Anti-inflammatory and anti-oxidative effects of the green tea polyphenol epigallocatechin gallate in human corneal epithelial cells

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Purpose: To determine the anti-inflammatory and anti-oxidant effects of epigallocatechin gallate (EGCG), the major polyphenol component of green tea, in human corneal epithelial cells (HCEpiC).
Methods: HCEpiC were challenged with interleukin-1β (IL-1β) for 18 h or hyperosmolarity (440 mOsm) for 24 h. Luminex technology was used to determine the effects of EGCG (0.3 – 30 µM) on IL-1β- or hyperosmolar-induced cytokine release into the medium. Cell metabolic activity was measured using the alamarBlue assay. Effects of EGCG on mitogen-activated protein kinase (MAPK) phosphorylation were determined by cell-based enzyme-linked immunosorbent assay (ELISA) and western blotting. Effects of EGCG on nuclear factor kappa B (NFκB) and activator protein-1 (AP-1) transcriptional activity were assessed by reporter gene assay. The effects of EGCG on glucose oxidase (GO)-induced reactive oxygen species (ROS) production was determined using the ROS probe CM-H$_2$DCFDA.
Results: Treatment of HCEpiC with 1 ng/ml IL-1β for 18 h significantly increased release of the cytokines/chemokines granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemotactic protein-1 (MCP-1), while hyperosmolarity-induced release of IL-6 and MCP-1. When cells were treated with IL-1β and EGCG or hyperosmolarity and EGCG there was a dose-dependent reduction in release of these cytokines/chemokines, with significant inhibition observed at 3–30 µM. There was no effect of EGCG on cell metabolic activity at any of the doses tested (0.3–30 µM). EGCG significantly inhibited phosphorylation of the MAPKs p38 and c-Jun N-terminal kinase (JNK), and NFκB and AP-1 transcriptional activities. There was a significant dose-dependent decrease in GO-induced ROS levels after treatment of HCEpiC with EGCG.
Conclusions: EGCG acts as an anti-inflammatory and anti-oxidant agent in HCEpiC and therefore may have therapeutic potential for ocular inflammatory conditions such as dry eye.

The corneal epithelium acts both as an initial physical barrier to insult and infection, and also as a key player in the ocular immune response by producing inflammatory cytokines [1]. Ocular surface inflammation can be induced by numerous insults including the presence of pathogens, allergic reaction, and dry eye disease. While acute inflammation is essential for healing, chronic inflammation contributes to epithelial damage and cell death [2].

Dry eye disease is associated with inflammation of the ocular surface, and hyperosmolarity of the tears is now recognized as an important mediator of this inflammatory response [3,4]. Increased tear osmolarity causes a signaling cascade which involves phosphorylation of the stress-activated mitogen activated protein kinases (MAPKs) p38 and c-Jun N-terminal kinase (JNK) [5-9], followed by activation of transcription factors such as activator protein-1 (AP-1) and nuclear factor kappa B (NFκB), resulting in increased levels of pro-inflammatory cytokines such as IL-1β, TNF-α,

interleukin-8 (IL-8), and interleukin-6 (IL-6) [8-14]. Increased levels of IL-1β at the ocular surface contributes to the epithelial damage observed in dry eye and also infectious eye diseases with an inflammatory component by inducing expression of other pro-inflammatory cytokines in corneal epithelial cells and other ocular cells [15-17].

Another contributor to the cellular damage which occurs during inflammation is oxidative stress [18,19]. It has been demonstrated that lipid peroxide and myeloperoxidase activity are increased in the tears of dry eye patients, indicating increased oxidative potential [20]. In addition, increased oxidative stress markers and reactive oxygen species (ROS) production in the corneal epithelia of blink-suppressed dry eyes has been reported [21]. The ability of antioxidants to reduce the inflammatory reaction in acute inflammation and experimental infectious keratitis indicates that reactive oxygen species play a role in corneal inflammatory diseases [22,23].

Epigallocatechin gallate (EGCG) is the major polyphenol found in green tea, which is produced from the *Carmellia sinensis* plant. EGCG has been demonstrated to have both anti-inflammatory and anti-oxidant properties in multiple cell

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types [24-29]. Because of these properties, it is thought that EGCG may have therapeutic benefit in numerous inflammatory diseases such as atherosclerosis, arthritis, and dry eye disease [30-32]. Therefore, the objective of the current study was to determine the effects of EGCG on pro-inflammatory and pro-oxidant stimuli in human corneal epithelial cells (HCEpiC). Our data demonstrate that EGCG exhibits both anti-inflammatory and anti-oxidant properties in HCEpiC. To investigate the mechanistic basis for the anti-inflammatory effects of EGCG in HCEpiC, we determined its effect on the activation of MAPKs p38 and JNK and transcription factors NFκB and AP-1.

METHODS

Reagents: EpiLife medium, human corneal growth supplement (HCGS), penicillin-streptomycin solution, alamarBlue solution, Lipofectamine LTX, and Opti-MEM were obtained from Invitrogen (Carlsbad, CA). EGCG was from DSM (Basel, Switzerland) and IL-1β was from R&D Systems (Minneapolis, MN). Human multiplex-cytokine kits were from Millipore (Billerica, MA). Fast Activated Cell-based ELISA (FACE) p38 and JNK ELISA kits were from Active Motif (Carlsbad, CA). Cignal AP1 reporter (luc) and Cignal NFκB reporter (luc) kits were purchased from SA Biosciences (Frederick, MD). Dual-Glo Luciferase assay system was from Promega (Madison, WI). All other reagents were purchased from standard commercial sources and were of the highest available purity.

Cells and treatments: SV40-transformed human corneal epithelial cells (HCEpiC) were received at passage 20 from ATCC (Manassas, VA), and maintained in EpiLife medium supplemented with HCGS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified incubator with 5% CO_2. Cells were cultured in glucocorticoid-free medium (EpiLife basal medium supplemented with 12.5 μg/ml bovine pituitary extract, 1.25 μg/ml bovine insulin, and 1.25 ng/ml EGF) for 48 h before exposure to IL-1β or hyperosmolar medium. Osmolarity was increased to 440 mOsM by the addition of 123 mM sucrose to basal EpiLife medium (at 317 mOsM). Osmolarity of the solutions was verified using an osmometer (Osmette; Advanced Intruments, Norwood, MA). EGCG stock solutions were prepared in DMSO. Each treatment was performed in at least triplicate, and appropriate dilutions were prepared to deliver a constant amount of the vehicle to each well. Lack of an effect of treatments on cell metabolic activity, an index of cell viability, was determined by the alamarBlue assay [33,34].

Cytokine release by multiplex Luminex: HCEpiC were seeded on 48-well plates at a density of 2×10^4 cells per well. After 72 h, cells were treated with vehicle (0.1% DMSO) or EGCG for 18 h with 1 ng/ml IL-1β or 24 h in 440 mOsM hyperosmolar basal media. Cytokine content in the culture medium was analyzed using multiplex Luminex bead technology [35,36] according to the manufacturer's instructions. Briefly, 25 μl of

medium samples were incubated with antibody-coated capture beads overnight at 4 °C. Washed beads were further incubated with biotin-labeled anti-human cytokine antibodies for 1 h at room temperature followed by incubation with streptavidin-phycoerythrin for 30 min. Samples were analyzed using Luminex 200™ (Luminex, Austin, TX) and Statlia software (Brendan Technologies Inc., Carlsbad, CA). Standard curves of known concentrations of recombinant human cytokines were used to convert median fluorescence intensity (MFI) to cytokine concentration in pg/ml. Only the linear portions of the standard curves were used to quantify cytokine concentrations.

Cell-based ELISA for determination of p38 and JNK phosphorylation: Cells were seeded on a 96-well plate at a density of 1×10^4 cells per well. After 48 h, the medium was replaced with basal medium for 18 h. Due to the short incubation time necessary for measurement of MAPK phosphorylation, cells were pre-treated with EGCG for 2 h in basal medium. Cells were then treated with 1 ng/ml IL-1β alone or with EGCG at 3 or 30 μM, or hyperosmolar media alone or with EGCG at 3 or 30 μM for 30 min. Levels of phosphorylated and total p38 or JNK were measured using a cell-based ELISA according to the manufacturer's instructions. Briefly, the cells were fixed with 4% formaldehyde in PBS. After blocking, cells were incubated with primary antibodies specific to the appropriate phosphorylated or total MAPK overnight at 4 °C. Negative controls were performed using secondary antibody only. After washing, cells were incubated with HRP-conjugated secondary antibody for 1 h followed by further washing. Chemiluminescent reagent was added to each well and the resulting luminescence was read on a Synergy plate reader (Biotek, Winooski, VT). To verify that cell number was unchanged by treatments, cells in each well were further stained with crystal violet. The absorbance was read on a Synergy plate reader at 595 nm. Data were expressed as the ratio of phosphorylated to total MAPK, and were normalized by cell number.

Western blotting for determination of p38 and JNK phosphorylation: HCEpiC were preincubated with EGCG or vehicle control in EpiLife medium without growth factors for 2 h, and then were stimulated for 30 min with 1 ng/ml IL-1β. The cells were washed with ice-cold PBS and lysed in cell lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 1:1,000 protein inhibitor cocktail [PIC; Sigma, St Louis, MO]). Cells were sonicated, centrifuged at 12,800× g, and protein amount was determined using a Micro BCA protein assay kit (Pierce, Rockford, IL). Aliquots of cell lysate (30 μg) were separated by SDS-polyacrylamide electrophoresis and transferred to nitrocellulose membranes for western analysis. Membranes were blocked with 5% BSA and exposed to phospho p38 or phospho JNK antibody (Cell Signaling, Danvers, MA). The blots were washed, and
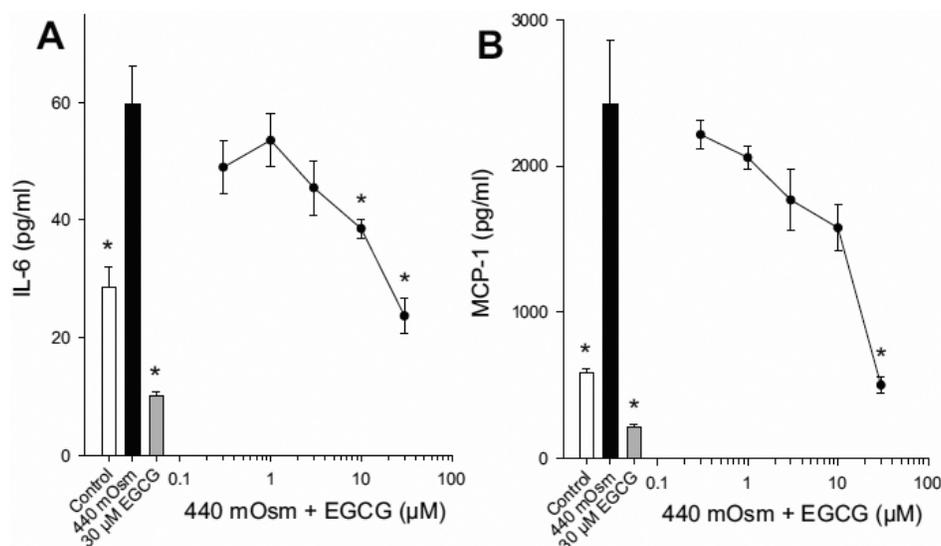
Figure 2. EGCG inhibits hyperosmolar-induced cytokine release in HCEpiC. Cells were cultured in complete (HCGS-containing) medium, followed by glucocorticoid-free medium for 18 h. Cells were then treated with 440 mOsm hyperosmotic basal media in the presence of EGCG for 24 h. IL-6 (**A**) and MCP-1 (**B**) release into the media was analyzed by Luminex. White bar represents control (317 mOsm); black bar represents hyperosmolarity (440 mOsm); gray bar represents EGCG alone; black circles + line represents EGCG. Lines are the linear interpolation between data points. Data are presented as mean±SEM, n=3. *versus 440 mOsm hyperosmotic media; $p < 0.05$.
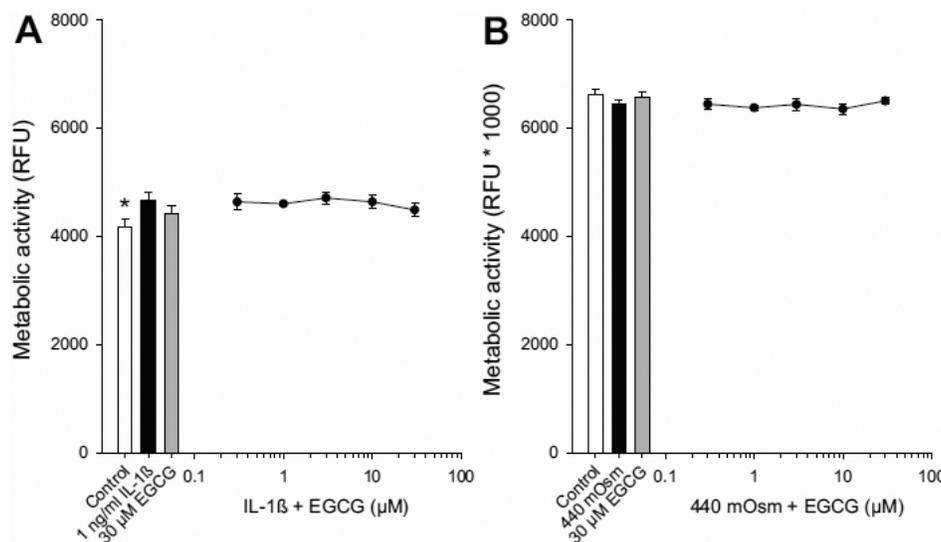

Figure 3. Effects of EGCG on cell metabolic activity in HCEpiC. Cells were cultured in complete (HCGS-containing) medium, followed by glucocorticoid-free medium for 18 h. Cells were then treated with IL-1β (**A**) or 440 mOsm hyperosmotic basal media (**B**) in the presence of EGCG for 18 or 24 h, respectively. Cell metabolic activity was determined using an alamarBlue assay. Data are presented as mean±SEM, n=3. *versus IL-1β for panel **A** and 440 mOsm media for panel **B**; $p < 0.05$.

treated with IL-1β and EGCG there was a dose-dependent decrease in release of these cytokines/chemokines, with significant inhibition observed at 3 – 30 μM (Figure 1).

Increased tear osmolarity is a key factor in dry eye disease [3,4], therefore the effect of EGCG on hyperosmolar-induced cytokine release was determined. There was a significant increase in IL-6 and MCP-1 release when the medium osmolarity was increased from 317 mOsm to 440 mOsm for 24 h. There was a dose-dependent inhibition of hyperosmolar-induced IL-6 and MCP-1 release, with significant inhibition observed at 10 and 30 μM for IL-6 and 30 μM for MCP-1 (Figure 2A,B).

To verify that the inhibitory effect of EGCG on cytokine release was not due to cellular toxicity an alamarBlue assay was performed. There was a small, yet significant increase in metabolic activity in cells treated with IL-1β as compared to

control (Figure 3A). However, there was no effect of IL-1β + EGCG or hyperosmolarity + EGCG on cell metabolic activity at any of the doses tested (0.3–30 μM; Figure 3A,B). These data demonstrate that EGCG can inhibit inflammatory cytokines induced by multiple stimuli in HCEpiC.

EGCG reduces IL-1β- and hyperosmolar-induced phosphorylation/activation of p38 and JNK MAPK: To investigate the possible mechanisms underlying the anti-inflammatory effects of EGCG, its effects on phosphorylation of the MAPKs p38 and JNK were determined. Quantitative cell-based ELISA and semi-quantitative western blotting were performed using phospho-specific antibodies against JNKs at Thr183/Tyr185, and against p38 at Thr180/Tyr182. These sites have been demonstrated to be associated with the activation state of these proteins [37]. Exposure of HCEpiC to 1 ng/ml IL-1β induced an increase in the ratio of
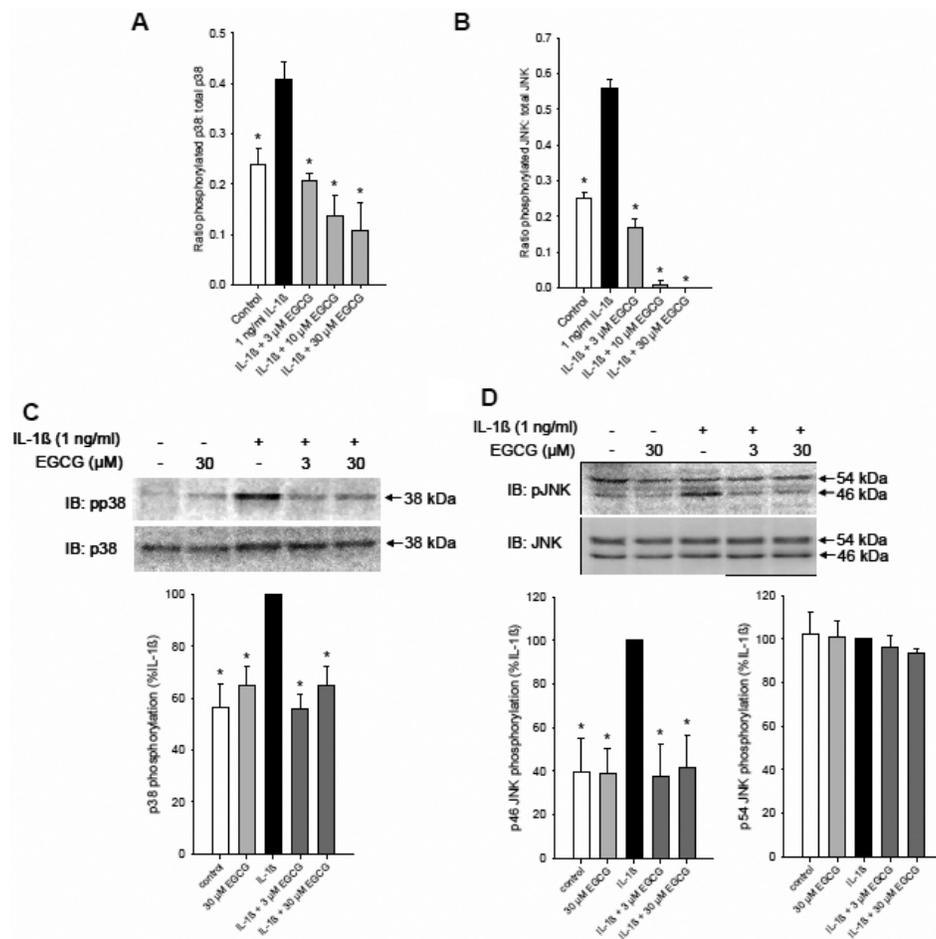
Figure 4. Effects of EGCG on IL-1β-induced phosphorylated p38 and JNK levels in HCEpiC. Cells were cultured in complete (HCGS-containing) medium, followed by basal medium for 18 h. Cells were pre-treated with EGCG for 2 h. Cells were then treated with IL-1β + EGCG for 30 min. Phosphorylated p38 and JNK levels were determined by cell-based ELISA (upper panels) or western blotting (lower panels). **A**: effect of EGCG on IL-1β-induced phosphorylated p38 determined by cell-based ELISA; **B**: effect of EGCG on IL-1β-induced phosphorylated JNK determined by cell-based ELISA; **C**: effect of EGCG on IL-1β-induced phosphorylated p38 determined by western blotting, Upper panel: upper blot shows phosphorylated p38. Lower blot is after stripping and probing with total p38 antibody. Lower panel: densitometric analysis of phosphorylated p38 normalized by total p38. D. effect of EGCG on IL-1β-induced phosphorylated JNK determined by western blotting, Upper panel: upper blot shows phosphorylated JNK. Lower blot is after stripping and probing with total JNK antibody. Lower left panel: densitometric analysis of phosphorylated p46 JNK normalized by total p46 JNK; lower right panel: densitometric analysis of phosphorylated p54 JNK normalized by total p54 JNK. For **A** and **B**, n=6, For **C** and **D**, n=3–4. Representative blots are shown. *versus IL-1β; p<0.05.

phosphorylated to total p38 and JNK, indicating an increase in activity of these inflammatory signaling mediators (Figure 4). Figure 4A,B shows that both p38 and JNK phosphorylation, as measured by cell-based ELISA and normalized to total p38 or JNK, were significantly inhibited by 3, 10, or 30 μM EGCG. Similar results were obtained using western blotting techniques. Both 3 and 30 μM EGCG significantly inhibited phosphorylation of IL-1β-induced p38 and p46 JNK with no change in total p38 or p46 JNK expression, however p54 JNK phosphorylation was unaltered by 1 ng/ml IL-1β or EGCG (Figure 4C,D).

The effect of EGCG on hyperosmolar-induced p38 and JNK phosphorylation was also determined by cell-based ELISA. There was a significant increase in p38 phosphorylation when cells were exposed to hyperosmolarity for 30 min, however the JNK phosphorylation increase did not reach significance. Hyperosmolar-induced phosphorylation of p38 was significantly decreased when cells were treated

with 3 and 30 μM EGCG, as compared to hyperosmolarity alone (Figure 5A). There was also a significant inhibition of JNK phosphorylation at 30 μM EGCG (Figure 5B).

EGCG suppresses IL-1β- and hyperosmolar-induced AP-1 and NFκB transcriptional activation: It has been reported that MAPK phosphorylation leads to activation of the transcription factors AP-1 and NFκB, and that furthermore, EGCG treatment leads to AP-1 and NFκB suppression in many cell types [30,31,38]. Therefore, the effect of EGCG on activation of AP-1 and NFκB was determined in HCEpiC. Luciferase reporter gene constructs containing tandem repeats of NFκB or AP-1 specific transcriptional response elements were transiently transfected into HCEpiC together with a constitutively expressing *Renilla* construct as a normalizer. Cells were then treated with 1 ng/ml IL-1β (Figure 6A,B) or 440 mOsm hyperosmolarity (Figure 6C,D) in combination with EGCG. Figure 6A,B shows that NFκB and AP-1 transcriptional activities were significantly increased by
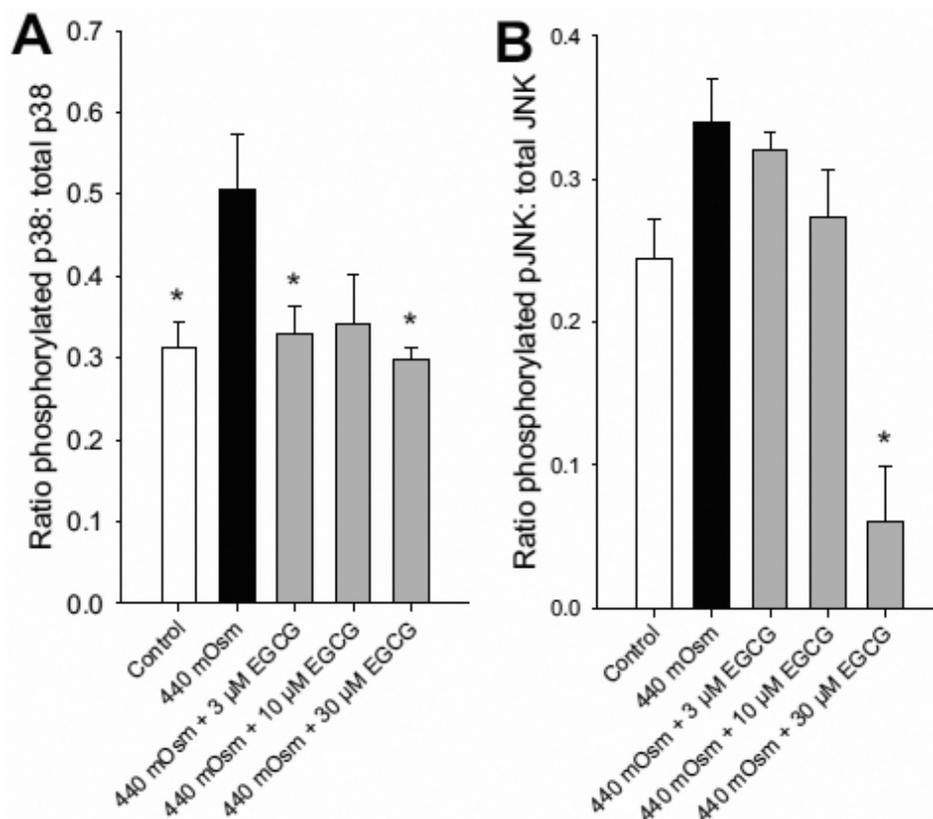
Figure 5. Effects of EGCG on hyperosmolar-induced phosphorylated p38 and JNK levels in HCEpiC. Cells were cultured in complete (HCGS-containing) medium, followed by basal medium for 18 h. Cells were pre-treated with EGCG for 2 h. Cells were then treated with 440 mOsm hyperosmotic media + EGCG for 30 min. Phosphorylated p38 and JNK levels were determined by cell-based ELISA. **A**: effect of EGCG on hyperosmolar-induced phosphorylated p38; **B**: effect of EGCG on hyperosmolar-induced phosphorylated JNK; n=6, *versus 440 mOsm media; $p < 0.05$.

IL-1β. EGCG at 3 or 30 μM reduced this IL-1β-induced transcriptional activity to control levels. Similarly, hyperosmolarity induced both AP-1 and NFκB activities (Figure 6C,D). Hyperosmolar-induced AP-1 activity was significantly decreased by 3 and 30 μM EGCG, while there was no inhibition of NFκB activity.

EGCG inhibits glucose oxidase-induced ROS in HCEpiC: EGCG is a well known anti-oxidant in many cell types, and since oxidative stress is a key component of the inflammatory cascade, the effect of EGCG on ROS in HCEpiC was determined. The ROS generator glucose oxidase significantly increased ROS levels in HCEpiC after 1 h, as determined using CM-H$_2$DCF-DA dye. There was a dose-dependent inhibition of glucose oxidase-induced ROS, with significant inhibition observed at 3, 10, and 30 μM EGCG (Figure 7), demonstrating that EGCG acts as an anti-oxidant in HCEpiC.

DISCUSSION

In this study we determined that the green tea polyphenol EGCG has both anti-inflammatory and anti-oxidant properties in HCEpiC. Two pro-inflammatory stimuli were selected to challenge the HCEpiC, IL-1β, and hyperosmolarity. The importance of IL-1β in the etiology of corneal inflammatory disease such as dry eye and keratitis has been demonstrated in multiple cell types [15-17,39-41]. An increase in osmolarity of the tear film is known to be one of the causative factors in

dry eye, resulting in inflammation and subsequent cell damage [3,4]. In addition, studies have demonstrated that increased osmolarity elevates levels of cytokines and matrix metalloproteases in corneal epithelial cells [5-7], in in vivo dry eye models [8,9] and in the tears of dry eye patients [11-14]. EGCG was effective in inhibiting multiple cytokines induced by both these inflammatory mediators, demonstrating it may have therapeutic potential in the treatment of corneal inflammatory diseases, including dry eye. These data are in agreement with an in vivo study which demonstrated that topical EGCG was efficacious for the treatment of dry eye disease [32]. Furthermore, we demonstrate that EGCG exhibited potent anti-oxidant activity in HCEpiC, inhibiting reactive oxygen species generated by the pro-oxidant glucose oxidase.

EGCG is reported to possess both anti-inflammatory and anti-oxidant properties in vitro and in vivo [24-27,30-32]. In the eye, green tea extract protects the lens and retina from UV-induced oxidation [28,29,42] and reduces retinal oxidative glutamate toxicity [43]. While the anti-inflammatory and anti-oxidant properties of EGCG are well described in multiple ocular and non-ocular cell types, to our knowledge, this is the first study demonstrating these properties and their mechanistic basis in corneal epithelial cells. The stress-activated MAPKs p38 and JNK are known to play a key role in regulating the production of pro-inflammatory cytokines
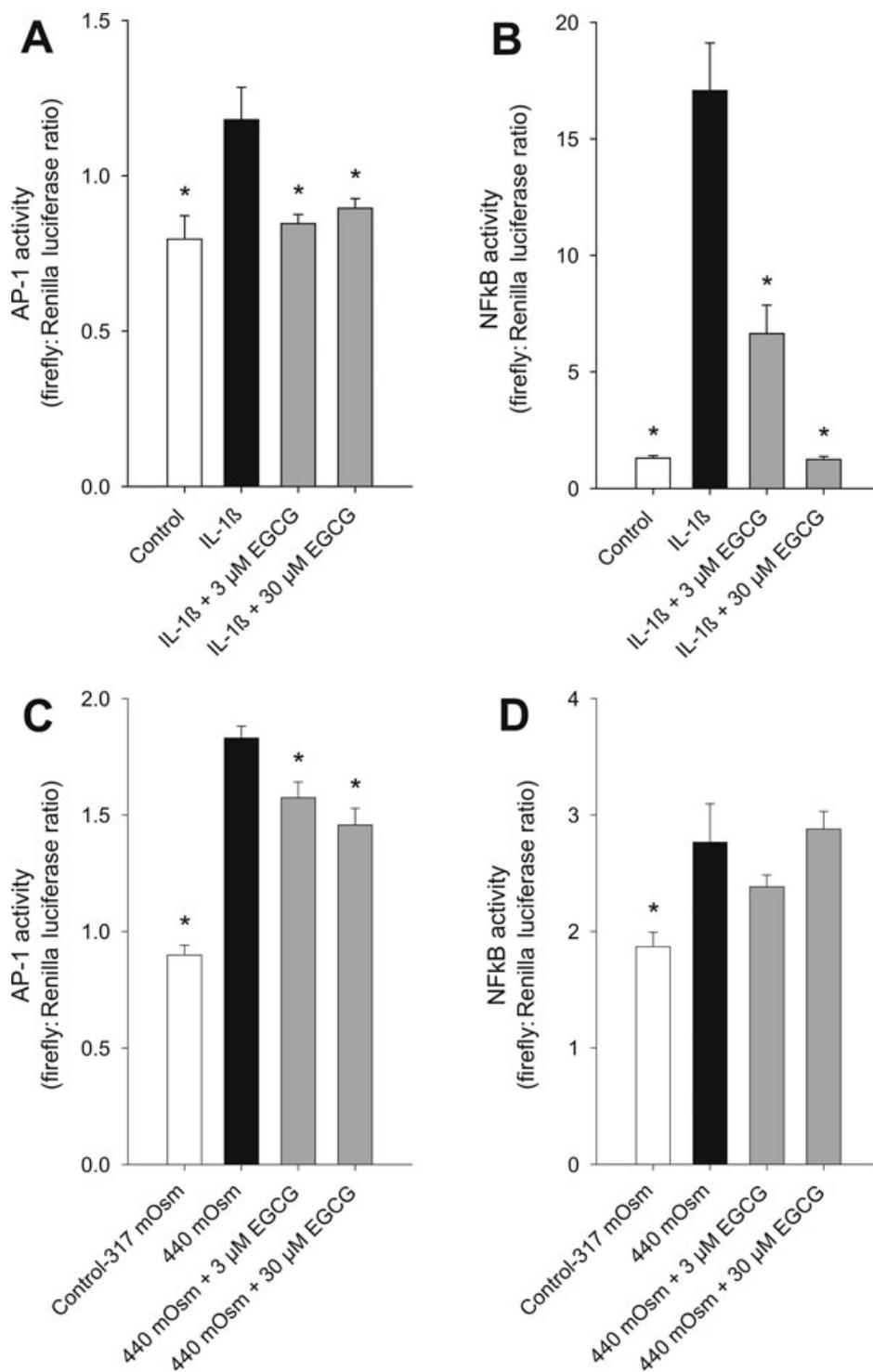
Figure 6. Effects of EGCG on IL-1β- and hyperosmolarity-induced HCEpiC NFκB and AP-1 activity. Cells were transfected with an AP-1 or NFκB reporter gene (expressing firefly) and a constitutively expressing *Renilla* construct and then placed in glucocorticoid-free medium for 24 h. Thirty h after transfection, cells were treated with IL-β (panels **A** and **B**) or 440 mOsM media (panels **C** and **D**) in the presence or absence of EGCG. Cells were assayed for firefly luciferase followed by *Renilla* luciferase luminescence 48 h after transfection. Data are expressed as normalized firefly: *Renilla* luciferase ratio. **A**: Effect of EGCG on IL-1β-induced AP-1 transcriptional activity; **B**: effect of EGCG on IL-1β-induced NFκB transcriptional activity. **C**: Effect of EGCG on 440 mOsm hyperosmolar-induced AP-1 transcriptional activity; **B**: effect of EGCG on 440 mOsm hyperosmolar-induced NFκB transcriptional activity. Data are mean ±SEM, n=6. For **A** and **B**, *versus IL-1β; for **C** and **D**, *versus 440 mOsm media; p<0.05.

leading to inflammation. EGCG has been shown to inhibit both JNK and p38 in multiple cell types [31,44]. We found that EGCG inhibited the IL-1β- and hyperosmolar-induced phosphorylation/activation of both p38 and JNK in HCEpiC. EGCG was comparably potent at inhibiting both IL-1β-induced p38 and JNK activation, with significant inhibition at 3 μM. However, EGCG appeared more efficacious at inhibiting hyperosmolar-induced p38 phosphorylation than JNK, with significant inhibition at 3 μM for p38 as compared to 30 μM for JNK.

Two transcription factors known to mediate the increases in cytokine production by both IL-1β and hyperosmolarity are AP-1 and NFκB [45,46]. AP-1 is regulated by multiple MAPKs – ERKs activate AP-1 after mitogenic stimulation of cells, in contrast JNK and p38 activate AP-1 after exposure of cells to environmental stress or proinflammatory cytokines [47]. NFκB is commonly made up of a heterodimer between the p65 subunit (also known as Rel A), which contains a transactivation domain mediating transcriptional induction and the p50 subunit. NFκB is predominantly activated through phosphorylation by inhibitory protein kappa B (IκB) kinase and subsequent degradation of the bound IκB, allowing it to translocate to the nucleus [2]. Our results indicate that inhibition of IL-1β-induced cytokine expression by EGCG is mediated, at least in part, by inhibition of both AP-1 and NFκB transcriptional activities. Inhibition of AP-1 activity by EGCG is presumably due to its effect on MAPK signaling and EGCG may also affect DNA binding activity of AP-1 as has been demonstrated in other cell types [31,48]. The effects of EGCG on NFκB in other cell types are through multiple

mechanisms, including inhibition of IκB kinase, IκB phosphorylation, p65NFκB phosphorylation, p65NFκB acetylation, and NFκB DNA binding activity [31,48,49].

While hyperosmolar-induced AP-1 activity was inhibited by hyperosmolarity, NFκB activity was not affected by EGCG. Incomplete inhibition of AP-1 activity and lack of effect on NFκB activity by EGCG suggests that EGCG inhibits hyperosmolar-induced cytokine levels at additional steps in the hyperosmolarity-induced signaling cascade. For example, the transcription factor nuclear factor of activated T-cell-5 (NFAT5) is induced and activated in limbal epithelial cells by hyperosmolarity [50]. Signal transducer and activator of transcription (STAT1) is another factor known to be activated by hyperosmolarity in several cell types [51,52], is expressed in human corneal epithelial cells [53], and is also a target of EGCG [54-56]. Another possibility is that EGCG could down-regulate cytokine gene expression at the post-transcriptional level through effects on mRNA stability [57]. Elucidating the additional mechanisms by which EGCG affects hyperomolar-induced cytokine release is an interesting area for future research.

In summary, these data demonstrate EGCG acts as an anti-inflammatory and anti-oxidant agent in HCEpiC challenged with either the pro-inflammatory cytokine IL-1β or hyperosmolarity. Therefore, EGCG has potential therapeutic application for the treatment of ocular disorders with an inflammatory component, including dry eye.
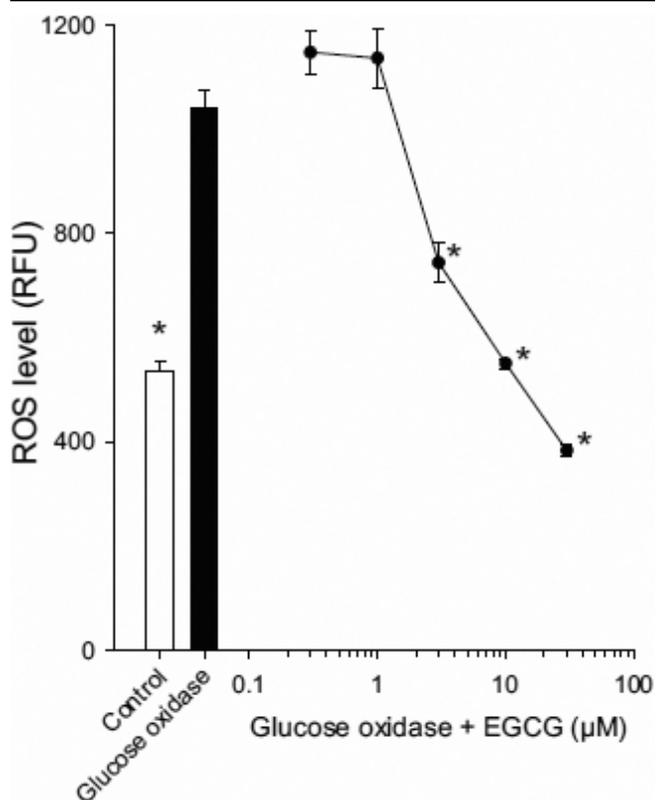

Figure 7. Effect of EGCG on glucose oxidase-induced ROS levels in HCEpiC. Cells were cultured in complete (HCGS-containing) medium, followed by glucocorticoid-free medium for 18 h. Cells were pretreated with EGCG for 1 h, loaded with CM-H₂DCFDA for 30 min, followed by treatment with 50 mU/ml glucose oxidase and EGCG for 1 h. White bar represents control; black bar represents glucose oxidase (GO) alone; black circles indicate glucose oxidase and EGCG. n=4, *versus GO, p<0.05.

REFERENCES

1. Ueta M, Kinoshita S. Innate immunity of the ocular surface. Brain Res Bull 2010; 81:219-28. [PMID: 19828129]
2. Wells CA, Ravasi T, Hume DA. Inflammation suppressor genes: please switch out all the lights. J Leukoc Biol 2005; 78:9-13. [PMID: 15774547]
3. Pflugfelder SC, Stern ME. Immunoregulation on the ocular surface: 2nd Cullen Symposium. Ocul Surf 2009; 7:67-77. [PMID: 19383275]
4. DEWS report. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007). Ocul Surf 2007; 5:75-92. [PMID: 17508116]
5. Li DQ, Chen Z, Song XJ, Luo L, Pflugfelder SC. Stimulation of matrix metalloproteinases by hyperosmolarity via a JNK pathway in human corneal epithelial cells. Invest Ophthalmol Vis Sci 2004; 45:4302-11. [PMID: 15557436]
6. Li DQ, Luo L, Chen Z, Kim HS, Song XJ, Pflugfelder SC. JNK and ERK MAP kinases mediate induction of IL-1beta, TNF-alpha and IL-8 following hyperosmolar stress in human limbal epithelial cells. Exp Eye Res 2006; 82:588-96. [PMID: 16202406]
7. Corrales RM, Luo L, Chang EY, Pflugfelder SC. Effects of osmoprotectants on hyperosmolar stress in cultured human corneal epithelial cells. Cornea 2008; 27:574-9. [PMID: 18520508]
8. Luo L, Li DQ, Doshi A, Farley W, Corrales RM, Pflugfelder SC. Experimental dry eye stimulates production of

inflammatory cytokines and MMP-9 and activates MAPK signaling pathways on the ocular surface. Invest Ophthalmol Vis Sci 2004; 45:4293-301. [PMID: 15557435]

9.   Luo L, Li DQ, Corrales RM, Pflugfelder SC. Hyperosmolar saline is a proinflammatory stress on the mouse ocular surface. Eye Contact Lens 2005; 31:186-93. [PMID: 16163009]

10. Corrales RM, Villarreal A, Farley W, Stern ME, Li DQ, Pflugfelder SC. Strain-related cytokine profiles on the murine ocular surface in response to desiccating stress. Cornea 2007; 26:579-84. [PMID: 17525655]

11. Yoon KC, Jeong IY, Park YG, Yang SY. Interleukin-6 and tumor necrosis factor-alpha levels in tears of patients with dry eye syndrome. Cornea 2007; 26:431-7. [PMID: 17457192]

12. Yoon KC, Park CS, You IC, Choi HJ, Lee KH, Im SK, Park HY, Pflugfelder SC. Expression of CXCL9, −10, −11, and CXCR3 in the tear film and ocular surface of patients with dry eye syndrome. Invest Ophthalmol Vis Sci 2010; 51:643-50. [PMID: 19850844]

13. Lam H, Bleiden L, de Paiva CS, Farley W, Stern ME, Pflugfelder SC. Tear cytokine profiles in dysfunctional tear syndrome. Am J Ophthalmol 2009; 147:198-205. e1. [PMID: 18992869]

14. Massingale ML, Li X, Vallabhajosyula M, Chen D, Wei Y, Asbell PA. Analysis of inflammatory cytokines in the tears of dry eye patients. Cornea 2009; 28:1023-7. [PMID: 19724208]

15. Narayanan S, Corrales RM, Farley W, McDermott AM, Pflugfelder SC. Interleukin-1 receptor-1-deficient mice show attenuated production of ocular surface inflammatory cytokines in experimental dry eye. Cornea 2008; 27:811-7. [PMID: 18650668]

16. Narayanan S, Glasser A, Hu YS, McDermott AM. The effect of interleukin-1 on cytokine gene expression by human corneal epithelial cells. Exp Eye Res 2005; 80:175-83. [PMID: 15670796]

17. Solomon A, Dursun D, Liu Z, Xie Y, Macri A, Pflugfelder SC. Pro- and anti-inflammatory forms of interleukin-1 in the tear fluid and conjunctiva of patients with dry-eye disease. Invest Ophthalmol Vis Sci 2001; 42:2283-92. [PMID: 11527941]

18. Wakamatsu TH, Dogru M, Tsubota K. Tearful relations: oxidative stress, inflammation and eye diseases. Arq Bras Oftalmol 2008; 71:72-9. [PMID: 19274416]

19. Shoham A, Hadziahmetovic M, Dunaief JL, Mydlarski MB, Schipper HM. Oxidative stress in diseases of the human cornea. Free Radic Biol Med 2008; 45:1047-55. [PMID: 18718524]

20. Augustin AJ, Spitznas M, Kaviani N, Meller D, Koch FH, Grus F, Gobbels MJ. Oxidative reactions in the tear fluid of patients suffering from dry eyes. Graefes Arch Clin Exp Ophthalmol 1995; 233:694-8. [PMID: 8566825]

21. Nakamura S, Shibuya M, Nakashima H, Hisamura R, Masuda N, Imagawa T, Uehara M, Tsubota K. Involvement of oxidative stress on corneal epithelial alterations in a blink-suppressed dry eye. Invest Ophthalmol Vis Sci 2007; 48:1552-8. [PMID: 17389484]

22. Alió JL, Artola A, Serra A, Ayala MJ, Mulet ME. Effect of topical antioxidant therapy on experimental infectious keratitis. Cornea 1995; 14:175-9. [PMID: 7743801]

23. Alio JL, Ayala MJ, Mulet ME, Artola A, Ruiz JM, Bellot J. Antioxidant therapy in the treatment of experimental acute

corneal inflammation. Ophthalmic Res 1995; 27:136-43. [PMID: 8538990]

24. Wu CH, Wu CF, Huang HW, Jao YC, Yen GC. Naturally occurring flavonoids attenuate high glucose-induced expression of proinflammatory cytokines in human monocytic THP-1 cells. Mol Nutr Food Res 2009; 53:984-95. [PMID: 19557821]

25. Zheng Y, Toborek M, Hennig B. Epigallocatechin gallate-mediated protection against tumor necrosis factor-alpha-induced monocyte chemoattractant protein-1 expression is heme oxygenase-1 dependent. Metabolism 2010; 59:1528-35. [PMID: 20580034]

26. Kim IB, Kim DY, Lee SJ, Sun MJ, Lee MS, Li H, Cho JJ, Park CS. Inhibition of IL-8 production by green tea polyphenols in human nasal fibroblasts and A549 epithelial cells. Biol Pharm Bull 2006; 29:1120-5. [PMID: 16755003]

27. Wheeler DS, Catravas JD, Odoms K, Denenberg A, Malhotra V, Wong HR. Epigallocatechin-3-gallate, a green tea-derived polyphenol, inhibits IL-1 beta-dependent proinflammatory signal transduction in cultured respiratory epithelial cells. J Nutr 2004; 134:1039-44. [PMID: 15113942]

28. Heo J, Lee BR, Koh JW. Protective effects of epigallocatechin gallate after UV irradiation of cultured human lens epithelial cells. Korean J Ophthalmol 2008; 22:183-6. [PMID: 18784447]

29. Xu JY, Wu LY, Zheng XQ, Lu JL, Wu MY, Liang YR. Green tea polyphenols attenuating ultraviolet B-induced damage to human retinal pigment epithelial cells in vitro. Invest Ophthalmol Vis Sci 2010; 51:6665-70. [PMID: 20702817]

30. Tipoe GL, Leung TM, Hung MW, Fung ML. Green tea polyphenols as an anti-oxidant and anti-inflammatory agent for cardiovascular protection. Cardiovasc Hematol Disord Drug Targets 2007; 7:135-44. [PMID: 17584048]

31. Singh R, Akhtar N, Haqqi TM. Green tea polyphenol epigallocatechin-3-gallate: inflammation and arthritis. Life Sci 2010; 86:907-18. [PMID: 20462508]

32. Lee H, Dana R. Topical EGCG for treatment of dry eye disease. ARVO Annual Meeting; 2010 May 2-6; Fort Lauderdale (FL).

33. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 2000; 267:5421-6. [PMID: 10951200]

34. Ahmed SA, Gogal RM Jr, Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. J Immunol Methods 1994; 170:211-24. [PMID: 8157999]

35. Vignali DA. Multiplexed particle-based flow cytometric assays. J Immunol Methods 2000; 243:243-55. [PMID: 10986418]

36. Morgan E, Varro R, Sepulveda H, Ember JA, Apgar J, Wilson J, Lowe L, Chen R, Shivraj L, Agadir A, Campos R, Ernst D, Gaur A. Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. Clin Immunol 2004; 110:252-66. [PMID: 15047203]

37. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002; 298:1911-2. [PMID: 12471242]

38. Shimizu M, Weinstein IB. Modulation of signal transduction by tea catechins and related phytochemicals. Mutat Res 2005; 591:147-60. [PMID: 15992833]

39. Matsumoto K, Ikema K, Tanihara H. Role of cytokines and chemokines in pseudomonal keratitis. Cornea 2005; 24:S43-9. [PMID: 16227823]

40. Thakur A, Barrett RP, McClellan S, Hazlett LD. Regulation of Pseudomonas aeruginosa corneal infection in IL-1 beta converting enzyme (ICE, caspase-1) deficient mice. Curr Eye Res 2004; 29:225-33. [PMID: 15590467]

41. Xue ML, Zhu H, Willcox M, Wakefield D, Lloyd A, Thakur A. The role of IL-1beta in the regulation of IL-8 and IL-6 in human corneal epithelial cells during Pseudomonas aeruginosa colonization. Curr Eye Res 2001; 23:406-14. [PMID: 12045890]

42. Zigman S, Rafferty NS, Rafferty KA, Lewis N. Effects of green tea polyphenols on lens photooxidative stress. Biol Bull 1999; 197:285-6. [PMID: 10573853]

43. Siu AW, Lau MK, Cheng JS, Chow CK, Tam WC, Li KK, Lee DK, To TS, To CH, Do CW. Glutamate-induced retinal lipid and protein damage: the protective effects of catechin. Neurosci Lett 2008; 432:193-7. [PMID: 18249068]

44. Kim SJ, Jeong HJ, Lee KM, Myung NY, An NH, Yang WM, Park SK, Lee HJ, Hong SH, Kim HM, Um JY. Epigallocatechin-3-gallate suppresses NF-kappaB activation and phosphorylation of p38 MAPK and JNK in human astrocytoma U373MG cells. J Nutr Biochem 2007; 18:587-96. [PMID: 17446059]

45. Dower SK, Qwarnstrom EE. Signalling networks, inflammation and innate immunity. Biochem Soc Trans 2003; 31:1462-71. [PMID: 14641090]

46. Cavet ME, Harrington KL, Ward KW, Zhang JZ. Mapracorat, a novel selective glucocorticoid receptor agonist, inhibits hyperosmolar-induced cytokine release and MAPK pathways in human corneal epithelial cells. Mol Vis 2010; 16:1791-800. [PMID: 20824100]

47. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol 2002; 4:E131-6. [PMID: 11988758]

48. Surh Y-J. NF-kB and AP-1 as molecular targets for chemoprevention with EGCG, a review. Environ Chem Lett 2006; 4:137-41.

49. Choi KC, Jung MG, Lee YH, Yoon JC, Kwon SH, Kang HB, Kim MJ, Cha JH, Kim YJ, Jun WJ, Lee JM, Yoon HG. Epigallocatechin-3-gallate, a histone acetyltransferase inhibitor, inhibits EBV-induced B lymphocyte transformation via suppression of RelA acetylation. Cancer Res 2009; 69:583-92. [PMID: 19147572]

50. Lee JH, Kim M, Im YS, Choi W, Byeon SH, Lee HK. NFAT5 induction and its role in hyperosmolar stressed human limbal epithelial cells. Invest Ophthalmol Vis Sci 2008; 49:1827-35. [PMID: 18436816]

51. Bode JG, Gatsios P, Ludwig S, Rapp UR, Haussinger D, Heinrich PC, Graeve L. The mitogen-activated protein (MAP) kinase p38 and its upstream activator MAP kinase kinase 6 are involved in the activation of signal transducer and activator of transcription by hyperosmolarity. J Biol Chem 1999; 274:30222-7. [PMID: 10514514]

52. Dudley AC, Thomas D, Best J, Jenkins A. The STATs in cell stress-type responses. Cell Commun Signal 2004; 2:8. [PMID: 15296508]

53. McInnis KA, Britain A, Lausch RN, Oakes JE. Human corneal epithelial cells synthesize ELR(-)alpha-chemokines in response to proinflammatory mediators. Ocul Immunol Inflamm 2007; 15:295-302. [PMID: 17763126]

54. Townsend PA, Scarabelli TM, Pasini E, Gitti G, Menegazzi M, Suzuki H, Knight RA, Latchman DS, Stephanou A. Epigallocatechin-3-gallate inhibits STAT-1 activation and protects cardiac myocytes from ischemia/reperfusion-induced apoptosis. FASEB J 2004; 18:1621-3. [PMID: 15319365]

55. Zykova TA, Zhang Y, Zhu F, Bode AM, Dong Z. The signal transduction networks required for phosphorylation of STAT1 at Ser727 in mouse epidermal JB6 cells in the UVB response and inhibitory mechanisms of tea polyphenols. Carcinogenesis 2005; 26:331-42. [PMID: 15550455]

56. Darra E, Shoji K, Mariotto S, Suzuki H. Protective effect of epigallocatechin-3-gallate on ischemia/reperfusion-induced injuries in the heart: STAT1 silencing flavonoid. Genes Nutr 2007; 2:307-10. [PMID: 18850185]

57. Sukhthankar M, Choi CK, English A, Kim JS, Baek SJ. A potential proliferative gene, NUDT6, is down-regulated by green tea catechins at the posttranscriptional level. J Nutr Biochem 2010; 21:98-106. [PMID: 19157820]