine the short-term action of sex hormones on physiological, inflammatory, and allergic agonists, 10⁻⁷ M DHT, E1, or E2 or vehicle were administered to goblet cells 30 min prior to addition of LTB₄ (10⁻⁸ M). In male cells, LTB₄ at 10⁻⁸ M significantly increased the [Ca²⁺]i, to 540.90 ± 171.20 nM (p < 0.05) (Fig. 5A,B). A 30 min treatment with DHT significantly decreased the [Ca²⁺]i, to 70.02 ± 8.70 nM (p < 0.05); with E1 significantly decreased the [Ca²⁺]i, to 76.70 ± 13.04 nM (p < 0.05); and with E2 significantly decreased the [Ca²⁺]i, to 74.32 ± 7.4 nM (p = 0.04) (Fig. 5A,B). In female cells, LTB₄ 10⁻⁸ M alone significantly increased the [Ca²⁺]i, to 559.7 ± 209.2 nM from basal (Fig. 5C,D). A 30 min treatment with DHT significantly decreased the [Ca²⁺]i, to 97.60 ± 35.37 nM (p < 0.05) (Fig. 5C,D); with E1 significantly decreased the [Ca²⁺]i, to 62.95 ± 5.11 nM (p < 0.05) (Fig. 5C,D); and with E2 significantly decreased the [Ca²⁺]i, to 65.15 ± 10.58 nM (Fig. 5C,D).

The short-term action of sex hormones was also determined for carbachol at 10⁻³ M and histamine at 10⁻⁵ M using the same experimental design used for LTB₄. Neither DHT, E1, nor E2 incubated for 30 min blocked the...
carbachol-induced $\text{Ca}^{2+}$ response in either males or females (Supplemental Data 3A,B,E). Similarly, neither DHT, E1, nor E2 incubated for 30 min blocked the histamine-induced $\text{Ca}^{2+}$ response (Supplementary Fig. 3C,D,F).

To determine the action of long-term stimulation with sex hormones, goblet cells were incubated with DHT, E1, or buffer for 24 h before adding LTB$_4$. In male cells, LTB$_4$ 10$^{-8}$ M significantly increased the $[\text{Ca}^{2+}]$, to 404.70 ± 135.90 nM from basal (Fig. 6A,B). Treatment for 24 h with DHT or E2 significantly decreased the $[\text{Ca}^{2+}]$, to 63.21 ± 11.24 nM ($p < 0.05$); and 55.78 ± 9.49 nM ($p < 0.05$), respectively. In contrast, 24 h treatment with E1 dampened but did not significantly decrease the $[\text{Ca}^{2+}]$, to 127.60 ± 41.24 nM (Fig. 6B). In cells from females, LTB$_4$ 10$^{-8}$ M significantly increased the $[\text{Ca}^{2+}]$, to 463.90 ± 122.60 nM (Fig. 6C,D) and to 458.6 ± 118.3 (Fig. 6E,F) from basal. Incubation for 24 h with DHT, E1, or E2 each significantly decreased the LTB$_4$-triggered $[\text{Ca}^{2+}]$, increase to 58.52 ± 6.54 nM ($p < 0.05$), 85.18 ± 21.16 nM ($p < 0.05$) (Fig. 6C,D), and 94.17 ± 44.96 nM ($p < 0.05$) (Fig. 6E,F), respectively.

The long-term action of sex hormones was determined for carbachol and histamine using the same experimental protocol as for LTB$_4$. Neither DHT, E1, nor E2 incubated for 24 h blocked the carbachol- or histamine-induced $\text{Ca}^{2+}$ response in either males or females (Supplemental Data 4).

In summary, sex hormones incubated for both the short-term and long-term inhibited the cellular $\text{Ca}^{2+}$ response induced by the eicosanoid inflammatory mediator LTB$_4$, but not the cholinergic agonist carbachol nor the autacoid histamine.

**Differential response of goblet cells from male compared to female conjunctiva to LXA$_4$, but not RvD1 or RvD2.** We next determined the $[\text{Ca}^{2+}]$, response of goblet cells to the specialized pro-resolving mediators LXA$_4$, RvD1, and RvD2. LXA$_4$ is produced earlier in the resolution response than the resolvins as it
is biosynthesized from arachidonic acid compared to DHA for the D-series resolvins. When goblet cells cultured from male and female conjunctiva were stimulated with arachidonic acid (10^{-5} M (A and B) or LTB_{4} 10^{-11}–10^{-8} M (C and D) or histamine (E) 10^{-6}–10^{-5} M (E and F)). [Ca^{2+}], measured by fura2 assay. The average [Ca^{2+}], level over time was shown in (A, C and E). Change in peak [Ca^{2+}], was calculated and shown in (B, D and F). Number of individuals is indicated on each graph. Data are mean ± SEM; the number of individuals is marked in each graph. Arrow indicates the addition of stimuli. Black lines and solid black bars indicate data from male cells, grey lines and slash-patterned bars indicate data from female cells. *Significance difference from basal. #Significance difference between male and female values.

Figure 4. Human conjunctival goblet cells cultured from males and females respond to a neurotransmitter, inflammatory mediator, and allergic mediator. Cultured goblet cells from both sexes were stimulated with carbachol (Cch) 10^{-5}–10^{-3} M (A and B) or LTB_{4} 10^{-11}–10^{-8} M (C and D) or histamine (His) 10^{-6}–10^{-5} M (E and F). [Ca^{2+}], measured by fura2 assay. The average [Ca^{2+}], level over time was shown in (A, C and E). Change in peak [Ca^{2+}], was calculated and shown in (B, D and F). Number of individuals is indicated on each graph. Data are mean ± SEM; the number of individuals is marked in each graph. Arrow indicates the addition of stimuli. Black lines and solid black bars indicate data from male cells, grey lines and slash-patterned bars indicate data from female cells. *Significance difference from basal. #Significance difference between male and female values.
difference was found when comparing the response from male and female cells stimulated with RvD1 at any concentration used (Fig. 7C,D). Similar results were obtained with RvD2 (10⁻¹⁰–10⁻⁸ M). RvD2-induced [Ca²⁺]ᵢ increase was concentration-dependent and maximum at 10⁻⁸ M at which concentration the peak [Ca²⁺]ᵢ was significantly increased to 573.5 ± 386.4 nM in males and 460.8 ± 144.1 nM in females (Fig. 7E,F). No significant sex-based differences were observed in the Ca²⁺ response to RvD2 at any concentration used.

In summary, human GCs cultured from male or female conjunctiva responded differently to LXA₄, but no difference was found with the response to another family of SPMs, the D-series.

Discussion
Several tear-producing ocular adnexa, including the lacrimal gland and the Meibomian glands, display sex-based differences in structure and function, resulting in sex-based differences in diseases such as dry eye and allergy. Herein we show that message and location of protein for the receptors for estrogens are differently present in cultured human conjunctival goblet cells from both males and females. No AR protein was detected in female samples even though the mRNA was detected, a possible explanation of this finding is the prolonged lack of testosterone in culture. ARs in females are more dependent on the testosterone-induction, and the same action could have occurred with ERβ in male samples. The presence of ERα immunoreactivity in the nucleus in females suggests that ERα could bind to DNA to regulate the expression of target genes. Although the presence of the three sex hormone receptors in biopsy specimens from both sexes has been reported by other studies, our results indicate there are differences with the expression of sex hormone receptors with the absence of sex hormones.

Even though the sex hormones themselves when tested did not alter the [Ca²⁺]ᵢ in cells from either sex, they did affect the response to LTB₄ and to the same extent in both males and females. Sex hormones have a variety of effects on cell function. The protective role of the androgen DHT has been observed, and is consistent
with the present study in which DHT decreased the action of the inflammatory mediator LTB₄. Interestingly, we found that estrogen also attenuates the action of LTB₄. The role of estrogen is controversial as reports demonstrate protective, disruptive, and neutral effects of estrogen in ocular surface diseases. Estrogen also has multiple types of actions. Estrogen regulates expression of a variety of genes involved in dry eye. In addition, estrogen has more rapid non-gene expression action on cells, such as regulation of vascular endothelium by activating mitogen-activated protein (MAP) kinase kinase. On the ocular surface, estradiol downregulates cyclic AMP (cAMP) signaling in human meibomian gland epithelial cells. As the inflammatory mediator LTB₄

Figure 6. Overnight treatment with sex hormones inhibits the Ca²⁺ response from human conjunctival goblet cells cultured from male and female cells to the inflammatory mediator LTB₄. Cultured goblet cells from male and female conjunctiva were treated with DHT, E1 or E2 (10⁻⁷ M) or vehicle overnight prior to addition LTB₄ (10⁻⁸ M). [Ca²⁺], was measured by fura2 assay. The average [Ca²⁺], level over time was shown in (A, C and E). Change in peak [Ca²⁺], was calculated and shown in (B, D and F). Data are mean ± SEM. Number of individuals for each experiment are marked in graphs. Black lines and solid bars indicate data from male cells. Grey lines and slash-patterned bars indicate data from female cells. *Significance difference from basal. #Significance difference between males and females.
activates the BLT1 receptor and is coupled to Gi, the BLT1 receptor could work through cAMP in goblet cells. Furthermore, the BLT1 receptor also carries phosphorylation site for MAPK according to Scansite 4.0 https://scansite4.mit.edu/4.0/#home, indicating that it is possible for estrogen to modulate the activity of LTB4 through the BLT1 receptor. In summary, as one major consequence of goblet cell activation is mucin secretion, the results of our study indicate that sex hormones may protect the ocular surface from the excess tears and itchiness caused by mucin over-secretion during inflammation.

Sex hormones also demonstrate a long-term effect on the LTB4-Ca2+ response, but not on the cholinergic or histaminergic activated Ca2+ response. As sex hormones have receptors in the cell nuclei, they could affect the response through the synthesis of proteins. How sex hormones affect the expression of the receptors of the stimuli of each category needs to be further investigated.

We also investigated if neural, histaminergic, eicosanoid, and pro-resolution mediators have differential effects on the Ca2+ response in male compared to female human conjunctival goblet cells. We initially hypothesized that the unequal prevalence of conjunctival inflammatory diseases in men and women was due to different cellular responses to allergens or pathogens. However, the results of our experiments indicate that goblet cells
from males and females are equally susceptible to histamine and LTB₄ when sex hormones are eliminated from the culture conditions. Carbachol, a parasympathomimetic agonist, has an increased response in females compared to male goblet cells. These results are in contrast to the effect of sex hormones on the cholinergic agonist and LTB₄, but not the histamine, responses. Cholinergic agonists have a sex-based difference in their action, but sex hormones do not affect this response. LTB₄ stimulation does not have a sex-based difference in cultured goblet cells without sex hormones, but the sex hormones strikingly decrease the LTB₄ response both short-term and long-term in cells from both sexes. These results suggest that very different cellular signaling mechanisms regulate sex-based differences in agonist-stimulated response compared to the effect of exogenously added sex hormones on these responses.

The distinct mechanisms facilitate sex-based differences in conjunctival goblet cells was also observed among pro-resolving mediators. When cultured goblet cells were activated with SPMs, there was a difference between goblet cells from males and females in their response to LX₄ only, with a higher Ca²⁺ response in males compared to females, among the three SPMs tested. LX₄ is one of the earliest discovered SPMs. Together with its receptor ALX/FPR2, LX₄ constitutes an essential protective eicosanoid circuit, regarded as the “jump start” of the resolution of inflammation⁴⁰. LX₄ can be produced by several ocular tissues/cells, including the neutrophils in the corneal limbus, lacrimal glands, and cervical lymph nodes⁴¹, as well as the corneal epithelial cells⁴², and is present in the tears of healthy individuals⁴³. The beneficial role of LX₄ in reducing chronic inflammation occurs by inhibiting the regulatory T cells in draining lymph nodes, which drive the inflammation towards chronicity⁴⁴. Interestingly, a substantial decrease in lymph node LX₄ biosynthesis was found in an animal model of dry eye specifically in females rather than males⁴⁵. An additional sex-based difference in LX₄ was found when estrogen was demonstrated to down-regulate the LX₄ circuit to induce delayed female-specific wound healing in the cornea⁴⁶. Our current findings add new aspects of the relationship between LX₄ and sex, that is, sex also determines the cellular response to LX₄ in the epithelial conjunctival goblet cells. As LX₄, is biosynthesized from the same precursor as the pro-inflammatory mediator LTB₄ and appears at an early stage of resolution of inflammation, we speculate that the active resolution of inflammation begins earlier in males than in females. This finding is consistent with increased disease found in female cells. The sex-based difference in the action of LX₄ on goblet cells also suggests that in future studies on LX₄, attention should be paid to the sex differences in tissues, as analysis of both sexes together could cover up sex-based differences.

The D series resolvins such as RvD₁ and RvD₂ used in the present study are biosynthesized from DHA, a process that occurs later in resolution of inflammation than biosynthesis of LX₄. RvD₁ and RvD₂ have pro-resolution activities as does LX₄ and these D-series resolvins have protective effects on the ocular surface⁴⁰,⁴¹. In the present study, however, we did not observe any sex-based differences in goblet cell responses to either RvD₁ or RvD₂. In many cell types RvD₁ and LX₄ can use the same receptor, however, in human conjunctival goblet cells RvD₁ and LX₄ use different receptors: RvD₁ uses GPR32 and LX₄ uses ALX/FPR2⁴⁴. RvD₂ uses the GPR18 receptor (unpublished work by Botten, et al.). This could explain the different results for RvD₁ and RvD₂ compared to LX₄ obtained in the present study. In healthy human tears, we found a significantly higher level of RvD₁ and RvD₂ in males⁴⁷. Evidence in mice peritonitis model showed elevated key enzymes in the SPMs synthesis, including 12/15-lipoxygenase in males with elimination of sex hormones by gonadectomy, but not in females⁴⁸. Thus we suggest that the sex-based differences of LX₄ are mainly in the cellular response to LX₄, while those of RvD₁/D₂ are mainly the amount of biosynthesis by local tissues. The mechanisms behind the sex-based difference in the amount of biosynthesis of RvD₁/D₂ needs further investigation.

In conclusion, there are specific mediators that are altered in male versus female conjunctival goblet cell Ca²⁺ responses and whose Ca²⁺ response is affected by exogenous sex hormones using the androgen and estrogen receptors present in goblet cells from both sexes. There is, however, no widespread or generalized effect of sex hormones on goblet cell function. The major effect of the sex hormones is on proinflammatory mediators; the major sex-based difference is in the cellular response to the pro-resolution mediator LX₄. That males have higher response to the ω-6-fatty acid derived SPM LX₄ indicates that males may terminate inflammation in conjunctival goblet cells faster than females. Sex-based differences should be taken into consideration when studying inflammation and its resolution in conjunctival inflammatory diseases.

Methods

Human materials. Human conjunctival tissue was obtained from Eversight Eye Bank (Ann Arbor, MI). All tissue was donated to the eye bank with the prior informed consent and authorization of the donors for use in scientific research. The tissues were placed in Optisol media within 6 h of death. Use of this tissue was reviewed by the Massachusetts Eye and Ear Human Studies Committee and determined to be exempt and does not meet the definition of research with human subjects. The age and sex of each individual donor are listed in Supplementary Table 1.

Goblet cell culture. Goblet cells from human conjunctiva were grown in organ culture. Pieces of minced conjunctival epithelium were placed on culture dishes with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 2 mM non-essential amino acids (NEAA), 2 mM sodium pyruvate, and 100 μg/ml penicillin–streptomycin. The tissue pieces were removed after nodules of cells were observed. Twenty-four hours before experiments, cells were transferred into FBS-free and phenol red-free media to minimize the potential effect of sex hormones contained in FBS. First passage goblet cells were used in all experiments. Identity of cultured cells was periodically checked by evaluating staining with antibody to cytokeratin 7 (detects goblet cell bodies) and the lectin Helix pomatia agglutinin (HPA) (detects goblet cell secretory product) to ensure that goblet cells predominated.
Immunofluorescence microscopy (IF). First passage goblet cells were grown on glass coverslips and fixed with paraformaldehyde. The coverslips were blocked in 1% bovine serum albumin (BSA) with 0.2% Triton X-100 in PBS for 45 min. Anti-estrogen receptor (ER)α antibody, anti-ERβ antibody or anti-androgen receptor (AR) antibody (Abcam, Cambridge, UK) were used at a 1:100 dilution overnight at 4 °C. HPA conjugated to Alexa Fluor 488 (Invitrogen, Eugene, OR) was used at a 1:200 dilution. Secondary antibodies conjugated either to Cy2 or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at 1:150 dilution for 1.5 h at room temperature. Negative control experiments included incubation with the absence of the primary antibody. Antibody specificity was determined by incubation with the primary antibody that was treated overnight with the corresponding immunizing peptide at a 1:5 ratio (Abcam, Cambridge, UK).

Western Blotting Analysis (WB). Cultured goblet cells were were homogenized in radio-immunoprecipitation assay buffer (RIPA, 10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 1 mM EDTA) in the presence of a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). The homogenate was centrifuged at 2000g for 30 min at 4 °C. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and processed for western blotting analysis. The same antibodies were used as in IF experiments, with 1:500 dilution. Immunoreactive bands were visualized by the enhanced chemiluminescence method. The specificity of the antibodies was confirmed using the corresponding peptide at a 1:5 ratio (Abcam, Cambridge, UK) as for IF.

RT-PCR. Cultured goblet cells were homogenized in TRIzol (Invitrogen, Carlsbad, CA), and total RNA was isolated. The total RNA was purified using a TURBO DNA-free kit (Thermo Fisher, Waltham, MA). Five micrograms of purified total RNA were used for complementary DNA (cDNA) synthesis using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The cDNA was amplified by the polymerase chain reaction (PCR) using primers specific to human Erα and Erβ and AR receptors using the Jumpstart REDTaq ReadyMix Reaction Mix (Sigma-Aldrich, St, Louis, MO) in a thermal cycler (Master Cycler, Eppendorf, Hauppauge, NY). The genome sequence was found in the NCBI database (https://www.ncbi.nlm.nih.gov) and primers were designed using primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Erα: 5′-GGAGGCAGGGTTGAA-3′; Erβ: 5′-GCAGCGCGGAGGCTTCTCTTAG-3′; AR: 5′-ACTTGCCCTGTTGAGAGAA-3′; KRB: 5′-GACAGCCGAGAAGTGACATC-3′). The conditions were as follows: 5 min at 95 °C followed by 35 cycles of 1 min at 94 °C, 30 s at an annealing temperature of 62 °C for Erα and Erβ, 59 °C for AR, and 1 min at 72 °C with a final hold at 72 °C for 10 min. Samples with no cDNA served as the negative control while the presence of β-actin was confirmed by ethidium bromide staining.

Measurement of [Ca2+]i. First passage human conjunctival goblet cells were plated onto 35-mm glass-bottom culture dishes and incubated at 37 °C overnight. Cells were then incubated for 1 h at 37 °C with Krebs–Ringer bicarbonate buffer containing 119 mM NaCl, 4.8 mM KCl, 1.0 mM CaCl2, 1.2 mM MgSO4, and 25 mM NaHCO3 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) plus 0.5% BSA containing 0.5 μM fura-2/AM (Invitrogen, Grand Island, NY, USA) and 250 μM sulfinpyrazone (Sigma-Aldrich, St Louis, MO, USA). Before calcium measurements were started, cells were washed with KRB-HEPES containing sulfinpyrazone. Ca2+ measurements were conducted using a ratio imaging system (In Cyt im2; Intracellular Imaging, Cincinnati, OH, USA) using wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. Cells were stimulated with agonists, antagonists, and inhibitors. The intracellular [Ca2+]i ([Ca2+]i) over time was recorded, and the change in peak [Ca2+]i, was calculated by subtracting the average of the basal value from the peak [Ca2+]i, value.

Statistical analysis. The data are presented as the fold-increase above basal as average ± SEM. Student’s t-test was used in two group comparisons, and One-way ANOVA with Dunnett correction was used in multiple group comparisons. P<0.05 was set as statistically significant.

Ethical approval. Institutional Review Board Statement: The use of human tissue was reviewed by the Massachusetts Eye and Ear Human Studies Committee and determined to be exempt and does not meet the definition of research with human subjects.

Data availability Raw data were generated at Schepens Eye Research Institute. Derived data supporting the findings of this study are available from the corresponding author D.A.D. on request.

Received: 25 March 2022; Accepted: 9 September 2022
Published online: 29 September 2022

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Author contributions
Conceptualization, M.Y. and D.A.D.; Investigation, M.Y.; student intern, H.F, K.F, N.W.; writing-original draft, M.Y.; writing-review and editing, D.A.D., C.N.S. and T.P.U.; funding acquisition, D.A.D.; supervision, D.A.D.; H.F and K.F contributed equally.

Funding
National Institutes of Health USA (R01 EY019470) (D.A.D). American Society for Investigative Pathology Summer Research Opportunity Program in Pathology (N.W). Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway (T.P.U). The Faculty of Medicine, University of Oslo, Norway and the Norwegian Research Council (T.P.U).

Competing interests
Dr. T. P. Utneim is a TFOS Global Ambassador for Norway, a board member of the International Ocular Surface Society and a Faculty Member of the European School for advanced studies in ophthalmology. The rest of the authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-20177-9.
Correspondence and requests for materials should be addressed to D.A.D.

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