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Original Article

Lipoteichoic acid of *Streptococcus sanguinis* induces the expression of cyclooxygenase-2 via MAPK signaling pathways in H9c2 cells

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Abstract: Bloodstream infections, including bacteremia and infective endocarditis, represent important medical conditions resulting in high mortality rates. *Streptococcus sanguinis* are dental plaque Gram-positive organisms associated to infective endocarditis. Lipoteichoic acid, is a component of the outer membrane of Gram-positive bacteria and can cause septic shock. H9c2 cells were exposed to lipoteichoic acid in order to determine levels of nuclear factor (NF)-κB, mitogen activated protein kinase (MAPK) and cyclooxygenase-2. The results are presented as mean ± SE obtained from three independent experiments. A p value of < 0.05 was considered statistically significant. In this study, we characterized lipoteichoic acid signal-transduction mechanisms on H9c2 cardiomyoblasts. When H9c2 cells were exposed to lipoteichoic acid (15 μg/ml) they induced time-dependent augmented phoshorylation of MAPK; they also enhanced cytosolic and nuclear NF-κB levels in time-dependent manner. Furthermore, lipoteichoic acid significantly increased LTA-induced COX-2. LTA-mediated COX-2 expression was inhibited by PD98059, SP-600125 and calphostin C. The present study showed that lipoteichoic acid obtained from *Streptococcus sanguinis* can increase cyclooxygenase-2 synthesis through protein kinase C, mitogen activated protein kinases and NF-κB activation in H9c2.

Keywords: Lipoteichoic acid, cardiomyoblasts, mitogen activated protein kinases, nuclear factor-κB and cyclooxygenase-2

Introduction

Streptococcus group species are Gram-positive microorganisms present in dental plaque; they are associated to dental caries [1-3]. Bacteria may be seeded into the bloodstream through brushing, chewing and oral surgery procedures. In the blood bacteria may colonize endocardium or cardiac valves that have been damaged by congenital conditions or degenerative processes resulting in infective endocarditis [4-6]. IE results fatal if not treated with antibiotics or surgery. *Streptococcus sanguinis* is a member of the viridans group of streptococci and is a primary colonizer of teeth. The viridans species, particularly *S. sanguinis* are a leading cause of infective endocarditis [5-7]. Damage is the result of the formation of sterile cardiac vegetations composed of platelets and fibrin that can be colonized by certain bacteria during periods of bacteremia [8-11].

The cell wall of Gram-positive bacteria encompasses multiple functions during bacterial growth, such as maintaining bacterial cell integrity and shape as well as resisting internal turgor pressure [12-14]. Moreover, the wall is composed of peptidoglucan surrounding the cytoplasmic membrane and it includes glycopolymers, such as teichoic acids, polysaccharides and proteins. The most common types of teichoic acids are comprised of either polyglycerol phosphate or polyribitol phosphate chains of variable length that are substituted either with glycosylresidues or D-alanyl esters or both [15-17]. Teichoic acids are covalently linked to peptidoglycan or anchored in the cytoplasmic membrane by their glycolipid moiety called lipoteichoic acids, which are the main constituents of Gram-positive bacteria surfaces, LTA have diverse biological functions such as autolysis, adhesion, biofilm formation and stimulation of

immune responses [15-20]. LTA binds to Toll-like receptor 2 to transduce the LTA signal into the cytoplasm [21-23]. It also triggers MyD-88-dependent signaling pathway leading to NF-κB activation and can stimulate production of cytokines, such as tumor necrosis factor-alpha (TNF-α), COX-2, NOS-2 and interleukin-6 (IL-6), and excessive immune response to LTA results in severe sepsis [24, 25]. Unlike *Staphylococcus aureus*, which can cause severe sepsis [26, 27], some Gram-positive lactic acid bacteria do not induce sepsis. However, the exact mechanism of cytokine expression remains unclear. The intracellular signaling pathways through which LTA cause NOS expression have been reported [28, 29], in macrophages through two separate pathways: the phosphatidylcholine-phospholipase/protein kinase C (PKC)/NF-κB cascade and the tyrosine kinase/phosphatidylinositol 3-kinase (PI3K)/AKT/p38 mitogen activated protein kinase (MAPK)/NF-κB cascade [30]. Other studies have shown a positive interaction between endogenous PGE2 synthesis and NOS-2 expression both *in vivo* and *in vitro* [31]. IL-1β plays a key role in host immune regulation against different pathogens [32]. The relevance of IL-1β has been reported in septic arthritis, brain infections and periodontal disease [33, 34]. During the initial phases of infection IL-1β attracts phagocytic cells and increases lymphocyte [35].

The results gathered in the present study showed that LTA can cause activation of MAPK pathway by induction of COX-2 protein and formation of PGE2, resulting in the activation of MAPKs and NF-κB, which in turn induce IL-1β expression. The signaling pathway explored in this study had a delayed onset, whereas two separate pathways which induce NF-κB activation in H9c2 cardiomyoblasts will be analyzed in the present study.

Materials and methods

Lipoteichoic acid (LTA obtained from *Streptococcus sanguinis*), Trizma base, dithiothreitol (DTT), Dulbecco's modified Eagle's medium (DMEM), glycerol, phenylmethysulphonyl fluoride (PMSF), leupeptin and sodium dodecylsulphate (SDS) were purchased from Sigma (St. Louis Mo, USA). Penicillin/streptomycin and fetal bovine serum were purchase from Life Technologies (Gaithersburg MD). A PGE2 enzyme immune-assay kit was obtained from Cayman (Ann Arbor, MI). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

H9c2 cardiomyoblasts, were cultured on 100 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg/ml penicillin, 100 μg/ml, 2 mM glutamine and 10% fetal bovine serum in humidified air (5% CO_2) at 37°C. H9c2 cells were incubated in serum-free essential medium overnight before LTA treatment.

Treatment of cells with LPS and inhibitors

Calphostin C (1 μM) was used as specific inhibitor of protein kinase C (PKC); PD98059 (10 μM) was used as a specific inhibitor of MEK; SB203580 (20 μM) was used as a specific inhibitor of p38 and SP600125 (10 μM) was used as a specific inhibitor of JNK. The inhibitors were dissolved in DMSO at a stock concentration of 1 mM and stored at -20°C until use. Cells were pretreated with inhibitors at the indicated concentration or with DMSO vehicle for 1 h before subjecting them to stimulation with LTA.

Western blot assay

Cells were plated onto 60 mm tissue-culture dishes and stimulated with LTA or inhibitors for the indicated time period. The cells were then washed with ice-cold PBS, lysed and then centrifuged at 13,000 g for 10 min. The whole cell lysates were separated by 10% SDS-PAGE and electro-transferred onto a PVDF membrane (Amersham Biosciences, Princeton, USA). After blocking with 5% skin milk in TBS containing 0.1% Tween 20, the membrane was incubated with antibodies against MAPKs, phosphorylated MAPKs (1:1000), COX-2 (1:1000) followed by incubation with HRP-conjugated secondary antibiodies. The immune-reactive bands were visualized with ECL reagents (Amersham Biosciences, Princeton, NJ, USA).

RT-PCR

H9c2 cells were plated onto six-well plates at 3×10^5 cells/ml and treated with LTA or inhibitors at concentrations for the indicated time period. Following treatment total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad,

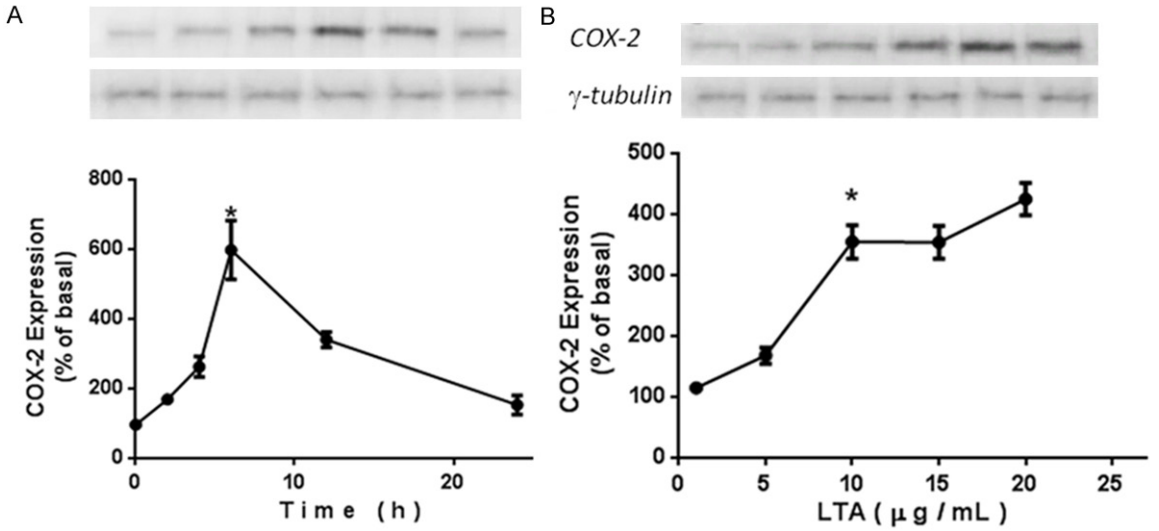

Figure 1. LTA-induced COX-2 expression in cardiomyoblasts. A: Cells were treated with vehicle or LTA at the indicated time periods. Cells were then harvested, COX-2 expression was determined by immunoblotting. Compiled results are shown at the bottom of the chart. Each column represents mean ± S.E.M. of at least three independent experiments. *P < 0.05 compared with control group. B: Cells were treated with vehicle or LTA at indicated concentrations for 6 h. After treatment, extent of COX-2 expression was determined by immunoblotting. Each column represented mean ± S.E.M. of at least three independent experiments. *P < 0.05 compared with control group.

CA, USA), according to manufacturer's instructions. Total RNA was reverse-transcribed to generate cDNA using OneStep kit (Invitrogen). The sequence of each primer are as follows: 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' (coding sense) and 5'-AGATCATCTCTGCCTGAGTATCTT-3' (anticoding sense) derived from COX-2 gene; 5'-ACAGGGAAGTCTGAAGCACTAG-3' (coding sense) and 5'-CATGCAAGGAAGGGAACTCTTC-3' (anticoding sense) derived from iNOS gene; 5'-TCCCTCAAGATTGTCAGCAA-3' (coding sense); 5'-GGCTGCAGTTCAGTGATCGTACAGG3' (sense) and 5'-AGATCTAGAGTACCTGA GCTCGCCAGTGAA3' (non-sense) derived from IL-1β and 5'-AGATCCACAACGGATACATT-3' (anticoding sense) derived from glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene For PCR cDNA was denatured initially at 94°C for 5 min and amplified with 35 cycles at 94°C for 30 s and 72°C for 1 min. PCR was completed with a final extension at 72°C for 10 min. The amplified PCR products were resolved on 1% agarose gel, visualized by staining with ethidium bromide, and subjected to densitometric analysis with Digi-Doc system.

Statistical analysis

Results are presented as the mean ± S.E. from at least three independent experiments. One-way analysis of variance (ANOVA) followed by, when appropriate, the Newman-Keuls test was used to determine the statistical significance of the difference between means. A p value of < 0.05 was considered statistically significant.

Results

LTA induced COX-2 expression in H9c2 cells

Immunoblotting analysis was conducted to examine COX-2 levels in H9c2 cells exposed to LTA. We therefore assessed COX-2 expression in cardiomyoblasts cells exposed to LTA, which increased in a time-dependent manner (**Figure 1A**). Maximal effect was observed after 6 h incubation with LTA. Protein COX-2 significantly decreased after exposure to LTA for 12 h, but returned to baseline level after treatment for 24 h (**Figure 1A**). In keeping with these results, treatment with LTA (1-20 μg/ml) over 6 h led to increase in COX-2 protein levels in H9c2 in a concentration-dependent manner (**Figure 1B**). Maximum effect of LTA in COX-2 induction was observed at doses ranging from 10 to 20 μg/ml. High concentrations (15 μg/ml) of LTA were thus selected to explore the signaling cascades involved in COX-2 induction in H9c2 in the ensuing reported experiments. We found that LTA

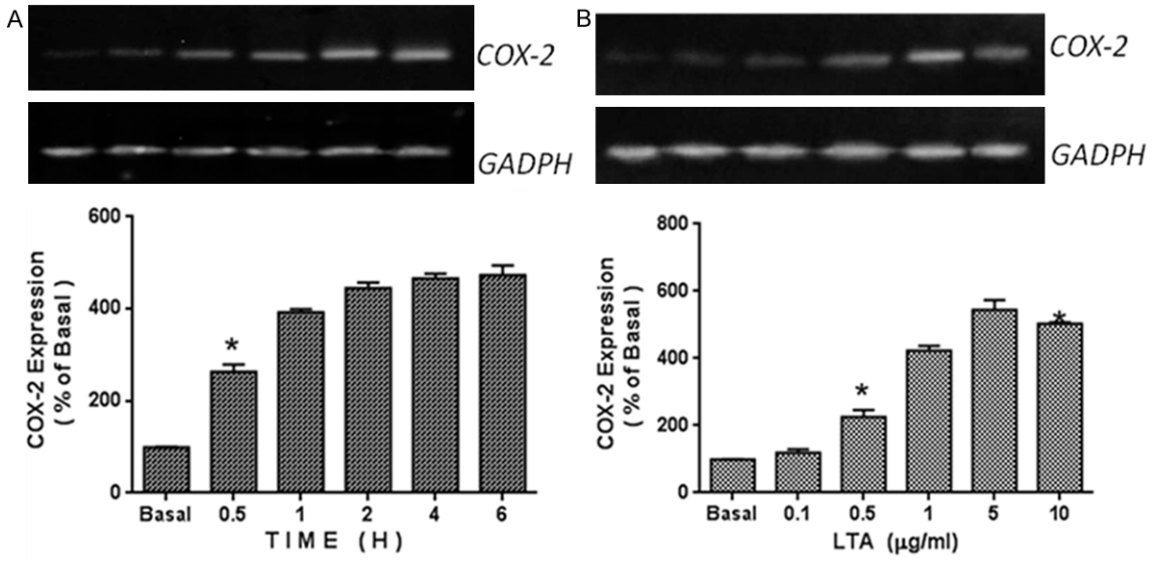

Figure 2. Concentration- and time-dependent increases in COX-2 expression by LTA. A: H9c2 cells were incubated with LTA (15 μg/ml) for 0.5, 1, 2, 4 and 6 h. B: H9c2 cells were incubated with various concentrations of LTA for 6 hrs, mRNA expression of COX-2 was examined by RT-PCR as described in Material and Methods section. Results of three independent experiments are expressed *P < 0.05 compared with the control group.

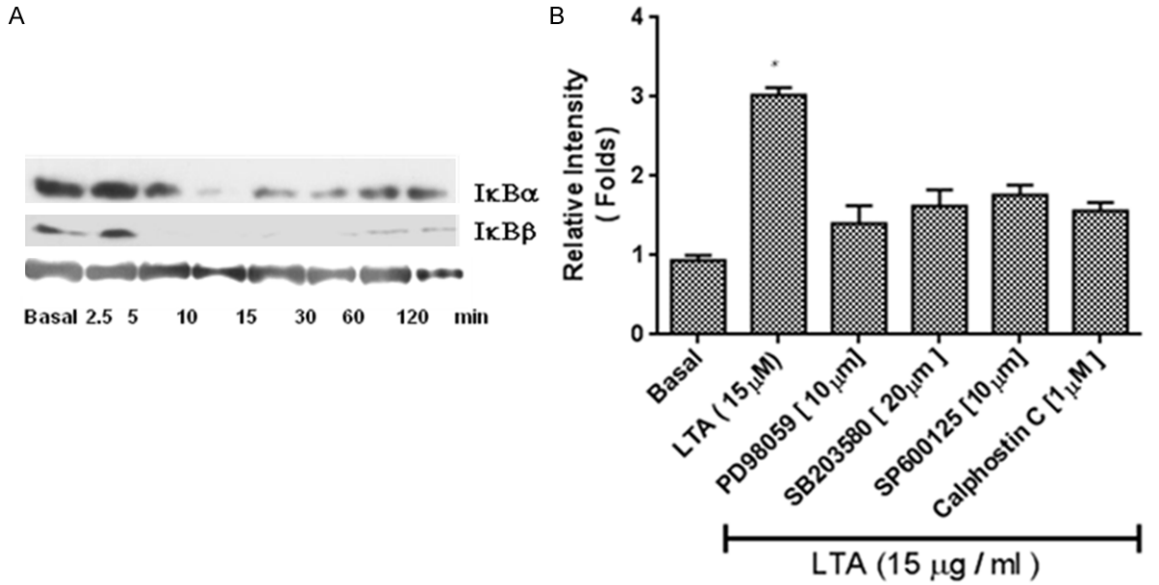

Figure 3. Kinetics of LTA-induced increases in NF-κB-specific DNA-protein formation and effects of various inhibitors on LTA-induced κB-luciferase activity in H9c2 cells. A: Cells were treated with LTA (15 μg/ml) for different time intervals. Whole cells extracts were then prepared and IκB-α or β degradation were evaluated by immunoblotting. Results were expressed as mean ± S.E.M (n=3). *P < 0.05 as compared with control group. B: Effect of various inhibitors on LTA-induced increase in κB-luciferase activity in H9c2 cardiomyoblasts. Cells were transiently transfected with 0.5 μg of pNifty-ELAM-Luc, before incubation with different inhibitors for 1 h and treated with 15 μg/ml LTA for another 24 h. Cells were then harvested for κB luciferase assay as described in Material and Methods. Results were expressed as mean ± S.E.M. *P < 0.05 as compared with LTA-treated group.

also significantly increased COX-2 mRNA levels in H9c2 after 4 h exposure. LTA induction was time (0.5-12 h) (**Figure 2A**) and dose dependent (1-20 μg/ml) (**Figure 2B**). Afore mentioned results confirmed the fact that protein level elevation is a result of increased transcription.

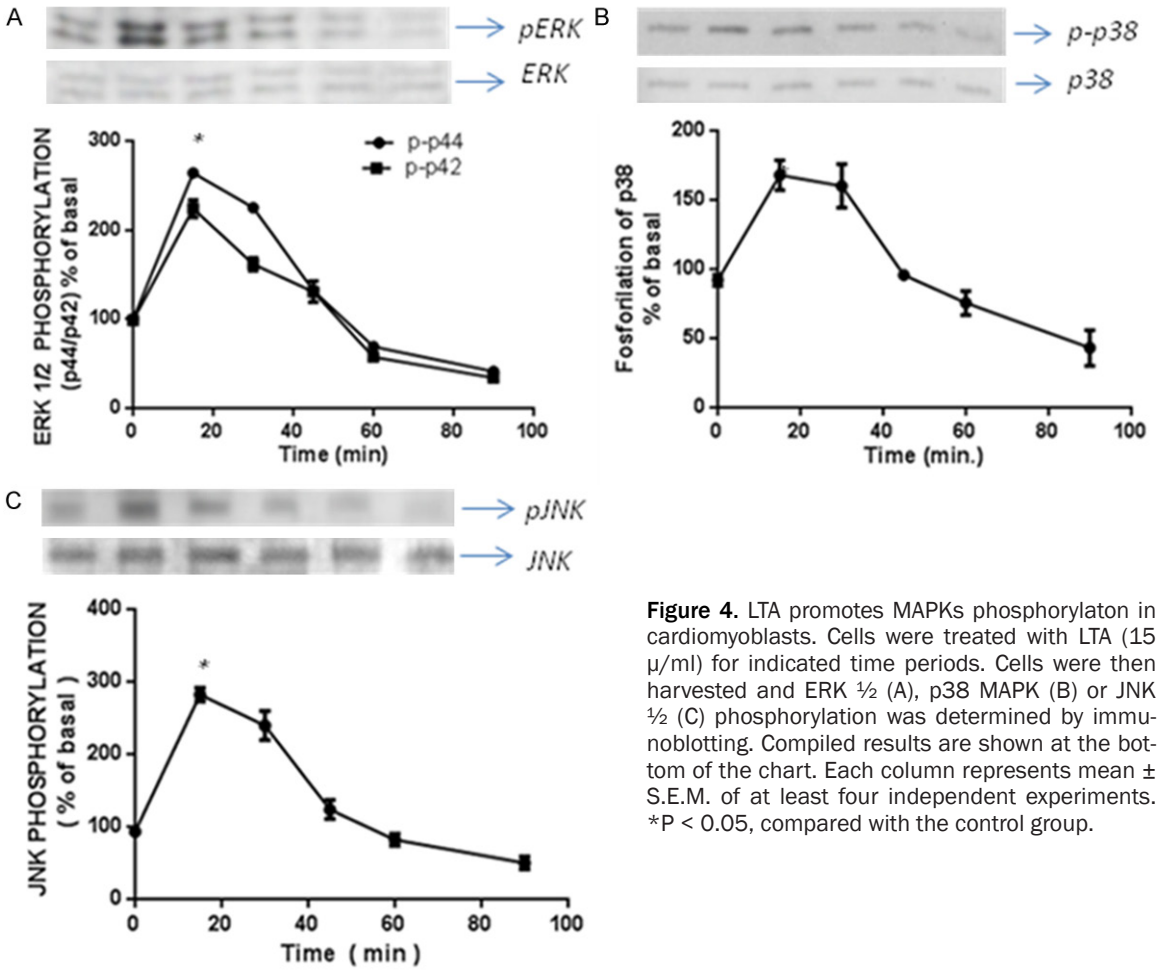

Figure 4. LTA promotes MAPKs phosphorylaton in cardiomyoblasts. Cells were treated with LTA (15 μ/ml) for indicated time periods. Cells were then harvested and ERK ½ (A), p38 MAPK (B) or JNK ½ (C) phosphorylation was determined by immunoblotting. Compiled results are shown at the bottom of the chart. Each column represents mean ± S.E.M. of at least four independent experiments. *P < 0.05, compared with the control group.

LTA promotes NF-κB activation in H9c2 cells

It is conceivable that LTA activates transcription factors, leading to COX-2 expression in H9c2. NF-κB have been reported to contribute to COX-2 elevation in different types of cells in response to various stimuli. Time course of NF-κB activation after treatment with 15 μg/ml LTA was studied. Whole cell extracts prepared from H9c2 cardiomyoblasts were analyzed for IκB by western blot assay. As shown in **Figure 3A**, cell-stimulation with 15 μg/ml LTA for 2.5 to 120 min resulted in marked degradation of I-κB α and β. After 15 min both isoforms are completely degraded and recovered after 80 to 120 min. To directly determine NF-κB activation after LTA treatment H9c2 cells were transiently transfected with pNifty-ELAM-κB-luciferase as an indicator of NF-κB activation. As shown in **Figure 3B**, LTA-induced increase in κB luciferase activity was inhibited by pretreatment of H9c2 cardiomyoblasts with SB203580 and PD98059. As a whole, these data suggest that LTA-induced NF-κB activation in cardiomyoblasts initiates pro-inflammatory cytokines.

LTA-induced phosphorylation of MAPKs in cardiomyoblasts

We next explored the signaling cascades that may contribute to LTA-induced COX-2 expression in H9c2. We examined whether ERK ½ MAPK (**Figure 4A**), p38 (**Figure 4B**) and JNK ½ phosphorylation (**Figure 4C**) were altered in H9c2 after LTA exposure. As shown in **Figure 4** p38, JNK and ERK ½ phosphorylation were increased in cells exposed to LTA.

Protein kinase C and AKT mediated LTA-induced ERK ½ mediated phosphorylation in H9c2 cells

To determine whether the activation of ERK occurs through PKC or AKT phosphorylation, cells were stimulated with 15 μg/ml LTA for 15,

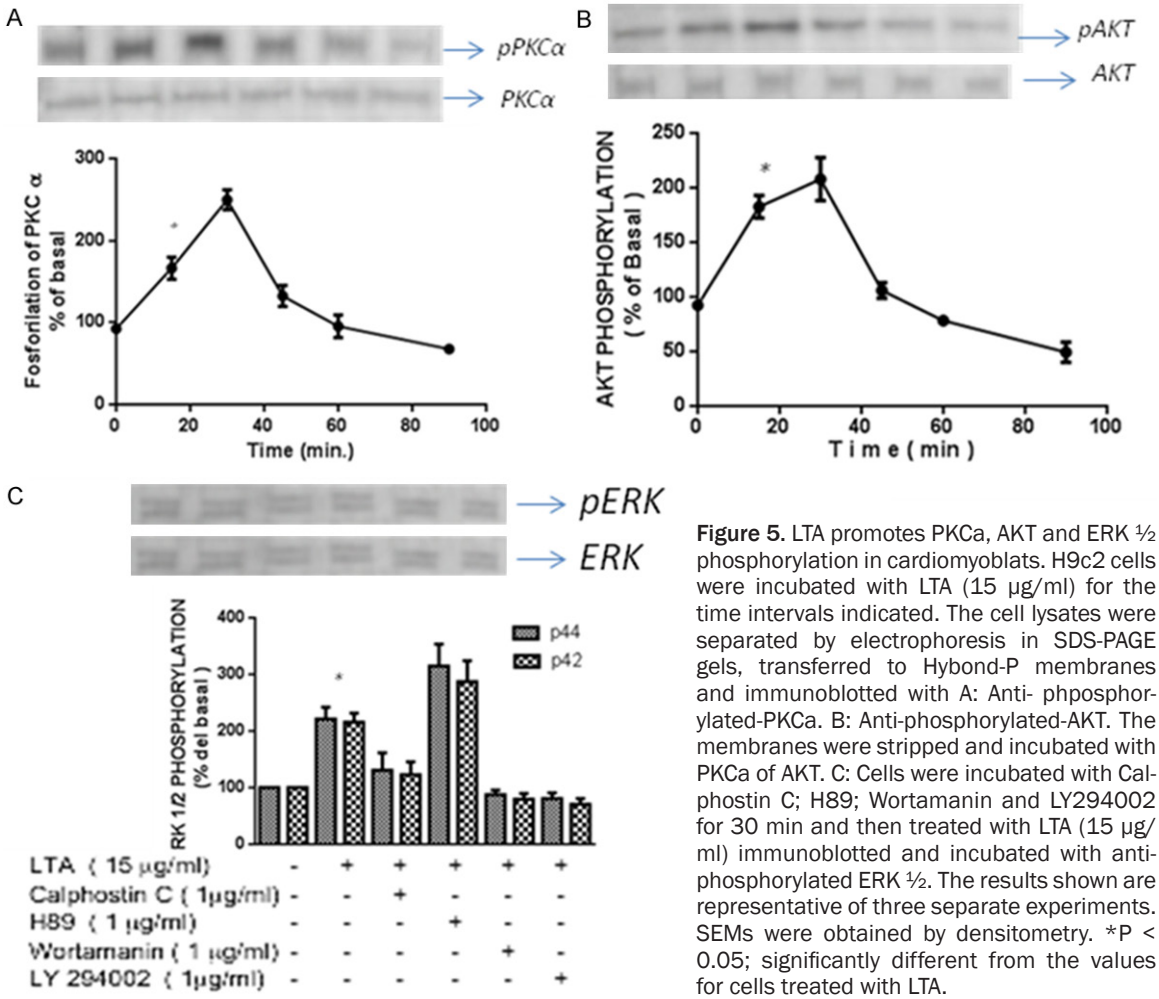

Figure 5. LTA promotes PKCa, AKT and ERK ½ phosphorylation in cardiomyoblats. H9c2 cells were incubated with LTA (15 µg/ml) for the time intervals indicated. The cell lysates were separated by electrophoresis in SDS-PAGE gels, transferred to Hybond-P membranes and immunoblotted with A: Anti- phposphorylated-PKCa. B: Anti-phosphorylated-AKT. The membranes were stripped and incubated with PKCa of AKT. C: Cells were incubated with Calphostin C; H89; Wortamanin and LY294002 for 30 min and then treated with LTA (15 µg/ml) immunoblotted and incubated with anti-phosphorylated ERK ½. The results shown are representative of three separate experiments. SEMs were obtained by densitometry. *P < 0.05; significantly different from the values for cells treated with LTA.

30, 45 and 90 minutes; this induced increases in PKCα phosphorylation in a time dependent manner. Response was elevated at 15 min and peaked at 30 min. However, after 45 min of LTA treatment, activation of PKC began to decline (**Figure 5A**), similar results were obtained with AKT (**Figure 5B**). Furthermore LTA's enhancing effects on ERK ½ phosphorylation were reduced in the presence of PKC and AKT inhibitors. As a whole, these results suggested PKC and AKT are upstream in LTA-stimulated ERK ½ phosphorylation pathway.

MAPKs and protein kinase C mediates LTA-induced COX-2 expression in H9c2

To determine whether the expression of COX-2 is mediated by MAPKs or PKC signaling pathways, cells were incubated with PD98059 (10 µM), SB203580 (20 µM); SP600125 (10 µM) or Calphostin C (1 µM) for 1 h and then treated with LTA (15 µM) for 6 hr (**Figure 6A**) or 4 hr (**Figure 6B**). MAPK's inhibitors and PKC inhibitor, significantly suppressed LTA-induced COX-2 expression. In general terms, these data suggest that COX-2 expression, MAPK's and PKC activation are involved in LTA-induced NF-κB activation in H9c2 cardiomyoblasts cells.

Discussion

In this study, we showed that LTA induced COX-2 expression in H9c2 cardiomyoblasts through a mechanism involving MAPK's and PKC activation stimulated with LTA.

LTA represents a class of amphiphilic molecules anchored to the outer face of the cytoplasmic membrane in Gram-positive bacteria [36]. LTA increase proinflammatory cytokines synthesis [37]. In addition LTA from *Streptococcus sanguinis* can lead to most of the clinical manifestations of infective endocarditis [37].

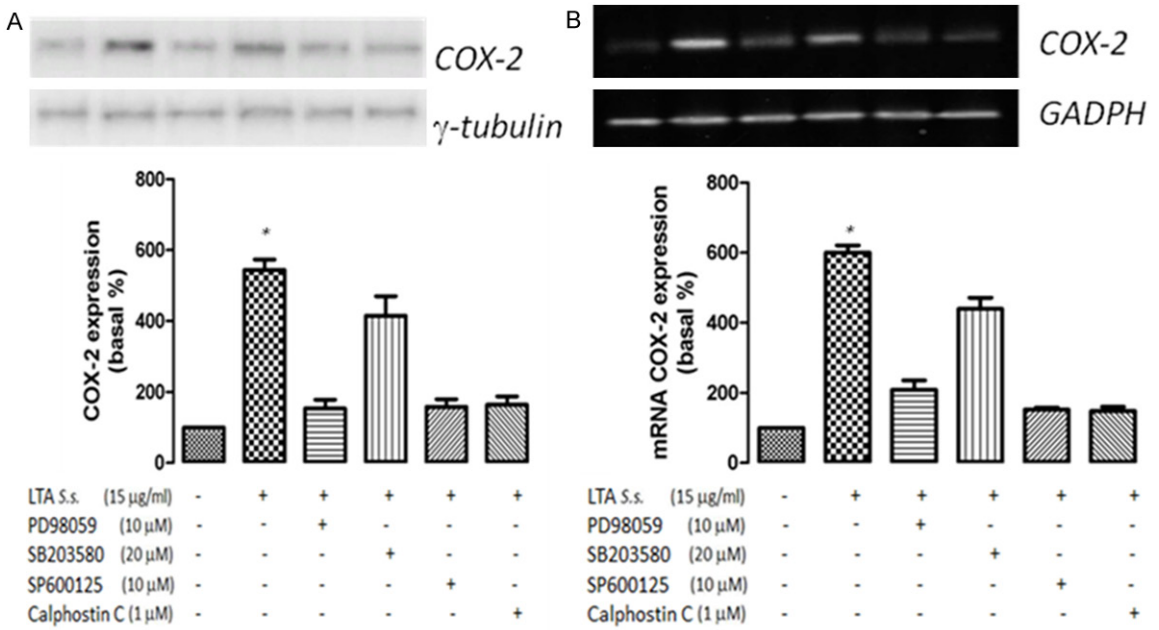

Figure 6. LTA promotes COX-2 expression. Cells were pretreated with PD 98059 (10 mM); SB203580 (20 mM); SP600125 (10 mM) or Calphostin C (1 mM) for 30 min before being treated with LTA (15 μg/ml) for 6 h. A: Whole cell lysates were obtained and processed by SDS-PAGE, and immunoblotted, membranes were blocked and incubated with antibodies that recognize COX-2. To corroborate that same protein concentration was used, membranes were denuded and incubated with g-tubulin. The blot is representative of three different experiments. COX-2 expression was quantified by densitometry using LabWorks 4.0 (Upland, CA, USA) commercial software. B: Total RNA was then extracted from H9c2 cells and the expression of COX-2 and GADPH mRNAs were analyzed by RT-PCR. The PCR product for COX-2 and GADPH were 305 and 506 bp respectively. The difference between the cultures treated with LTA is significant; *P* > 0-05.

This study showed that LTA increased the expression of NF-κB and IκB degradation. NF-κB is a transcription factor activated in response to stimulation by LTA. LTA binds to TLR2, and induces activation of cell signaling pathway conducted to promote NF-κB translocation from cytosol to nucleus, which can be activated by protein kinase and thus initiate different physiological responses such as cell proliferation, differentiation, death or inflammatory responses. Several reports show that LTA induced cyclooxygenases-2 expression in macrophages [38], human gingival fibroblats [39] and epithelial cells [40] via IκB degradation, in the present study we found that LTA promotes a rapid and transient degradation of IκB -α and -β. Results revealed that the phosphorylation of MAPK's and PKC were associated with NF-κB activation. Sequentially PKC activated ERK ½ kinase can phosphorylate IκB at two conserved serine residues in the amino terminus triggering the degradation of this inhibitor and allowing for the rapid translocation of NF-κB into nuclei [40]. Nevertheless, p38, JNK and AKT are also involved in its activation. There is a growing evidence pointing out that ERK signaling pathway, which contributes to regulating inflammatory response [40]. ERK activation is mediated by at least two different pathways: a Raf/MEK-dependent pathway, a PI3K/Raf-independent pathway. This study showed that LTA time dependently increased levels of phosphorylated MAPK. Thus, one of the possible reasons explaining why LTA stimulates ERK ½ phosphorylation is the increase in PKC activaton.

Conclusion

In summary, we used H9c2 model to study cell signal pathway responses of LTA obtained from *Streptococcus sanguinis*. Results revealed that LTA can induce inflammatory responses in H9c2 cells by means of enhancing MAPKs and PKC phosphorylation. Moreover, signal-transducing mechanisms of LTA caused regulation of COX-2 expression through a phosphorylation cascade of PKC and MAPKs pathways. At a

later stage LTA increased NF-κB expression and translocation. However, this study faced limitations, and exhaustive analysis of the specific role of each kinase in the regulation of the inflammatory response would be required. Nevertheless LTA-induced inflammatory response may indicate status of Gram-positive bacteria present in dental plaque. This article purported the aim of examining the impact exerted by biodental film on infective endocarditis among other heart diseases.

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Disclosure of conflict of interest

None.

Authors' contribution

GGV carried out the cell culture, RT-PCR, statistical analysis and drafted the manuscript, the conception and design of the study, IFH carried out western blots. All authors read and approved the final manuscript.

Abbreviations

COX-2, cyclooxygenase-2; ERK, extracellular regulated kinase; LTA, Lipoteichoic acid; MAPK's, Mitogen activated protein kinases; PKC, Protein kinase C; TLR, Toll like receptors.

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