

Apigenin reduce lipoteichoic acid-induced inflammatory response in rat cardiomyoblast cells

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Received: 14 September 2015 / Accepted: 8 May 2016 / Published online: 18 May 2016
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Abstract Infective endocarditis is caused by *Streptococcus sanguinis* present in dental plaque, which can induce inflammatory responses in the endocardium. The present study depicts research on the properties of apigenin in embryonic mouse heart cells (H9c2) treated with lipoteichoic acid (LTA) obtained from *S. sanguinis*. Interleukin-1 β and cyclooxygenase (COX)-2 expression were detected by reverse transcriptase polymerase chain reaction. In addition, western blot assays and immuno-fluorescence staining were used to assess translocation of nuclear factor kappa beta (NF- κ B), degradation of I κ B, as well as activity of the mitogen activated protein kinases: extracellular signal-regulated kinase (ERK)1/2, p38, and c-Jun N-terminal kinase (JNK). Effect of apigenin on cell viability was equally assessed in other experimental series. Our results showed that apigenin blocked activation of ERK, JNK, and p38 in cardiomyocytes treated with LTA in a dose-dependent fashion. Moreover, apigenin showed no cytotoxic effects; it blocked NF- κ B translocation and I κ B degradation. Our findings suggested that apigenin possessed potential value in the treatment of infectious endocarditis.

Keywords Cardiomyocytes · Lipoteichoic acid · Flavonoid · Apigenin · Cyclooxygenase-2 · Mitogen activated protein kinases

Introduction

The most prevalent oral diseases are periodontitis and caries; these diseases are caused by microorganisms existing in dental biofilm (Haffajee et al. 2008; Hodgson et al. 2001; Olsson et al. 1992; Shu et al. 2000; Stenudd et al. 2001). Caries development is associated to microorganisms such as *Streptococcus mutans* and *Streptococcus sanguinis* (Ge et al. 2008a, b; Nishikawara et al. 2006; Zijngje et al. 2010). The virulence and molecular mechanisms of viridians streptococci in the pathogenesis of bacterial infective endocarditis has not yet been fully clarified (De Moor et al. 1972; Mayo et al. 1995; Ge et al. 2008a, b; Zhu et al. 2011). Nevertheless, *S. mutans* and *S. sanguinis* are believed to enter the bloodstream as a result of trauma or oral hygiene manipulations (Kinane et al. 2005; Strom et al. 2000). This bacteremia may infect sites of underlying pathological changes of heart valves causing infective endocarditis (IE), which is a rare and severe infectious disease that has been extensively described in North America (Lockhart et al. 2009). IE is characterized by a high rate of microorganisms, which lead to cardiac complications and embolic events. Current therapies with antibiotic coverage, and vasopressors as well as early surgery have become a mainstay in the treatment of IE, nevertheless, more effective and specific treatment of sepsis is still lacking and mortality remains high (Hirschman 1987; Lockhart and Schmidtke 1994; Lockhart et al. 2004, 2009; van der Meer et al. 1992); therefore, a better understanding of the molecular pathogenesis of sepsis is clearly needed in order to develop novel and more effective therapeutic strategies.

Nevertheless local innate immune responses are crucial in order to limit establishment of an infectious focus and reducing disease severity. Microbial sensing by the innate

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immune system is mediated by pattern recognition receptors such as Toll-like receptors (TLR). TLRs are evolution-preserved innate receptors with specificity to microbial determinants; they are expressed in different immune and non-immune cells. TLR's main function is recognition of pathogen-associated molecular patterns (PAMPs) of invading microbes. After binding, TLRs induce downstream activation of protein kinases and cytokine production in a MyD88-dependent pathway (Michelsen et al. 2001; Zhang et al. 1999).

Lipoteichoic acid (LTA), a major component of Gram-positive bacteria cell membrane, is a well-characterized inducer of inflammatory responses. TLR2 recognizes and responds to the Gram-positive bacterial cell wall component, LTA [22]. LTA is a diacylated, glycerophosphate polymer, which is recognized by a TLR2/6 heterodimer complex (Gutiérrez-Venegas and Cardoso-Jiménez 2011; Kang et al. 2009). Activation of cardiomyocytes by LTA leads to a increased secretion of a large set of pro-inflammatory cytokines such as tumor necrosis factor (TNF) α , interleukin-(IL)-1 β , IL-6, nitric oxide (Gutiérrez-Venegas et al. 2013) and cyclooxygenase (COX)-2, all aforementioned molecules were induced through phosphorylation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases (MAPK) (Gutiérrez-Venegas and Bando-Campos 2010; Kao et al. 2005). Over-production of these inflammatory mediators were involved in myocardium damage and in permeability increase, resulting thus in many inflammation-associated disorders.

Flavonoids are a family of polyphenolic compounds that are widely distributed in the plant kingdom, and are consumed in significant amounts as part of the human diet. Different studies have shown that flavonoids in a healthy diet exert potentially beneficial effects in regulation of inflammatory responses. Apigenin (4',5,7-trihydroxyflavone) is a non-toxic dietary flavonoid present in fruits and vegetables such as oranges, onions, chamomille and grapefruits (Smolinski and Pestka 2003).

Some reports have targeted to demonstrate that apigenin exerts certain effect on nitric oxide regulation (Liang et al. 1999; Raso et al. 2001; Smolinski and Pestka 2003; Shukla and Gupta 2004) but, to this date no research has been conducted to assess the inhibitory effect of apigenin on LTA-induced inflammatory response in cardiomyocytes. In a previous work we found that apigenin regulated nitric oxide synthase expression and nitric oxide production (Gutiérrez-Venegas et al. 2013), for this reason the purpose of the present study was to examine the possible role of apigenin in LTA-induced inflammatory response in cardiomyocytes as well as to characterize molecular events involved in its anti-inflammatory response, particularly in IL-1 and COX-2 expression.

Methods

Materials Apigenin (purity > 98 %), Dulbecco's modified Eagle medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), were obtained from Sigma-Aldrich (St. Louis, USA). Antibodies were purchased from Santa Cruz (Santa Cruz, CA).

Cell culture and treatment

H9c2 cardiomyocytes cells (ATCC, Cat # CRL-1446) were cultured in Dulbecco's modified Eagle medium supplemented with 10 % FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37 °C with 5 % CO₂. Apigenin was dissolved in dimethylsulfoxide (DMSO), the concentrations of apigenin used in this research were 2, 5, 10 and 15 μ M. For each result, three independent experiments were performed.

Cell viability by MTT assay

Cell viability was measured by the quantitative colorimetric assay using MTT, as described in literature (Carmichael et al. 1987). After treatment, in a time and dose dependent fashion, media were removed, and fresh media containing MTT (0.5 mg/mL) were added to each well, followed by incubation for 4 h at 37 °C. MTT solution was later removed and cells were lysed with DMSO. The absorbance at 595 nm was measured using a microplate reader. Control cells were treated in the same way and the value was calculated as % of cell viability. All experiments were conducted three times in a sixfold sequence.

IL-1 β and PGE2 expression

H9c2 culture supernatants were recovered; IL-1 β expression was quantified with a Quantitative ELISA kit for PGE₂ (Titer Zyme ELISA Kit Assay Designs) (n = 3, threefold).

Immunocytochemistry

Cells were grown on glass cover-slips and fixed for 30 min with 2 % formaldehyde in PBS at 4 °C. After this, cells were permeabilized during 5 min with Triton 0.1 % in PBS and washed five times with PBS. For protein-kinase visualization, cells were treated for 1 h with primary antibodies, diluted 1:100 in PBS, and then washed five times with PBS. Cells were incubated 45 min with goat anti-mouse IgG-conjugated rhodamine, bovine anti-goat-conjugated rhodamine and goat anti-rabbit-conjugated fluorescein-5-isothiocyanate (FITC) (all from Santa Cruz Biotechnology) diluted 1:100 in PBS. Samples were mounted on resin and

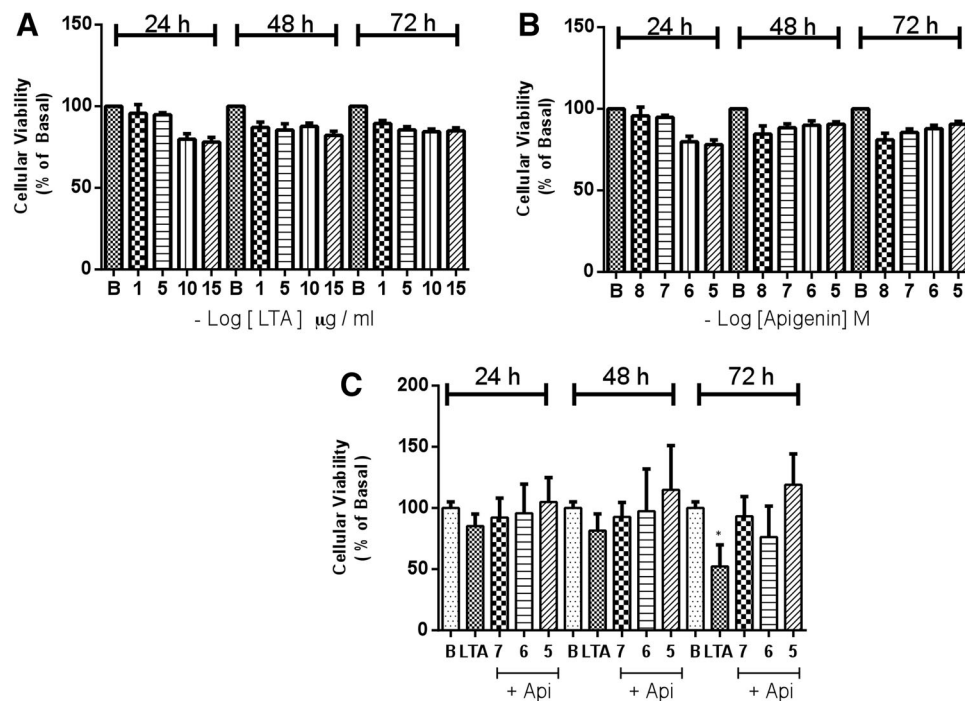


Fig. 1 Effect of apigenin on LTA-cell viability. H9c2 cells were seeded in 96-well plates (1×10^4 cells/well) and **a** treated with different concentrations of LTA, **b** treated with different doses of apigenin, **c** pre-incubated with apigenin for 30 min and then treated with LTA for 24, 48 and 72 h, followed by MTT assay as described in Experimental Section. Each bar represents mean \pm SD calculated from three independent experiments. * $p < 0.05$, which is significantly different from the basal value

examined with a confocal photomicroscope. Secondary antibodies were used as a control. All experiments were repeated at least three times.

RT-PCR analysis

RNA isolation was prepared using TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA) according to protocol. One microgram of RNA was used along with One Step kit (Invitrogen). PCR was performed using oligonucleotides 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' (coding sense) and 5'-AGATCATCTCTGCCTGAGTATCTT-3' (anticoding sense) derived from COX-2 gene (O'Neill and Ford-Hutchinson 1993); or IL-1 β 5'-GGCTGCAGTT CAGTGATCGTACAGG-3' and 5'-AGA TCT AGA GTA CCT GAG CTC GCC AGT GAA-3' (Gutiérrez-Venegas et al. 2013), and 5'-AGATCCACAACGGATACATT-3' (anticoding sense) derived from glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene (Fort et al. 1985). PCR amplification conditions included denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min; PCR was carried out for 35 cycles. GAPDH was used as internal control. Electrophoresis was carried out using agarose gel electrophoresis; ethidium bromide was used for staining. Stained gels were analyzed using LabsWorks 4.0 (Upland, CA, USA) commercial software. Each densitometric value was expressed as

mean \pm SD. All experiments were repeated at least three times.

Western blot analysis

H9c2 cells (1×10^6 /well) were grown in 6-well plates (Corning, NY, USA). Cells were treated with apigenin for 30 min prior to treatment with LTA (15 μ g/ml). Thereafter, the medium was aspirated, washed twice with phosphate-buffered saline (PBS), and PBS was replaced with 50 μ l of cold lysis buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 1 % Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]), 10 μ g/ml leupeptin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate (all obtained from Sigma Chemical Co, St. Louis, MO, USA). Cells were scraped off, and the lysate was transferred to a microcentrifuge tube, to be then pulse-sonicated (1 s \times 30) on ice. Western analysis was performed on 50 μ g of proteins mixed 1:1 with 2 \times sample buffer (20 % glycerol, 4 % SDS, 10 % 2-mercaptoethanol, 0.05 % bromophenol blue, and 1.25 M Tris-HCl pH 6.8, all from Sigma Chemical Co to be then loaded onto a 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and run at 40 V for 2 h. Cell proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen) 30 min at 10 V. Equal

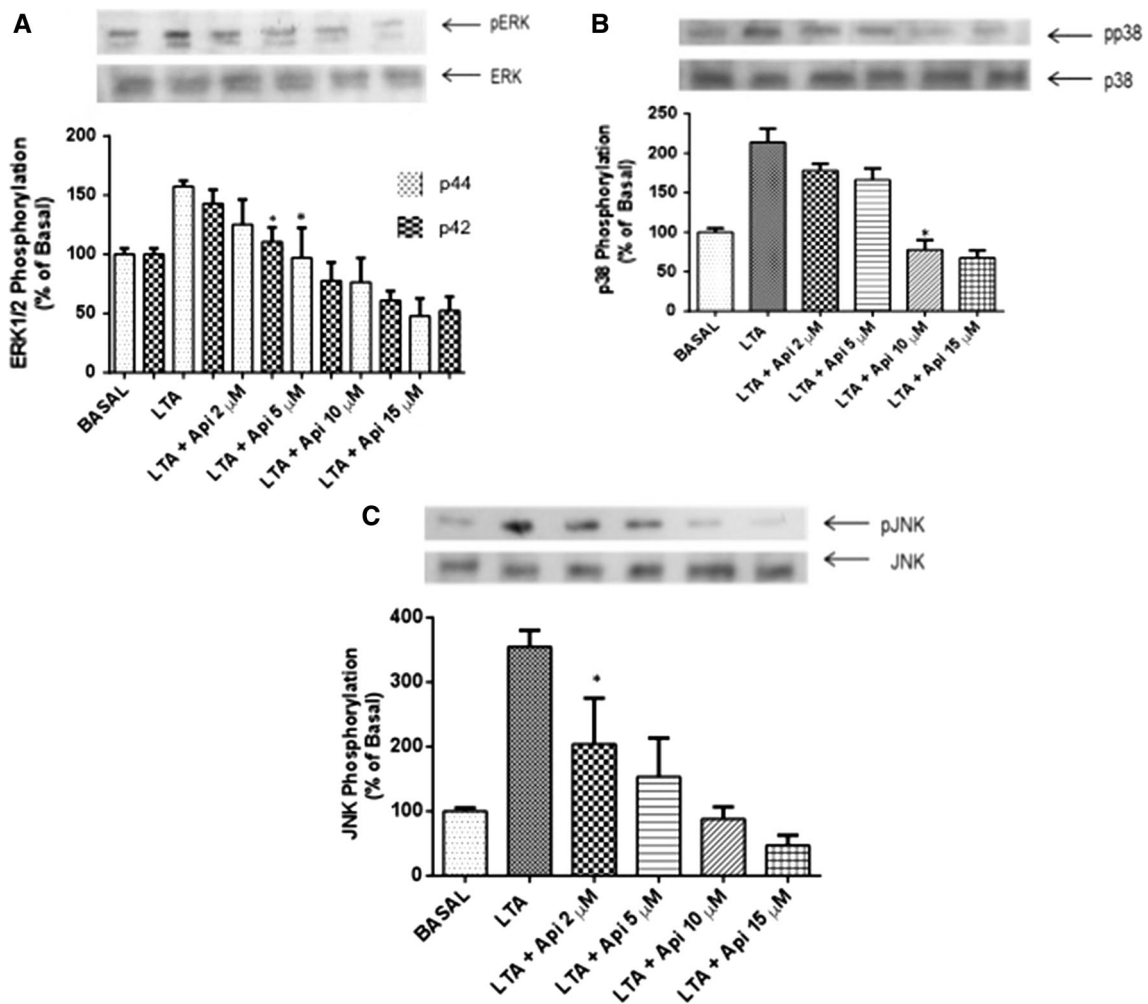


Fig. 2 Effect of apigenin on LTA-induced phosphorylation of ERK 1/2, p38 and JNK in H9c2 cells. Cells were incubated with apigenin (2, 5, 10 and 15 μ M) for 30 min, followed by treatment with LTA (15 μ g/ml) for 15 min. **a** Phospho-ERK 1/2; **b** phospho-p38; **c** phospho-JNK. Cell lysates were separated in sodium dodecyl-sulfate polyacrylamide (SDS-PAGE gels), transferred to HybondTM membranes and incubated with phospho-ERK 1/2, phospho-p38 and phospho-JNK. Membranes were stripped and incubated with total ERK 1/2, p38 and JNK. Results are representative of three separate experiments. Densitometric analysis revealed mean of three separate experiments as well as standard error of the mean (SEM). *Significantly different from LTA alone $p < 0.05$

loading of protein groups on the blot was assessed using Ponceau (Sigma Chemicals Co.). Membranes were blocked with Tris-buffered saline (TBS) and 5 % bovine serum albumin for 1 h, washed, and then incubated overnight at 4 °C with primary antibody p-ERK 1/2 mouse monoclonal IgG (1:1,000), p-JNK, p-p38 (1:1000), or p-Akt (1:1000), or I κ β (1:1000). Blots were washed three times with TBS and incubated for 1 h with horseradish peroxidase (HPR)-conjugated anti-IgGAb (1:1,000) (Santa Cruz Biotechnology, Inc., USA). Immuno-reactive bands were developed using a chemiluminescent substrate (Santa Cruz Biotechnology, Inc., USA). Autoradiographs were obtained with a 10-min exposure. Three different experiments were carried out for each figure. Equal loading of blots was demonstrated by stripping blots and re-probing with antibodies for total ERK 1/2, p38, AKT, and γ -tubulin.

Statistical analysis

All data were presented as mean \pm SEM. Statistical significance of differences between treated and untreated groups were determined by a One-Way ANOVA test using the PRISMA software. A value of $p < 0.05$ was considered significant.

Results

Effect of apigenin on cell viability

We first evaluated the effect of apigenin on H9c2 cells at cell viability; (a) H9c2 cells were incubated with serial concentrations of LTA (1–15 μ g/ml) for 24, 48 and 72 h. Whereupon treatment of H9c2 cells with apigenin was

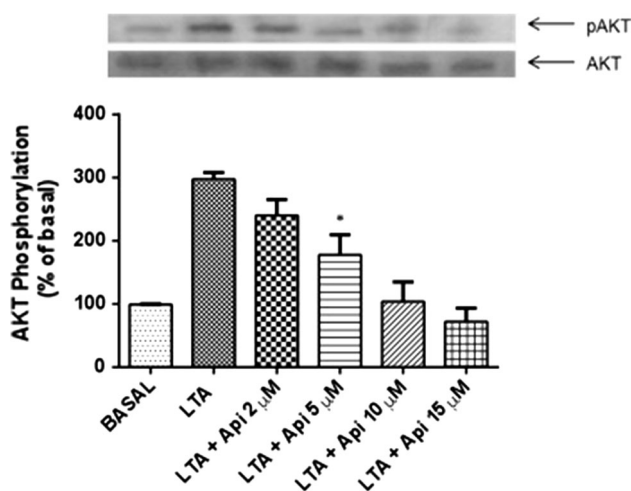


Fig. 3 Effect of apigenin on phosphorylation of AKT. Cells were incubated with apigenin (2, 5, 10 and 15 μ M) for 30 min followed by treatment with LTA (15 μ g/ml) for 15 min. Cell lysates were separated in sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels and transferred to Hybond membranes and incubated with phospho-AKT antibodies. Membranes were stripped and treated with total AKT. The results are representative of three separate experiments. By densitometry we obtained standard error of the mean (SEM). * Significantly different from LTA alone $p < 0.05$

found to exert no significant change in cell viability in presence of LTA (Fig. 1a). In order to examine the sensitizing effect of apigenin on viability, cells were treated with apigenin in the range of 1×10^{-6} – 1×10^{-4} M, since this range of apigenin, when used as sole treatment, did not influence viability of H9c2 (Fig. 1b). Nevertheless, treatment of LTA (15 μ g/ml) with apigenin (1×10^{-6} – 1×10^{-4} M) for 24, 48 and 72 h, resulted in a significant increase of cell viability in a 72 h (Fig. 1c).

Effect of apigenin on LTA-induced MAPK phosphorylation

To determine flavonoid action on LTA-induced ERK $\frac{1}{2}$ phosphorylation, cells were pre-incubated with apigenin 30 min at dosages of 2, 5, 10 and 15 μ M and treated with LTA. Our results showed that apigenin blocked LTA-induced MAPK activation (15 μ g/ml) from doses of 2 μ M onwards (Fig. 2a). Assessment of apigenin effect on p38 phosphorylation revealed blocked p38 phosphorylation induced by LTA in a dose-dependent fashion (Fig. 2b). Similar results were obtained with JNK phosphorylation (Fig. 2c). It was found that apigenin promoted a significant effect from 2 μ M doses onwards.

Effect of apigenin on LTA-induced AKT activation

We next determined the effect of apigenin on LTA-induced AKT activation. Our results showed that apigenin blocked LTA-induced AKT phosphorylation (Fig. 3).

Effect of apigenin on LTA-induced NF κ B activation

To further elucidate the effect of apigenin on LTA-induced NF κ B translocation and I κ B degradation, we proceeded to observe the effect under immunocytochemistry. We found that LTA (15 μ g/ml) induced I κ B degradation and NF κ B translocation, and that the process was inhibited by apigenin. We equally observed maximal effect at 15 μ M (Fig. 4).

Effect of apigenin on IL-1 β expression

LTA treatment increased the levels of IL-1 β (Fig. 4a, b) in H9c2 cells. This response was attenuated by apigenin in a concentration-dependent manner, we found that LTA administration induced IL-1 β expression, furthermore apigenin significantly decreased IL-1 β level in a concentration-dependent manner (Fig. 5a, b). Our experimental data indicated that apigenin pre-treatment reduced LTA-induced expression of IL-1 β by inhibiting MAPK and NF κ B activation in H9c2 cells treated with LTA.

Effect of apigenin on COX-2 transcription and translation

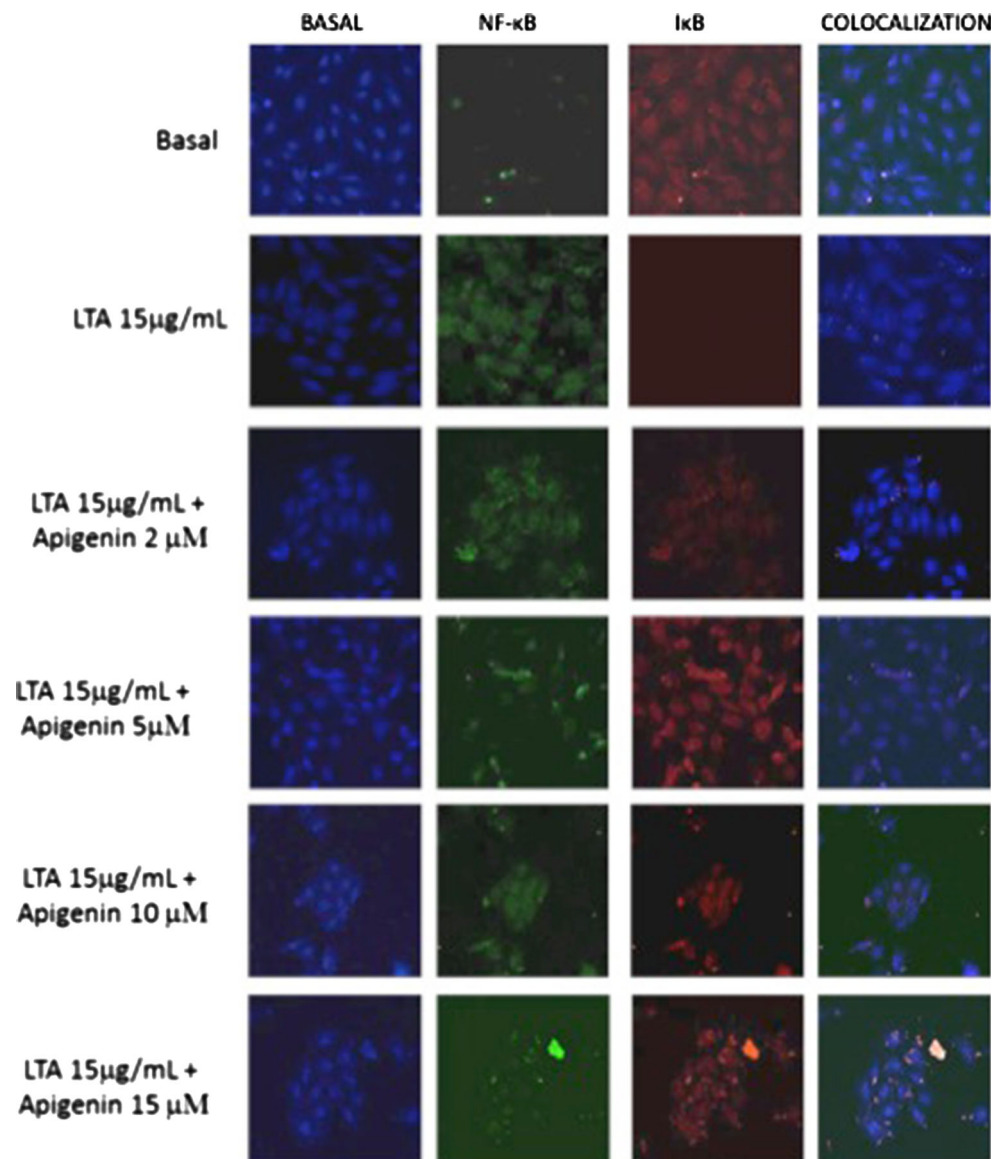
Incubation of H9c2 cells with LTA significantly increased COX-2 transcription and translation. Apigenin treatment, markedly attenuated COX-2 transcription induced by LTA (Fig. 6a). We next evaluated the effect of apigenin on PGE2 synthesis in H9c2 cells, we found that LTA administration induced PGE2 expression, furthermore apigenin significantly decreased PGE2 level in a dose-dependent fashion (Fig. 6b).

Likewise, as shown in Fig. 6c and d, LTA stimulated COX-2 expression up to 2.3 times over basal level. This figure additionally shows that apigenin significantly attenuated LTA-mediated COX-2 expression. We next assessed COX-2 expression, regulated by different kinase inhibitors. H9c2 cells were pretreated with ERK $\frac{1}{2}$ inhibitor PD98059 (10 μ M), p38 MAPK inhibitor SB203580 (20 μ M), JNK inhibitor SP600125 (10 μ M), PKC inhibitor Calphostin C (1 μ M) and C) with Phospholipase C inhibitor U73122 (1 μ M), LY294002 (1 μ M), H89 (1 μ M) and Curcumin (1 μ M) for 30 min, then exposed to LTA (15 μ M) for 6 h. LTA-stimulated H9c2 cells COX-2 expression was significantly attenuated ($*p < 0.05$) after treatment with all different inhibitors, we found that JNK inhibitor did not inhibit COX-2 expression.

Discussion

The main purpose of the present research was to evaluate cellular mechanisms underlying anti-inflammatory effect of apigenin induced by LTA in cardiomyocytes. The

Fig. 4 Apigenin inhibits NF κ B translocation and I κ B degradation in LTA-stimulated H9c2 cells. Cells were pre-treated with or without apigenin (10 μ M) and stimulated with LTA (15 μ g/ml) for 1 h. Cells were fixed in 2 % formaldehyde in PBS, stained with DAPI, and anti-NF κ B p65 (FITC) and I κ B (Rhodamine). Immunostained cells were then mounted with medium containing DAPI and visualized under a Bio-Rad confocal microscope



present study revealed that apigenin inhibited LTA-induced inflammatory response, by decreasing ERK $\frac{1}{2}$, p38, JNK and AKT phosphorylation in a dose-dependent fashion; thus reducing NF κ B translocation and modulation of COX-2 and IL-1 β transcription and translation in H9c2 cardiomyocytes. Cardiomyocytes are the main cardiac cells involved in inflammatory response and can produce numerous pro-inflammatory cytokines (Damås et al. 2001; Schilling et al. 2011; Wang et al. 2004). Chronic inflammatory response will result in severe organ damage and infective endocarditis, thus, reduction of chronic inflammation is an effective strategy to prevent pathological progression (Cognasse et al. 2014; Weinstock et al. 2014). In recent years, new approaches on the use of herbal

products for the treatment of inflammatory diseases are being practiced in traditional medicine. Our study focuses on the action mechanism of flavonoid apigenin which is used in alleviating inflammatory diseases by inhibiting cytokines and TNF α expression (Kang et al. 2011; Kowalski et al. 2005). Several studies showed that apigenin inhibited NO production induced by lipopolysaccharide (LPS) in macrophages and cytokine synthesis in monocytes as well as LPS treated macrophages (Hougee et al. 2005; Nicholas et al. 2007).

In the present study, we demonstrated that apigenin showed no effect on the reduction in cell viability as measured by MTT assay; our data suggest that flavonoids do not exhibit cytotoxic effect on cardiomyocytes, similar

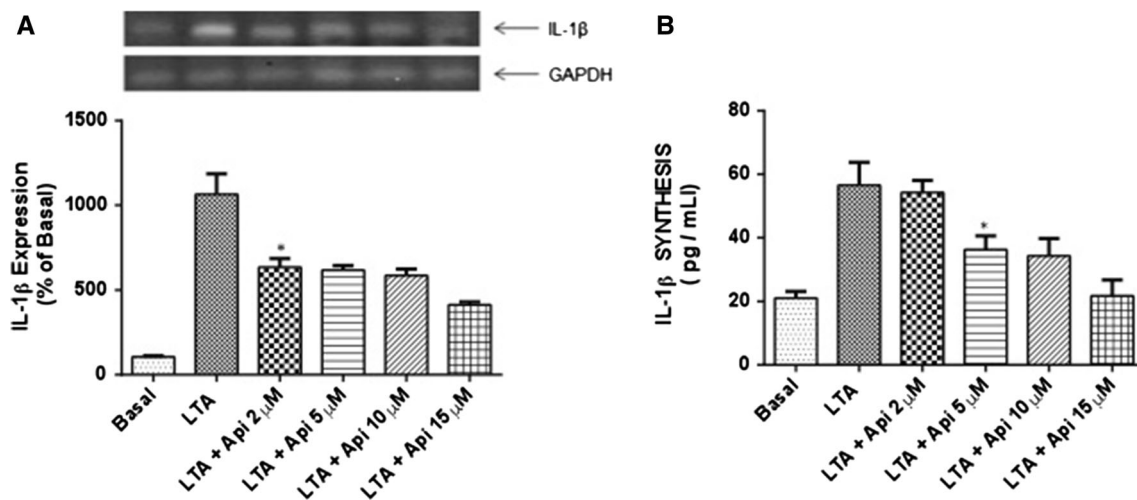


Fig. 5 Apigenin inhibit the effects of LTA on IL-1 β expression and Synthesis. **a** H9c2 cells were treated with apigenin at the doses indicated in this figure for 30 min and subsequently treated with LTA (15 μ g/ml) for 4 h. Total RNA was extracted and COX-2 mRNA expression was determined by RT-PCR, GAPDH was used as a control. Densitometric analyses represent the means and SEM of three separate experiments. **b** IL-1 β synthesis was measured by ELISA in the presence of LTA. H9c2 cells were grown in a 6-well plate then were incubated with apigenin for 30 min, followed by stimulation with LTA (15 μ g/ml) for 24 h, then the supernatants were collected and assayed for IL-1 β ELISA. The graph represents mean and SEM of three separate experiments. * Significantly different from LTA alone $p < 0.05$

results were reported on macrophages and A459 cells (Hougee et al. 2005; Nicholas et al. 2007; Patil et al. 2015). However on lung cells, apigenin decreased the percentage of viable cells (Liu et al. 2005; Lu et al. 2010).

COX-2 enzyme is involved in the synthesis of prostaglandin E₂; its chronic activation may lead to various inflammatory diseases. Hence, the decreased expression of inducible COX-2 is considered a therapeutic target for inflammation. In our study H9c2 cells treated with LTA promoted COX-2 expression, treatment with apigenin induced by LTA suppressed COX-2 expression in a dose-dependent fashion. These data suggest presence of apigenin anti-inflammatory activity as reported by other authors in monocytes and macrophages (Seo et al. 2014).

In many studies the production of interleukin 1-beta participates in the immune response to many inflammatory stimuli; overproduction of these mediators is detected in several inflammatory disorders. Our results showed that within 4 h of treatment of H9c2 cells with LTA expression of IL-1 β was induced. A 30 min pre-treatment of H9c2 cells with apigenin significantly inhibited the LTA induced expression of mRNA levels of IL-1 β .

A certain variety of mechanisms have been proposed in order to determine the effect of flavonoids as neuro-protector, chemo-preventive and cardio-protector agents (Tang et al. 2014; Sloley et al. 2000; Wang et al. 2001). It has been reported that apigenin plays an important role as anti-inflammatory activity agent.

Previous studies reported that apigenin significantly decreases mRNA levels of TNF α , IL 6 and IL-1 β in mice

macrophages. Nevertheless, apigenin-induced molecular mechanisms in regulation of LTA-stimulated inflammatory response have not yet been characterized. In the present study, we found that apigenin blocks ERK 1/2, p38, JNK and AKT phosphorylation in a dose-dependent manner. We additionally found that these kinases are involved in COX-2 expression. These results would suggest the fact that apigenin not only inhibits LTA actions, but would rather tend to carry on its effects without eliciting cytotoxic effects. Additionally apigenin inhibits NF κ B translocation; due to the aforementioned reasons it could be suggested that apigenin regulates expression of other inflammatory molecules associated to NF κ B response.

LTA used in the present study was extracted from caries-associated *S. sanguinis* bacteria. The surface of inflamed and ulcerated gingival crevicular tissue surrounding teeth could be considered an access for streptococci bacteria's entry to blood, which would then cause IE. It would be worth mentioning that dental procedures foster IE-associated bacteremia. LTA from these bacteria would be associated to the inflammatory response exhibited by many different cells.

In conclusion, several studies point out that plants are an important providing source of medicinal products. For thousands of years, use of plants to protect health has been duly documented. Advances experienced in the understanding of human diseases have allowed the development of plant-derived drugs. In the present study we were able to show that apigenin inhibited LTA-induced inflammatory

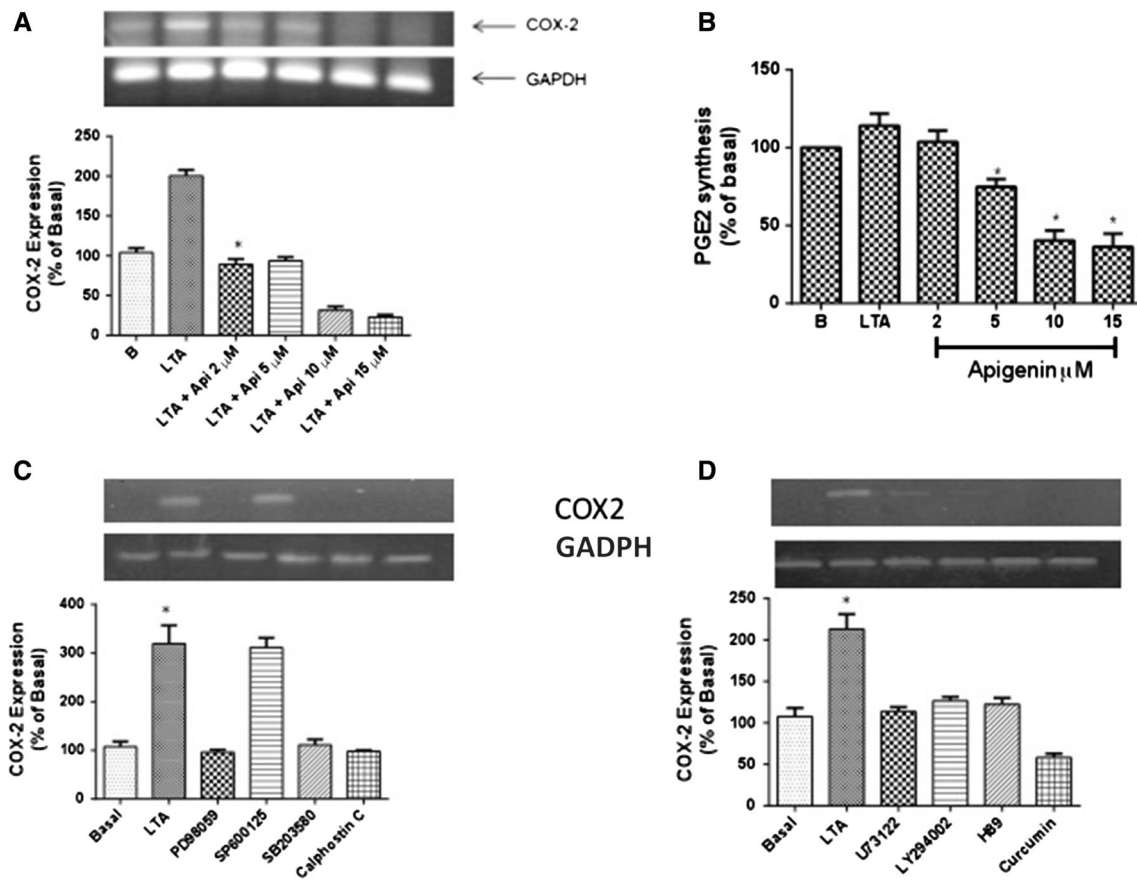


Fig. 6 Apigenin inhibit the effects of LTA on transcription and translation of COX-2 in H9c2 cells. **a** Cells were treated with apigenin (2, 5, 10 and 15 μM) for 30 min and subsequently treated with LTA (15 μg/ml) for 4 h. Total RNA was extracted and COX-2 mRNA induction was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as control. Densitometric analyses represent standard error of the mean ± (SE) of five separate experiments. **b** PGE2 synthesis was measured by ELISA in the presence of LTA. H9c2 cells were grown in a 6-well plate then were incubated with different doses of apigenin for 30 min, followed by stimulation with LTA (15 μg/ml) for 24 h, then the supernatants were collected and assayed for PGE2 ELISA. The graph represents mean and SEM of three separate experiments. * Significantly different from LTA alone $p < 0.05$. **c** Cells were pre-incubated with PD98059 (10 μM), SP600125 (10 μM), SB203580 (20 μM) and Calphostin C (1 μM) for 30 min, and then stimulated with LTA (15 μM) for 4 h. After incubation, mRNA was isolated and amplified by RT-PCR as described in the “Methods” section. Results are presented as mean ± SEM of three independent experiments. * $p < 0.05$. **d** H9c2 cells were pre-incubated with U73122 (1 μM), LY294002 (1 μM), H89 (1 μM) and Curcumin (20 μM) for 30 min and then incubated with LTA (15 μg/ml) for 4 h. Total RNA was extracted and COX-2 mRNA expression was determined by RT-PCR, GAPDH was used as a control. Densitometric analyses represent means and SEM of three separate experiments. * Significantly different from basal $p < 0.05$

responses in cardiomyocytes, therefore, in inflammatory diseases, it could be used as a therapeutic agent.

Acknowledgments We wish to express our thanks to the Dirección General de Asuntos del Personal Académico (DGAPA) PAPITT-IN 201816 for their financial support, and to Carmen Muñoz-Seca for editorial assistance.

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