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# NOD2 triggers PGE2 synthesis leading to IL-8 activation in *Staphylococcus aureus*-infected human conjunctival epithelial cells

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ABSTRACT

We previously showed that *Staphylococcus aureus* and *Pseudomonas aeruginosa* stimulate IL-8 expression in human conjunctival epithelial cells through different signal transduction pathways. As in some cell types both the bacteria may induce the release of prostaglandin E2 (PGE2) and PGE2 may affect the expression of IL-8, we aimed at investigating whether in human conjunctival cells infected with *S. aureus* or *P. aeruginosa* the activation of IL-8 transcription was mediated by PGE2 and which were the underlying molecular mechanisms. We found that *S. aureus*, but not *P. aeruginosa*, triggered IL-8 activation by increasing COX-2 expression and PGE2 levels in a time-dependent manner. Overexpression of nucleotide-binding oligomerization domain-2 (NOD2) resulted to be essential in the enhancement of IL-8 induced by *S. aureus*. It dramatically activated c-jun $NH_2$-terminal kinase (JNK) pathway which in turn led to COX2 upregulation and ultimately to *IL-8* transcription. The full understanding of the *S. aureus*-induced biochemical processes in human conjunctival epithelium will bring new insight to the knowledge of the molecular mechanisms involved in conjunctiva bacterial infections and develop novel treatment aiming at phlogosis modulation.

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## 1. Introduction

The epithelium of the ocular surface, and particularly the conjunctival epithelium, is positioned at the interface between the internal milieu and the external environment and helps to prevent the entrance of microbes into the eye. It serves a critical function in the front-line defense of the innate immune system against microbes through non-specific mechanisms, among which the production of a variety of proinflammatory cytokines/chemokines [1]. This feature enables a swift immune response to invaders but may create the danger of over-reaction that may induce the initiation and perpetuation of inflammatory responses [1].

The overproduction of the chemokine IL-8, through transcriptional or epigenetic mechanisms, may lead to exacerbation of tissue injury through the amplification of inflammation [2]. IL-8 was shown to be activated as in *Staphylococcus aureus*- as in *Pseudomonas aeruginosa*-triggered human conjunctivitis albeit through different signal transduction pathways: *S. aureus* via JNK activation and *P. aeruginosa* [3–5] via the cooperative binding of Rel-A and C/EBP-β to IL-8 promoter. As in a variety of cells these two bacteria increase the production of PGE2 [6,7], which at times may induce IL-8 production [8–10], here we investigated whether in conjunctival epithelial cells they activate IL-8 through PGE2. Furthermore, we attempted to identify the signaling components upstream PGE2 secretion. Elucidating these mechanisms may be of interest, as in several instances an increase of PGE2 synthesis may be responsible for exaggerated host defense reactions against bacterial infections [11–13]. In this light, a setting of PGE2 signaling may have potential as a form of adjuvant therapy for bacterial conjunctivitis.

## 2. Materials and methods

### 2.1. Cell cultures and treatment

The investigation adhered to the Declaration of Helsinki and it was approved by the Ethics Committee of the University Hospital of Messina. Primary and Chang conjunctival epithelial cells were cultured and challenged with *S. aureus* and *P. aeruginosa*, as previously reported [4,5,14]. Where indicated, cells were transfected with COX-2 or NOD2 siRNAs (0.5 μg; Santa-Cruz Biotechnology, CA), or with NOD2 plasmid (2 μg) [15], or treated with SP600125 (SP; 20 μM; Calbiochem, Milan, Italy); U0126 (1 μM; Calbiochem); SB203580 (SB; 1 μM; Calbiochem); CAPE (10 μM; Calbiochem),

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SC-506 (300 μM, Cayman Chemical, Ann Arbor, MI), NS-398 (300 μM, Cayman Chemical) 1 h before *S. aureus* [$3 \times 10^6$ CFU; multiplicity of infection (MOI) of 1] or muramyldipeptide (MDP; 10 μg/ml; Sigma–Aldrich Milan, Italy) stimulation.

### 2.2. IL-8 and PGE2 production

IL-8 and PGE2 were measured by enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical).

### 2.3. RNA extraction and reverse transcription

1 μg of total RNA extracted with TRIZOL (Invitrogen, Milan, Italy) was reverse-transcribed with IMProm-II™ kit (Promega, Milan, Italy).

### 2.4. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed using ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Milan, Italy). Primers and probes were as follows: for IL-8, *5′-CTCACTGTGTGTAAACATGACTTCCA-3′* (forward primer), *5′-TTCACACAGAGCTGCAGAAATCA-3′* (reverse primer) and *5′-FAM-CCGTGGCTCTCTTTGGCAGCCTTC-MGBNFQ-3′* (probe); for COX-1, *5′-AGCAGCTTTTCCAGACGACC-3′* (forward primer), *5′-CGGTTGCGGTATTGGAACTG-3′* (reverse primer) and *5′-FAM-CGTGCAGCAGCTGAGTGGCTATTTCC-MGBNFQ-3′* (probe); for COX-2, *5′-CCAGCACTTCACGCATCAGT-3′* (forward primer), *5′-ACGCTGTCTAGCCAGAGTTTCAC-3′* (reverse primer) and *5′-FAM-GGCTGGGCCATGGGGTGGACTTAAAT-MGBNFQ-3′* (probe); for NOD2, *5′-CCGAGGCATCTGCAAGCTCA-3′* (forward primer), *5′-TGCAAGGCTCTGTATTTGC-3′* (reverse primer) and *5′-FAM-CTCGCAGTGAAGAGCACATT-MGBNFQ-3′* (probe). Thermal cycling conditions included activation at 95 °C (10 min) followed by 40 cycles each of denaturation at 95 °C (15 s) and annealing/elongation (1 min) at 60 °C. Each sample was analyzed with beta-actin (Applied Biosystems) as housekeeping gene.

### 2.5. Transient transfections

Cells were transiently transfected with IL-8 [8], COX-2 [16] or JNK (PathDetect Kit, Stratagene) promoter/luciferase reporter constructs. Cells were harvested, and protein extracts were prepared for the luciferase activity using luciferine (Promega, Milan, Italy) as substrate.

### 2.6. Western blotting

The membranes were probed with anti-NOD2 (Santa Cruz Biotechnology, CA), anti-COX-2 (Cell Signaling Technology, Milan, Italy) and phosphorylated JNK (Cell Signaling Technology). Immunoreactive bands were detected by autoradiography (SuperSignal West Pico Chemiluminescent Substrate System, Pierce).

### 2.7. Statistical analysis

Data are expressed as means ± S.D. from four determinations. Results were analyzed by two-tailed Student's *t* test. *p* values less than 0.05 were considered significant.

## 3. Results

### 3.1. S. aureus, but not P. aeruginosa, increases COX-2 expression and PGE2 production in conjunctival epithelial cells

Primary and Chang conjunctival epithelial cells were infected with $3 \times 10^6$ CFU live *P. aeruginosa* or *S. aureus* for 2, 3, 4, 6, 8, 12, 18, and 24 h and PGE2 amounts were determined. Fig. 1A shows that PGE2 rose at 2 h after *S. aureus* treatment and backed to basal levels within 12–24 h. On the contrary, *P. aeruginosa* did not affect PGE2 production. To examine whether the *S. aureus*-induced increase of PGE2 was correlated to the activation of the expression of cyclooxygenase-1 and -2 (COX-1 and COX-2), quantitative real-time PCR (qRT-PCR) analysis was performed. The results reported in Fig. 1B and C show that, as compared to untreated cells, COX-1 mRNA expression remained unchanged after *S. aureus* stimulation, while the levels of COX-2 significantly increased by 2 h, peaked at 3 h, and were still elevated at 8 h. In contrast, *P. aeruginosa* challenge had no effect on either COX-1 or COX-2 mRNA abundance. To confirm that the PGE2 produced by *S. aureus* was through COX-2, cells were treated with the specific COX-1 inhibitor SC-506 and the COX-2-selective inhibitor NS-398. As shown in Fig. 1D, NS-398 suppressed PGE2 production induced by *S. aureus* in a dose-dependent manner, whereas SC-506 did not affect the PGE2 synthesis induced by the bacterium. These data show a key role for COX-2 in the PGE2 response to *S. aureus* in conjunctival epithelium and point to any contribution of COX-1.

### 3.2. S. aureus-induced IL-8 increase is mediated by COX-2 and PGE2

To verify whether the *S. aureus*-induced increase of COX-2 transcript was responsible for the induction of IL-8 upregulation, we investigated the effects of either NS-398, or a synthetic siRNA targeting COX-2, on IL-8 activation in *S. aureus*-treated cells. Both the treatments significantly reduced the upregulation of IL-8 promoter (Fig. 2B), the overexpression of IL8 mRNA (Fig. 2C), and the overproduction of IL-8 protein (Fig. 2D) induced by *S. aureus*. On the contrary, the inhibition of COX-2 expression had no effect on *P. aeruginosa*-triggered IL-8 activation (data not shown). To determine the involvement of PGE2 in *S. aureus*-induced IL-8 production, cultures were treated for 6 h with various concentrations of this prostaglandin in the presence or absence of the bacterium. As seen in Fig. 2E, progressively elevated levels of IL-8 were observed when the cells were treated with PGE2 from $10^{-6}$ M up to $10^{-4}$ M, and even more so when the cells were also infected by *S. aureus*. Therefore, it can be assumed that *S. aureus* led to IL-8 release through PGE2 enhancement in conjunctival epithelial cells.

### 3.3. S. aureus induced enhancement of COX-2 and IL-8 through upregulation of nucleotide-binding oligomerization domain-2 (NOD2)

As previous studies indicated that multiple intracellular pathogens, including *S. aureus*, are sensed by NOD2 [17], we investigated whether NOD2 is involved in *S. aureus*-induced conjunctivitis. Conjunctival epithelial cells treated with the pathogen exhibited a significant increase in NOD2 mRNA and protein levels with a peak at 2 h and 3 h after treatment, respectively (Fig. 3A). Then we investigated whether the overexpression of NOD2 affected COX2 levels. Cells overexpressing NOD2 led to enhanced promoter activation, mRNA levels and protein amount of COX-2 and stimulation of NOD2-transfected cells with *S. aureus* resulted in significantly higher levels of COX-2 expression (Fig. 3B). To confirm the involvement of NOD2 in the *S. aureus*-induced COX-2 activation, we performed gene silencing experiments using a synthetic NOD2 siRNA. Inhibition of NOD2 expression completely suppressed the *S. aureus*-induced COX-2 expression at promoter, mRNA and protein level (Fig. 3C), thus providing definitive evidence of the functional role of NOD2 in *S. aureus*-induced COX-2 gene expression in conjunctival cells. Next we investigated the signaling pathway involved in the NOD2-mediated enhancement of COX-2 expression in response to *S. aureus* challenge. Fig. 3D shows that either *S. aureus* or the NOD2 agonist MDP increased COX-2 mRNA levels through JNK, as their effects were antagonized by treatment

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