Induction of Cyclooxygenase 2 by *Streptococcus pyogenes* Is Mediated by Cytolysins

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**Abstract**
Prostaglandin E\(_2\) (PGE\(_2\)), an arachidonic acid metabolite regulating a broad range of physiological activities, is an important modulator of the severity of infection caused by *Streptococcus pyogenes*. Here, we investigated the role of streptococcal cytolysin S (SLS) and streptococcal cytolysin O (SLO) in the induction of cyclooxygenase-2 (COX-2), the rate-limiting enzyme in the synthesis of prostaglandins, in vitro cultured macrophages and during in vivo infection. Macrophages were infected with *S. pyogenes* wild type or with the isogenic mutant strains deficient in SLS (ΔSLS), SLO (ΔSLO), or both (ΔSLS/ΔSLO), and the expression of COX-2 was determined at the transcriptional and the protein level. The results indicated that *S. pyogenes* induced expression of COX-2 and concomitant synthesis of PGE\(_2\) in macrophages mediated by the synergistic activity of both SLS and SLO, and involved calcium and the PKC/JNK signaling pathway. These results were validated using recombinant cytolysins. In a murine skin infection model, COX-2-positive cells were found more abundant at the site of *S. pyogenes* wild-type infection than at the site of infection with ΔSLS/ΔSLO mutant strain. These findings suggest that inhibitory targeting of SLS and SLO could ameliorate the adverse effects of high levels of prostaglandins during *S. pyogenes* infection.

**Introduction**
Prostaglandin E\(_2\) (PGE\(_2\)) is an arachidonic acid metabolite produced by several cell types and elicits a wide range of biological activities, including the modulation of inflammation and immune responses [1, 2]. PGE\(_2\) modulates the function of immune cells such as macrophages [2, 3] and dendritic cells [4, 5], which play an important role in the host response to infection. Synthesis of PGE\(_2\) begins with the release of arachidonic acid from cell membranes, which is then transformed by the enzyme cyclooxygenase (COX) in prostaglandin H\(_2\) (PGH\(_2\)) that can be further converted into PGE\(_2\) by the activity of PGE\(_2\) synthase [6]. There are two different...
isoforms of COX enzymes, COX-1 and COX-2. While COX-1 is constitutively expressed in most tissues, COX-2 is an inducible enzyme [7, 8]. It has been shown that COX-2 is induced by several pathogens [9–16] and that it is expressed upon stimulation with bacterial products [6, 17]. We have previously reported that COX-2 is induced by the pathogen Streptococcus pyogenes during infection and that the levels of this enzyme correlate with the severity of infection in patients as well as in murine experimental models [11]. S. pyogenes is an important human pathogen and a frequent cause of mild infections such as pharyngitis or impetigo [18]. However, this pathogen can also cause very severe life-threatening diseases, such as necrotizing fasciitis, characterized by progressive necrosis of skin, subcutaneous tissue, and fasciae that can lead to septic shock [18]. The morbidity and mortality associated with these severe S. pyogenes infections have raised the need to understand the mechanisms underlining disease manifestations in order to develop therapies that are more effective. In this regard, the identification of molecular pathways that influence the ability of the host to mount an appropriate immune response to S. pyogenes, which are prone to pharmacological manipulation, will facilitate the development of these therapeutic approaches.

In our previous study [11], we demonstrated that the detrimental effect of high COX-2 levels in the course of S. pyogenes infections was associated with a stronger suppressive effect of PGE₂ on the capacity of professional phagocytic cells to ingest and kill S. pyogenes. The suppressive effect of PGE₂ was mediated by enhanced intracellular cAMP production and activation of protein kinase A resulting in the reduction of antimicrobial oxygen radical species [11]. Although a considerable amount of information has been gathered concerning the molecular basis underlying the inhibitory effect of PGE₂ on the bactericidal activity of phagocytic cells during S. pyogenes infection, little is known about the bacterial factors triggering the production of this prostaglandin.

Since bacterial toxins such as Clostridium difficile toxin A [13] and pneumolysin [19] have been reported to be strong inducers of COX-2 expression and S. pyogenes expresses two cytolysins, streptococcal cytolsin S (SLS) and streptococcal cytolsin O (SLO), which are among the most important virulence factors produced by this pathogen [20–22], the purpose of this study was to assess the role of SLS and SLO in the induction of COX-2 and concomitant production of PGE₂ by S. pyogenes in in vitro cultured macrophages and during in vivo infection.

Material and Methods

Bacteria

S. pyogenes strain NZ131 (M49), its derivatives SLO-deficient (ASLO), SLS-deficient (ASLS), and the double-mutant strain deficient in the production of both SLO and SLS (ΔSLS/ΔSLO) [23] were cultured at 37°C in Todd-Hewitt broth (Oxoid, Basingstoke, UK), supplemented with 1% yeast extract. Bacteria were collected during mid-log phase, washed twice with sterile phosphate-buffered saline (PBS), diluted to the required concentration, and the number of viable bacteria determined by counting colony forming units (cfu) after plating on agar plates (BD Pharmingen) containing 5% sheep blood.

Mice

C57BL/6 female mice were purchased from Harlan-Winkelmann (Borchen, Germany) and used in experiments when they were between 8 and 10 weeks of age. They were housed in microisolator cages and given food and water ad libitum. Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). All experiments were approved by the ethics board LAVES (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany; permit No. 33.9-42502-04-10/0296).

Subcutaneous Infection of Mice and Immunohistochemistry

Mice were infected subcutaneously with 10⁷ cfu of either S. pyogenes wild-type (WT) strain or with its ΔSLS/ΔSLO derivative and killed 24 h after infection by CO₂ asphyxiation. Skin samples from the local focus of infection were excised, fixed in 10% formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. Paraffin sections were rehydrated through graded alcohols. To block endogenous peroxidase, formalin-fixed, paraffin-embedded tissue sections were treated with 0.5% H₂O₂ diluted in methanol for 30 min at room temperature (RT). Subsequently, sections were incubated in 10 mM sodium citrate buffer (pH 6.0) for 20 min in a microwave oven (800 W). Following incubation with 20% goat serum each for 30 min to block nonspecific binding sites, sections were incubated with rabbit anti-mouse COX-2 antibodies (Cayman Chemicals) for 1.5 h at RT. Rabbit serum was used as a negative control. Sections were then incubated for 30 min at RT with the secondary goat anti-rabbit antibody (Vector Laboratories) followed by incubation with the peroxidase-conjugated avidin-biotin complex (Vector Laboratories) for 30 min at RT. After visualization of the positive antigen-antibody reaction by incubation with 3,3-diaminobenzidine-tetrachloride for 5 min, sections were counterstained with hematoxylin. Positive cells were counted in 5 randomly selected high-power fields (×40 magnification) per slide.

Immunohistochemical Double Labeling

Sequential immunohistochemical double labeling was performed using the ABC method. For this purpose, the monoclonal, biotinylated rat anti-mouse MAC-3 antibody (AbD Serotec) and the polyclonal rabbit anti-mouse COX-2-specific antibody (Cayman Chemicals) were used. Formalin-fixed and paraffin-embedded mouse skin sections were deparaffinized by Roticlear (Roth) and hydrated through graded alcohols. Endogenous peroxidase
COX-2 and PGE$_2$ in S. pyogenes Infection

was blocked by 85% ethanol with H$_2$O$_2$ (0.5%) for 30 min at RT. Antigen retrieval was performed by heating the sections for 20 min in a microwave oven (800 W) in citrate buffer (pH 6.0). Tissue sections were then incubated with the monoclonal, biotinylated rat anti-mouse MAC-3 antibody (dilution 1:200) for 90 min at RT. After washing the sections with PBS, they were treated with peroxidase-conjugated avidin-biotin complex (Vector Laboratories) for 30 min and the 3,3′-diaminobenzidine tetrahydrochloride chromogen for the visualization of positive antigen–antibody reactions. Sections were washed in PBS buffer for 5 min and nonspecific binding sites were blocked by incubation with 20% goat serum for 20 min and further incubated with the polyclonal rabbit anti-mouse COX-2-specific antibody (dilution 1:200) overnight at 4°C. Negative controls were incubated with rat and rabbit normal serum, respectively. Sections were then treated with the secondary goat anti-rabbit antibody (Vector Laboratories, dilution 1:200) for 30 min and further incubated for another 30 min with ABC solution. COX-2-specific reactions were visualized using the HistoGreen chromogen kit (Linaris). Colocalization of MAC-3 protein (brown) and COX-2 (green) were identified by the presence of both colors in 1 cell.

**Isolation of Bone Marrow-Derived Macrophages**

Murine bone marrow macrophages were obtained from bone marrow extruded from the femur and tibia of female mice and cultivated and differentiated as previously described [11]. On day 7, differentiated macrophages were harvested, counted, adjusted to 10$^6$ cells/mL, and used for infection.

**In vitro Infection of Bone Marrow-Derived Macrophages**

Macrophages were seeded in 24-well plates at a concentration of 10$^4$ cells/mL and infected with S. pyogenes WT or mutant strains at a multiplicity of infection (MOI) of 10:1 and incubated at 37°C for 1 h at 5% CO$_2$. Macrophages were then washed twice with sterile warm PBS, resuspended in complete medium containing 100 μg/mL of gentamicin, and further incubated at 37°C for 2 h at 5% CO$_2$. Cells were then washed again and lysed in RIPA-buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM MgCl$_2$, 10% glycerol, 1% NP-40, 200 mM EDTA, PMSF, and Mini Complete) for Western blotting. In some experiments, 5 mM of the extracellular calcium chelator EGTA, 100 nM of the protein kinase C (PKC) inhibitor GF 109203X, 50 μM of the selective inhibitor of MEK1 activation and the mitogen-activated protein kinase (MAPK) inhibitor PD 98059, as well as 100 nM of the inhibitor of the c-Jun N-terminal kinase (JNK) inhibitor II were added to the cell culture medium.

**Western Blotting**

Macrophage lysates were loaded into a 10% SDS-PAGE separation gel (4% SDS-PAGE collecting gel), run at 100 mA for 1.5 h, and transferred to a nitrocellulose membrane by wet blot. The membrane was blocked with 5% nonfat dried milk in PBS for 2 h at RT or overnight at 4°C. After washing twice for 10 min with PBS containing 0.1% Tween 20, blots were incubated for 2 h at RT or overnight at 4°C with rabbit anti-COX-2 or anti-β-actin antibodies (Cayman Chemicals) diluted 1:2,000 in 2.5% nonfat dried milk in PBS. After two washes for 10 min with PBS containing 0.1% Tween 20, blots were incubated for 2 h at RT or overnight at 4°C with HRP-conjugated goat-anti-rabbit IgG, diluted 1:2,000 in 2.5% nonfat dried milk in PBS. Blots were washed again and developed with ECL solution (Amersham).

**Flow-Cytometric Analysis**

To stain intracellular COX-2, macrophages were incubated with anti-CO16/CD32 antibody for 15 min at RT to block the FcγRIII/II receptors and permeabilized with 0.05% Triton X-100. Macrophages were then incubated with rabbit-anti-COX-2 antibodies for 1 h at 4°C, washed, and further incubated with goat anti-rabbit IgG Alexa Fluor 568 for 1 h at 4°C. After incubation, macrophages were washed and analyzed with a BD FACS Calibur (BD Bioscience).

**Immunofluorescence Microscopy**

Macrophages were seeded on glass cover slides and infected with FITC-labeled S. pyogenes to a MOI of 10:1 at 37°C for 2 h at 5% CO$_2$. Cells were fixed with 3% formaldehyde overnight at 4°C, washed, permeabilized with 0.05% Triton X-100 in PBS, and incubated for 5 min at RT. Cells were blocked with PBS + 10% fetal calf serum (FCS) and incubated for 1 h at RT with rabbit anti-COX-2 antibody diluted 1:150 in PBS + 10% FCS. After washing, goat anti-rabbit IgG Alexa Fluor 568 diluted 1:200 in PBS + 10% FCS was added, and macrophages were further incubated for 1 h at RT. Cover slides were washed again and placed on glass slides with Mowiol containing DAPI (ProLong Gold; Promega), sealed with nail polish, and analyzed by fluorescence microscopy.

**RT-PCR**

Total RNA was prepared using the GeneJet RNA purification kit (Thermo Scientific). The RNA was then subjected to RT-PCR under standard reaction conditions using a SensiFAST SYBR NO-ROX one step kit according to the manufacturer’s instructions. The PCR primer sequences are as follows: murine COX-2 forward 5′-AAGCGAGGACCTGGGTTCA-3′ and reverse 5′-AAGGCGGACCTGGTGTTCA-3′ and reverse 5′-TGGATCTGTCGATCAGATGCC-3′. Thermal cycling for macrophage transcriptional response conditions of cox-2 and β-actin consisted of reverse transcription for 15 min at 45°C and activation of polymerase for 5 min at 95°C, followed by 35 cycles of 20 s at 95°C, 20 s at 58°C, and 20 s at 72°C, which correspond to denaturation, annealing, and extension, respectively.

**Statistical Analysis**

Comparisons between groups were performed using either ANOVA and or the Student T test. p < 0.05 was considered to be statistically significant.

**Results**

S. pyogenes Induction of COX-2 in Macrophages Is Mediated by SLS and SLO

To investigate the contribution of SLS and SLO to the induction of cox-2 expression in murine macrophages by S. pyogenes, macrophages were infected with a MOI...
of 10:1 of either viable or heat-killed *S. pyogenes* WT or with the isogenic ΔSLS ± ΔSLO mutant strains, and the expression levels of *cox-2* were analyzed at the indicated time points by quantitative RT-PCR (Fig. 1a). The expression levels of *cox-2* in macrophages infected with the ΔSLS/ΔSLO mutant strain or with heat-killed *S. pyogenes* WT were found to be significantly lower than the expression levels observed in macrophages infected with the isogenic WT strain (Fig. 1a). These data clearly demonstrate that bacterial viability and production of SLS
COX-2 and PGE2 in *S. pyogenes* Infection

COX-2 and SLO by *S. pyogenes* contribute to the induction of the inducible form of COX-2 in macrophages. Western blot analysis was then performed to validate the obtained expression data at the protein level. For this purpose, macrophages were infected with either viable or heat-killed *S. pyogenes* WT or with the isogenic ΔSLS/ΔSLO mutant strain, total protein fractions were isolated after 24 h of infection, and the levels of COX-2 determined by Western blotting were compared with those of uninfected control macrophages. Whereas high levels of COX-2 protein were observed in macrophages infected with *S. pyogenes* WT, COX-2 protein levels in macrophages infected with either heat-killed *S. pyogenes* WT or with the isogenic ΔSLS/ΔSLO mutant strain were marginal (Fig. 1b). COX-2 protein was absent in uninfected macrophages (Fig. 1c), and the expression of COX-2 quantified in the different groups of treated macrophages by flow cytometry (Fig. 2a, b).
SLS and SLO Have a Synergistic Effect on the Induction of COX2 Expression in Macrophages Infected with S. pyogenes

To investigate the extent to which each single cytolysin contributed to the expression of COX-2, the expression of COX-2 was determined in macrophages infected with either WT or the ΔSLS/ΔSLO, ΔSLS, or ΔSLO mutant strains by Western blotting 24 h after infection. COX-2 expression in uninfected macrophages was used as a negative control. As shown in Figure 2c, the protein level of COX-2 in macrophages infected with the ΔSLS and ΔSLO single-mutant strains was higher than the level of COX-2 in macrophages infected with the ΔSLS/ΔSLO double-mutant strain but still lower than the level of COX-2 in macrophages infected with S. pyogenes WT. These data indicate a synergistic but independent effect of both cytolysins in the induction of COX-2 by S. pyogenes in macrophages.

Production of PGE2 by Bone Marrow-Derived Macrophages after in vitro Infection with S. pyogenes Is Mediated by the Streptococcal SLS and SLO

Since COX-2 catalyzes the rate-limiting step of prostaglandin biosynthesis that leads to the production of PGE2 [8], the role of SLS and SLO in the production of PGE2 by macrophages in response to S. pyogenes infection was investigated. For this purpose, the levels of PGE2 were determined by EIA in the supernatant of macrophages infected with either S. pyogenes WT or the ΔSLS/ΔSLO, ΔSLS, or ΔSLO mutant strains at different times after infection. Whereas PGE2 was significantly increased in the supernatant of macrophages infected with S. pyogenes WT mainly 24 h after infection, the PGE2 levels in the supernatant of macrophages infected with the ΔSLS/ΔSLO strain were below the detection limit (Fig. 3a). Although macrophages infected with the ΔSLS or ΔSLO single-mutant strains released significantly greater amounts of PGE2 into the culture supernatant than those infected with the ΔSLS/ΔSLO double-mutant strain, the PGE2 levels did not reach the maximal levels observed in macrophages infected with the WT strain (Fig. 3a). Thus, the synthesis of PGE2 by macrophages in response to S. pyogenes infection was mediated by both SLS and SLO. These data were confirmed by experiments where macrophages were treated with recombinant SLS (rSLS) and/or recombinant SLO (rSLO) at a concentration of 1 μg/mL. It was observed that each cytolysin was able to induce the release of PGE2 by macrophages (Fig. 3b). This effect was, however, potentiated when both cytolysins (0.5 μg/mL each) were combined (Fig. 3b). To ensure that the cytolysin concentrations used in these experiments do not have a cytotoxic effect, the release of lactate dehydrogenase, which is an indication of cell death, was determined in the cytolysin-treated macrophages. No necrotic cell death was observed at the indicated cytolysin concentrations (data not shown). These data underline the already described synergistic effect of both toxins for the induction of COX-2.

Ca2+ Is Involved in Cytolysin-Mediated S. pyogenes Induction of PGE2 Release by Macrophages

Since the induction of PGE2 synthesis by cytolysins such as pneumolysin of Streptococcus pneumoniae has been shown to be calcium dependent [19], the involvement of calcium in the induction of PGE2 by S. pyogenes in macrophages was investigated. To this end, the release of PGE2 by macrophages infected with S. pyogenes WT or with the ΔSLS/ΔSLO mutant strain was determined in the presence or absence of the calcium-chelating agent EGTA. The results depicted in Figure 4a show that the amount of PGE2 released by S. pyogenes-infected macrophages was significantly reduced in the presence of EGTA, indicating that PEG2 synthesis in these cells is calcium dependent. Interestingly, while induction of PGE2 synthesis by rSLO was fully calcium dependent, induction of PGE2 by rSLS was only partially dependent on calcium (Fig. 4b).

The PKC/JNK Signaling Pathway Is Involved in the Induction of COX-2 by Streptococcal Cytolysins

To identify the signaling pathways involved in the induction of COX-2 by the streptococcal cytolysins SLO and SLS, the effect of specific inhibitors of PKC, MAPK, and JNK signaling pathways was evaluated. Macrophages were treated with either GF 109203X, a potent, cell-permeable, ATP-competitive PKC inhibitor, PD-98059, a highly selective in vitro inhibitor of MEK1 activation and the MAPK cascade, or JNK inhibitor II, a potent, cell-permeable, selective, and reversible inhibitor of JNK prior to infection with S. pyogenes WT. RNA was isolated from differently treated, infected macrophages and induction of COX-2 gene expression was assessed by RT-PCR. A significant reduction in the COX-2 mRNA level was observed after blocking PKC, MAPK, or JNK signaling pathways (Fig. 5). These findings indicate the involvement of the PKC/JNK signaling pathway in the induction of COX-2/PGE2 expression by streptococcal cytolysins.
**Fig. 3.** *Streptococcus pyogenes*-induced synthesis of PGE$_2$ in macrophages is mediated by streptococcal cytolysin S (SLS) and streptococcal cytolysin O (SLO). *p* < 0.05; **p* < 0.01; ***p* < 0.001. **a** Levels of PGE$_2$ released by macrophages after infection with either *S. pyogenes* WT, ΔSLS/ΔSLO double-mutant strains, and ΔSLS or ΔSLO single-mutant strain strain 2, 6, and 24 h after infection determined in the cell supernatant by EIA. A summary of 3 independent experiments is shown. **b** Levels of PGE$_2$ release by macrophages after treatment with 1.0 μg/mL rSLS, 1.0 μg/mL rSLO, and 0.5 μg/mL rSLS + 0.5 μg/mL rSLO, or left untreated (Medium). Each bar represents the mean ± SD of *n* = 5. One of 3 representative experiments is shown.

**Fig. 4.** Calcium is involved in cytolysin-mediated *Streptococcus pyogenes*-induced PGE$_2$ release. A summary of 3 independent experiments is shown. **p** < 0.01; ***p** < 0.001. **a** Levels of PGE$_2$ in the supernatant of macrophages infected with *S. pyogenes* WT strains in the presence or absence of 5 mM of the calcium chelator EGTA 24 h after infection. **b** Levels of PGE$_2$ in the supernatant of macrophages treated with 1.0 μg/mL of rSLS or rSLO, 0.5 μg/mL rSLS + 0.5 μg/mL rSLO, or left untreated (Medium) in the presence or absence of 5 mM of the calcium chelator EGTA 24 h after treatment.
Induction of COX-2 by S. pyogenes during in vivo infection

To determine the induction of COX-2 by S. pyogenes during in vivo infection, mice were subcutaneously infected with 10^7 cfu of S. pyogenes WT or ΔSLS/ΔSLO mutant strain or injected with PBS as a control, and skin biopsies were taken 24 h after infection and subjected to immunohistochemical staining for the determination of COX-2. The micrographs displayed in Figure 6a revealed significantly more abundant COX-2-positive cells in skin tissue of S. pyogenes WT-infected mice (Fig. 6ii) than in the tissue of uninfected mice (Fig. 6i) or mice infected with ΔSLS/ΔSLO mutant strain (Fig. 6iii). Quantification of the amount of COX-2-positive cells in these samples is shown in Figure 6b. Double staining with antibodies against COX-2 and MAC-3 indicated that about 85–90% of cells expressing COX-2 in the infected tissue were macrophages (MAC-3+) (Fig. 6c).

Discussion

Prostaglandins are an early and important host response to microbial infections. We have previously shown that S. pyogenes is able to induce COX-2 and PGE₂ during in vivo and in vitro infection and that macrophages are among the first cells responding to the bacteria by massive upregulation of COX-2 with concomitant release of PGE₂ [11]. However, very little is known regarding the underlying molecular mechanism as well as the bacterial factors involved in this process.

Evidence has been provided that different bacterial toxins such as LPS [24], C. difficile toxin A [13] and pneumolysin [19, 25] are strong inducers of the expression of the inducible form of COX-2. This has been also shown for certain bacterial cytolysins like the ACT (adenylate cyclase toxin) of Bordetella pertussis [26]. However, the pathway mediating the effect of bacterial toxins in the expression of cyclooxygenases and, therefore, the production and release of prostaglandins is still poorly understood. The streptococcal cytolysins SLS and SLO are among the most important virulence factors of S. pyogenes [27, 28]. SLS and SLO belong to the family of pore-forming toxins and exert a cytotoxic effect on various cell types, including epithelial cells, keratinocytes, and leukocytes [20–22]. In the current study, we provide evidence that SLS and SLO are critically involved in the induction of COX-2 and the synthesis of PGE₂ during S. pyogenes infection, extending thus the list of functional activities attributed to these cytolysins. We have demonstrated that both SLS and SLO were capable to induce COX-2 expression and PGE₂ synthesis and that the amount of COX-2 and PGE₂ induced by S. pyogenes in macrophages resulted from the synergistic activity of both cytolysins. Also, in vivo, in a murine skin infection model, we observed increased amounts of COX-2-producing cells at the site of infection when mice were inoculated with WT S. pyogenes compared to animals infected with the S. pyogenes mutant strain deficient in the expression of both SLS and SLO.

Toxin signaling in immune cells is often mediated by pore formation in the cell membrane resulting in influx of extracellular Ca^{2+}. This process is a typical feature of cholesterol-dependent toxins [29] and has also been described for the streptococcal cytolysin SLO [30]. It has been proposed that bacterial toxins are able to potentiate the production of PGE₂ and leukotriene B₃ in a Ca^{2+}-dependent manner in human neutrophils [18]. For example, removal of extracellular Ca^{2+} with the chelator EGTA has been shown to completely attenuate the pneumolysin-mediated production of PGE₂ [18]. It can be argued that a similar
Ca²⁺-dependent mechanism might be involved in the induction of PGE₂ production by SLS and SLO. Indeed, we were able to demonstrate that Ca²⁺ was involved in this process because addition of EGTA to macrophages significantly attenuated the production of PGE₂ induced by S. pyogenes or recombinant cytolysins. Interestingly, the effect of Ca²⁺ chelation on the attenuation of cytolysin-induced PGE₂ synthesis was more pronounced for rSLO than rSLS. The reason for this difference in Ca²⁺ dependency between both cytolysins is not yet clear, but differences in the mechanism and structure between the pores formed by SLS and SLO might be a key issue. In this regard, SLO is an oxygen-labile secreted protein that binds to cholesterol in eukaryotic cell membranes where it oligomerizes and forms large pores [31]. SLS is an oxygen-stable oligopeptide with a very potent hemolysin activity, and the mechanism of hemolysis was reported to be closer to that mediated by complement [32].

In general, the signaling pathways involved in the up-regulation of COX-2 are not fully understood. It has been reported that elevated levels of Ca²⁺ in the cell cytoplasm are able to stimulate PKC through diacylglycerol resulting in the translocation of PKC through the plasma membrane [33]. PKC undergoes rapid activation and regulates the extracellular signal-regulated protein kinase (ERK)/nuclear factor (NF)-κB pathway by inducing the phosphorylation of ERK1/2, MAPK, and JNK [34], leading to the activation of NF-κB, which has been reported to mediate COX-2 activation and expression [33]. Using specific inhibitors of the PKC-mediated signaling pathway, we could demonstrate the involvement of the Ca²⁺-PKC signaling axis in the induction of COX-2 expression by SLS and SLO.
In summary, using different experimental in vitro and in vivo approaches, this study demonstrated that cyto-
sins produced by \textit{S. pyogenes} are critical bacterial factors
involved in the induction of COX-2 and synthesis of
PG\textsubscript{E}\textsubscript{2} by host cells, and that Ca\textsuperscript{2+}
and the PKC signaling pathway were involved in this process. The induction of
high levels of the proinflammatory lipid PG\textsubscript{E}\textsubscript{2} by strepto-
coccal cytolysins can lead to increased vascular permea-
bility, dysregulation of the inflammatory response, as well
as to decreased phagocytic bacterial killing, as previously
reported [11].

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Disclosure Statement

The authors declare no competing financial and other conflicts of interests.

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