

Rutin Prevents LTA Induced Oxidative Changes in H9c2 Cells

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ABSTRACT: Lipoteichoic acid (LTA), a component of Gram-positive bacteria cell walls is involved in infective endocarditis (IE), a life-threatening disease. We evaluated for the first time, whether flavonoid rutin (quercetin-3-rutinoside) can block LTA-induced pro-inflammatory response and reactive oxygen species (ROS) production, and reduction of antioxidant enzymes. We found that rutin suppresses LTA effects on the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase, as well as the pro-inflammatory enzyme cyclooxygenase-2, preventing phosphorylation of the mitogen-activated protein kinases (MAPKs), p38, and c-Jun N-terminal kinase, and the increase of ROS production induced by LTA. Taken together, these findings suggest that rutin prevents oxidative damage, inflammation, and MAPKs activation induced by LTA. Rutin may exert a protective effect in IE. These data provide novel insights for future use of rutin to prevent the mechanisms of LTA-related pathogenesis, inflammatory processes, and antioxidant enzyme levels in diseases such as IE.

Keywords: antioxidant enzyme, infective endocarditis, lipoteichoic acid, oxidative stress

INTRODUCTION

Lipoteichoic acid (LTA) is a major cell-wall component of Gram-positive bacteria composed of a backbone of repeating glycerophosphate units with *D*-alanine or *N*-acetylglucosamine substituents and a lipophilic anchor (Kochanowski et al., 1993; Percy and Gründling, 2014; Schneewind and Missiakas, 2014; Ma et al., 2018). LTA binding of Toll-like receptor (TLR)-2 and TLR-4 triggers immune responses that affect adaptive immunity development (Durand et al., 2006; Hong et al., 2014; Thapa et al., 2015). On cardiomyocytes, LTA stimulation of TLR-2 and TLR-4 leads to the activation of the pro-inflammatory cytokines, including tumor necrosis factor, interleukin (IL)-1 β , and IL-6, as well as activation of the pain/inflammation-associated enzyme cyclooxygenase-2 (COX-2), the nitric oxide (NO) biosynthesis enzyme inducible NO synthase (iNOS), and mitogen-activated protein kinases (MAPKs) (Ha et al., 2010; Tian et al., 2013; Lei et al., 2018; Meng et al., 2018; Zhou et al., 2018).

Infective endocarditis (IE) is a bacterial LTA-associated inflammatory pathology in which vegetations form in the heart, potentially affecting heart valves, septal defects,

tendon cords, and the endocardium. IE has been associated with Gram-positive bacteria, including *Staphylococcus aureus*, *Streptococcus bovis*, and *Streptococcus viridians*, as well as Gram-negative bacteria commonly found in dental plaque. A quarter of IE cases are attributed to *Streptococcus* that inhabit the oral cavity (Ballet et al., 1995; Ge et al., 2008; Cunha et al., 2010; Heilbronner et al., 2013). A potentially pathogenic state of oxidative stress occurs when reactive oxygen species (ROS) production exceeds the capacity of antioxidant enzymes, such as superoxide dismutase (SOD), which reduces the superoxide anion (O₂^{•-}), catalase (CAT), which reduces hydrogen peroxide (H₂O₂), and glutathione peroxidase (GPx), which reduces H₂O₂ as well as peroxide radicals (Turdi et al., 2012; Tang et al., 2014; Zhou et al., 2015). Moderation of ROS levels can be further supported by exogenous antioxidants, including flavonoids, which are found ubiquitously in plants (Liao et al., 2016; Yao et al., 2017; Chen and Fan, 2018; Liu et al., 2018). Good sources of dietary flavonoids include vegetables, fruits, nuts, seeds, stem, flowers, tea, and wine (Tapas et al., 2008).

Rutin is a flavonoid glycoside comprised of the flavonol quercetin and the disaccharide rutinoside. It is considered

Received 7 November 2019; Accepted 20 March 2020; Published online 30 June 2020

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a nutraceutical owing to its various beneficial properties including its anti-inflammatory, antitumor, antibacterial, and antioxidant effects. Rutin is found in citrus fruits (e.g., oranges, lemons, and limes) and berries (e.g., mulberries and cranberries) (Dar and Tabassum, 2012; Li et al., 2014; Wang et al., 2015; Yu et al., 2015; Zhou et al., 2016). Because other flavonoids (e.g. apigenin, kaempferol, luteolin, myricetin, and quercetagenin) (Gutiérrez-Venegas and Bando-Campos, 2010; Gutiérrez-Venegas et al., 2013; Gutiérrez-Venegas et al., 2014a; Gutiérrez-Venegas et al., 2014b; Gutiérrez-Venegas and González-Rosas, 2017) have been shown to suppress the pro-inflammatory effects of LTA, we hypothesized that the flavonoid rutin may prevent LTA activation of pro-inflammatory processes, including the activation of MAPKs, COX-2, and iNOS. We further hypothesized that rutin may favor ROS reduction by modulating the expression of the antioxidant enzymes CAT, SOD, and GPx. The aims of the present study were firstly to examine whether rutin inhibits LTA-induced activation of MAPKs, including p38 and c-Jun N-terminal kinases (JNKs), and secondly to examine whether rutin can modulate COX-2 expression and antioxidant enzymes SOD, CAT, and GPx expression.

MATERIALS AND METHODS

Reagents

LTA (*Streptococcus sanguinis* >97%), 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA), trichloroacetic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheniltetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), phenylmethylsulfonyl fluoride, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid, rutin (>94%), Tris-HCl, NaCl, Nonidet P-40, phenyl-methylsulfonyl fluoride, leupeptin, sodium orthovanadate, sodium fluoride, and sodium pyrophosphate were purchased from Sigma-Aldrich Co. (St. Louis, MO,

USA). Fetal bovine serum (FBS) and polyvinylidene fluoride (PVDF) membranes were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Antibodies against GPx 1/2, SOD-1, CAT, p38, JNK, COX-2, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were of analytical grade and commercially available.

Cell culture

Cells were grown in DMEM with 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine (Invitrogen Life Technologies), incubated at 37°C in a humidified atmosphere with 5% CO₂.

Culture treatment

Studies were performed in rat embryonic heart-derived H9c2 myogenic cells (lot # 63781507; American Type Culture Collection, Manassas, VA, USA) grown overnight on 6-well plates at a concentration of 20,000 cells/well. After the culture medium was replaced with 2% FBS medium, rutin was dissolved in ethanol (4 mM), cells were pretreated (or not) with rutin (10 μ M) for 30 min and then were treated with LTA at 0.1, 1, 5, 10, and 15 μ g/mL for 30 min (for JNK and p38 assays), 6 h (for COX-2 expression analysis), and for 6, 18, and 24 h (for antioxidant enzymes expression analysis) in DMEM with 2% FBS. The following specific kinase inhibitors were applied 1 h before LTA exposure where experimentally indicated: p38 MAPK inhibitor (50 μ M SB203580), MAPK kinase (MEK) 1/2 inhibitor (10 μ M PD98059), and JNK (10 μ M SP600125) (Sigma-Aldrich Co.).

Determination of intracellular ROS generation

Cells were plated in 24 well plates and allowed to attach by overnight incubation at 37°C. Cells were incubated with rutin (1, 5, 10, and 15 μ M) and treated with LTA (10 μ g/mL) for 2 h. After the LTA treatment, the cells were incubated for 30 min with 10 μ M carboxy-H₂DCFDA, a reduced fluorescein analog that converts

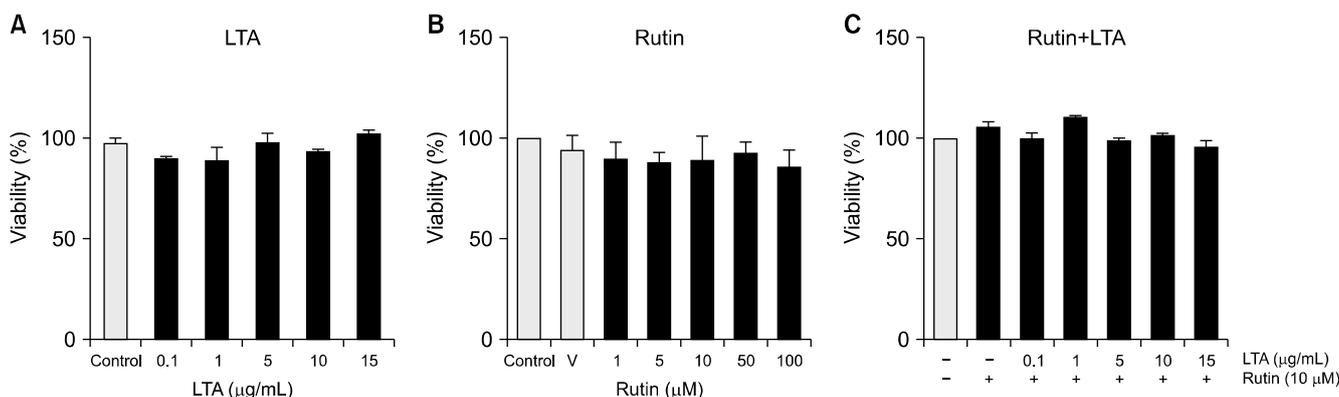


Fig. 1. Cell viability in the presence of (A) lipoteichoic acid (LTA), (B) rutin, and (C) LTA and/or rutin. V, vehicle (ethanol). Data are mean \pm SD (n=3).

from its non-fluorescent form to an immediately after incubation with carboxy-H₂DCFDA, cells were submitted to fluorescence measurement (excitation 492~495 nm/emission 517~527 nm) in a Synergy MTX multi-mode microplate reader (BioTek Instruments Inc., Winooski,

VT, USA).

Determination of cell viability

The number of viable cells (percentage of control) was estimated with a colorimetric MTT assay, in which MTT

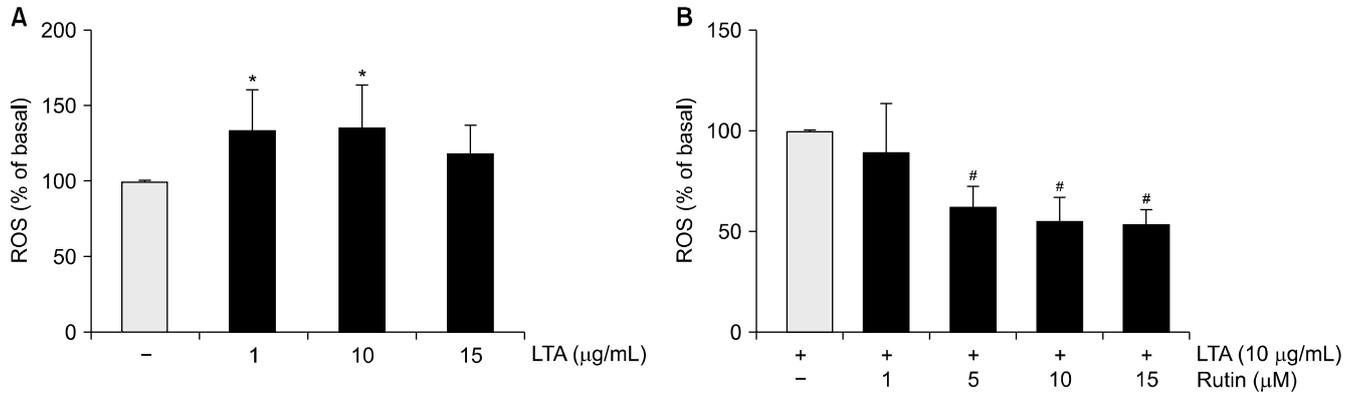


Fig. 2. Levels of reactive oxygen species (ROS) in the presence of (A) lipoteichoic acid (LTA) and (B) LTA and/or rutin. Data are mean±SD (n=3). Significantly different from *LTA and #rutin (*P*<0.05).

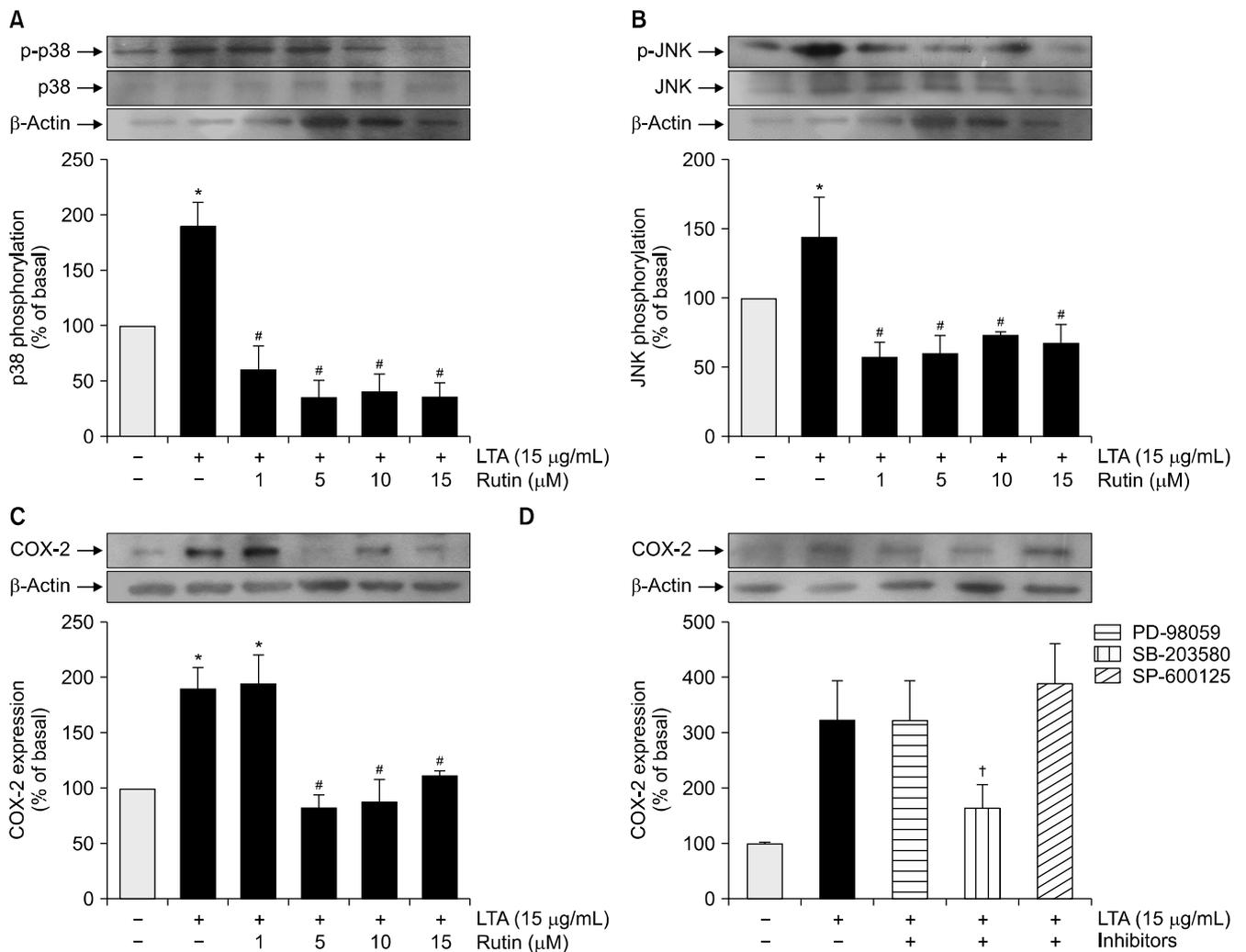


Fig. 3. Effect of rutin on the lipoteichoic acid (LTA) induced phosphorylation and/or expression of (A) p38, (B) c-Jun N-terminal kinase (JNK), and (C) cyclooxygenase-2 (COX-2), and (D) effects of the kinase inhibitors on the expression of COX-2. Data are mean±SD (n=3). Significantly different from *LTA, #rutin, and †LTA 15 µg/mL (*P*<0.05).

is transformed to formazan blue by the activity of mitochondrial dehydrogenases and absorbance is directly proportional to the number of viable cells. After the cells were pretreated (or not) with rutin for 30 min and cultured with LTA (0.1~15 µg/mL) for 24 h, MTT (5 mg/mL) was added to the culture medium and cells were incubated for 4 h at 37°C. After removal of the medium containing the remaining MTT, the blue formazan product was extracted with 2-propanol and quantified spectroscopically at 540 nm.

Western blot

Cells were lysed in 50 µL of lysis buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 0.5 M phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, 0.4 mM so-

dium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). Protein concentration was determined with a standard Bradford assay. Thirty micrograms protein samples were separated by SDS-polyacrylamide gel electrophoresis and then transferred electrophoretically to PVDF membranes. Membranes were blocked in 5% skim milk for 2 h, washed in phosphate buffered saline (pH 7.4) with 0.05% Tween 20, and then incubated with primary antibody overnight at 4°C. The next day membranes were washed and probed with secondary antibody incubation for 2 h at room temperature. The bands were visualized with an enhanced chemiluminescent advance kit (GE Healthcare, Little Chalfont, UK) according to manufacturer's instructions. The films were visualized, scanned, and subjected to quantitative densitometry anal-

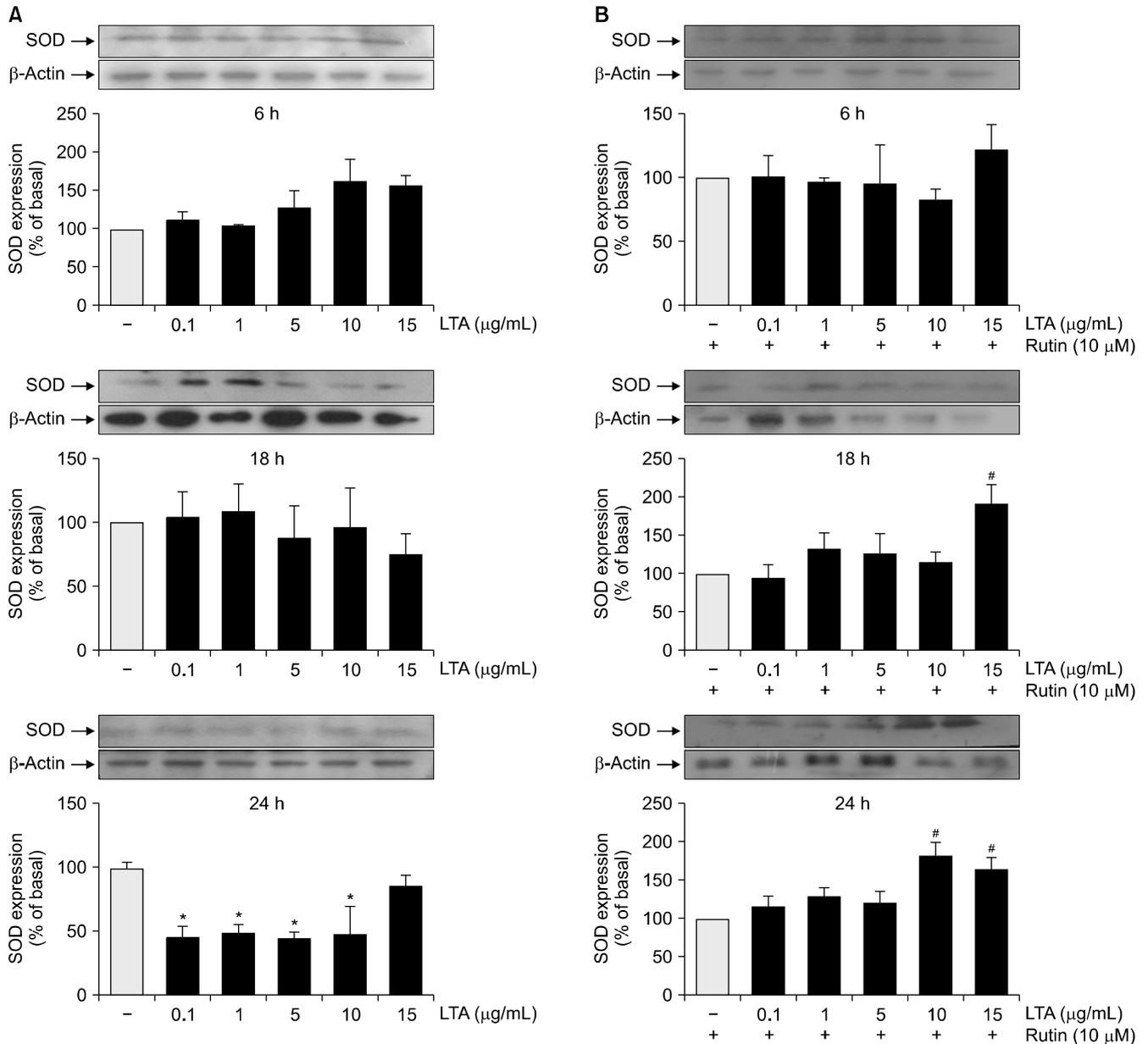


Fig. 4. Effect of lipoteichoic acid (LTA) on the expression of (A) superoxide dismutase (SOD) and (B) effect of rutin on the SOD induced by LTA at 6, 18, and 24 h. Data are mean±SD (n=3). Significantly different from *LTA and #rutin ($P<0.05$).

ysis using DigiDoc-It software (Thomas Scientific, Swedesboro, NJ, USA).

Statistical analysis

The data are expressed as mean±standard deviation (SD). One-way analyses of variance (ANOVA) and Bonferroni analyses were used to compare the data in Sigma Plot version 11.0 (Systat Software, San Jose, CA, USA). *P* values <0.05 were considered significant.

RESULTS

Cell viability

A MTT assay experiment showed no significant effects of

LTA exposure (serial concentrations, 0.1~15 µg/mL, 24 h) alone, rutin treatment (1~100 µM, 30 min) alone, or rutin treatment in the context of LTA exposure on the viability of H9c2 cells (Fig. 1).

Effects of LTA on intracellular ROS generation

Carboxy-H₂DCFDA fluorometry analysis showed that LTA exposure (0.1~15 µg/mL, 24 h) promoted an increase in ROS generation in a dose-dependent fashion (Fig. 2A). Rutin pretreatment (1~15 µM, 30 min) diminished LTA (10 µg/mL) induced-ROS generation markedly (Fig. 2B).

MAPK phosphorylation and COX-2 activation

Rutin (1~15 µM) pretreatment 30 min prior to LTA (15 µg/mL) exposure prevented LTA-induced activation of

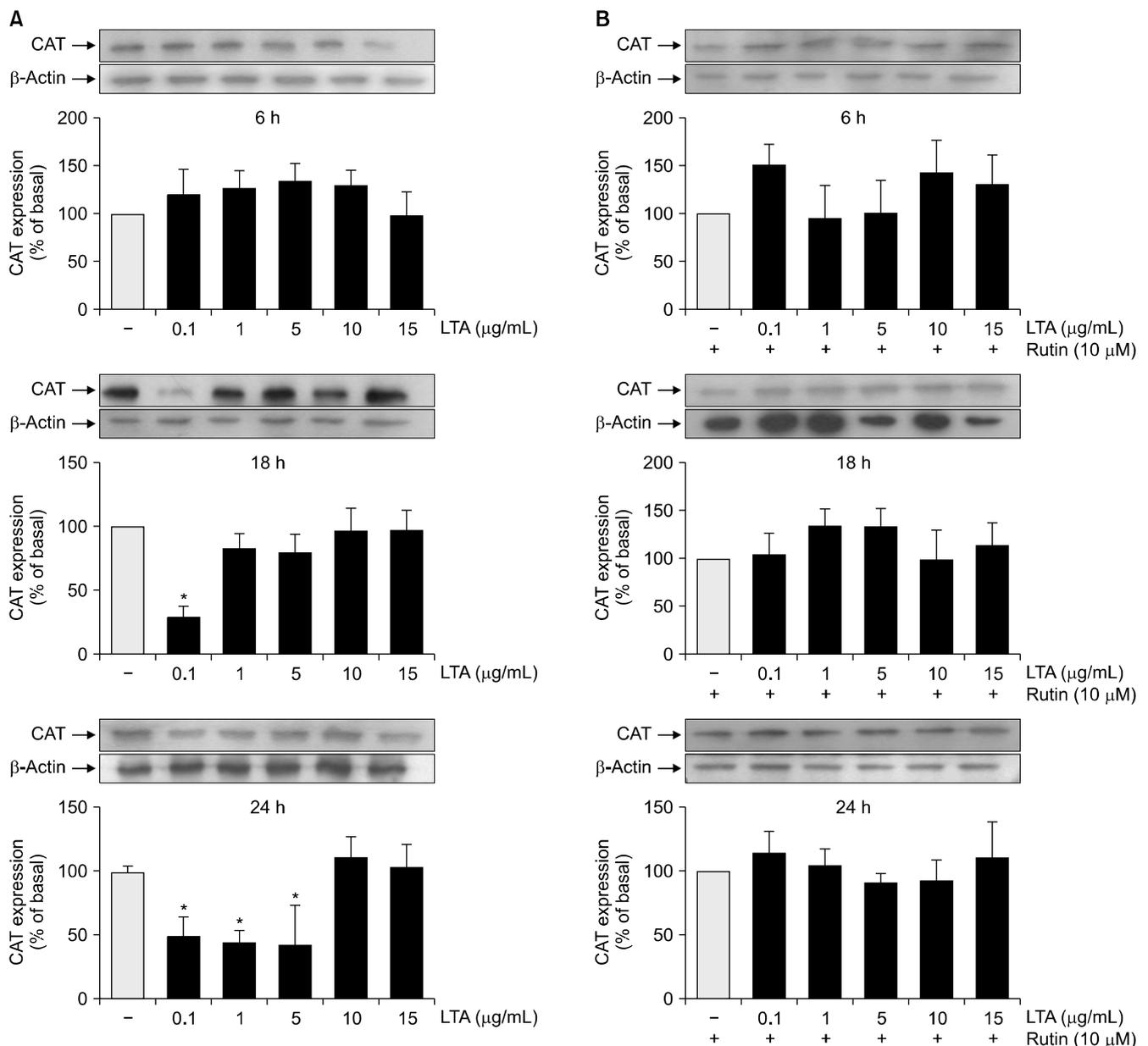


Fig. 5. Effect of lipoteichoic acid (LTA) on the expression of (A) catalase (CAT) and (B) effect of rutin on the expression of CAT induced by LTA at 6, 18, and 24 h. Data are mean±SD (n=3). Significantly different from *LTA (*P*<0.05).

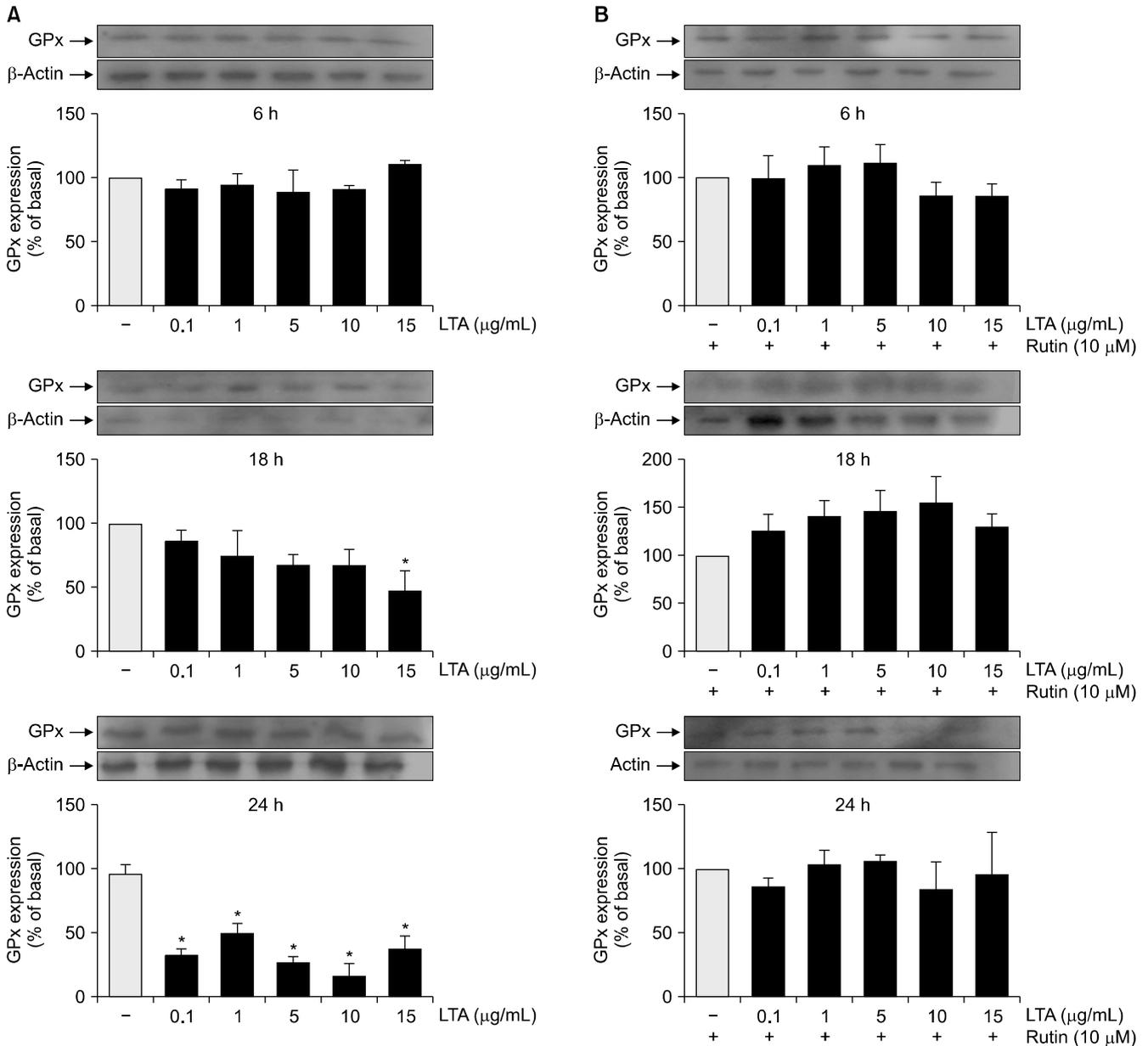


Fig. 6. Effect of lipoteichoic acid (LTA) on the expression of (A) glutathione peroxidase (GPx) and (B) effect of rutin on the expression of GPx induced by LTA at 6, 18, and 24 h. Data are mean±SD (n=3). Significantly different from *LTA ($P<0.05$).

MAPKs, as reflected by levels of phosphorylated (p)-p38 and p-JNK at all tested concentrations (Fig. 3A and 3B). Rutin pretreatment (Fig. 3C) or p38 MAPK inhibition (Fig. 3D) prevented LTA from increasing levels of the pro-inflammatory enzyme COX-2.

Expression of antioxidant enzymes

Western blots showed that 0.1~10 μg/mL LTA exposure resulted in significantly decreased SOD-1 protein expression after 24 h, and this effect was blocked by 10 μM rutin pretreatment (Fig. 4). Likewise, 0.1 μg/mL LTA reduced CAT protein expression significantly in cells after 18 h, and 0.1~5 μg/mL LTA reduced CAT expression significantly after 24 h. These reductions in CAT expression were blocked by rutin pretreatment (Fig. 5). Finally,

GPx protein expression was significantly reduced after 18 h of 15 μg/mL LTA treatment as well as after 24 h of 0.1~15 μg/mL LTA treatment. These reductions in GPx expression were also prevented in cells pretreated with rutin (Fig. 6).

DISCUSSION

In the present study, we found that the antioxidant flavonoid rutin did not affect cell viability, indicating that it is not cytotoxic, consistent with the results of Jeong et al. (2009). Rutin pretreatment 30 min before LTA exposure prevented LTA-induced phosphorylation of p38 and JNK in a dose-dependent manner. This is consistent with our

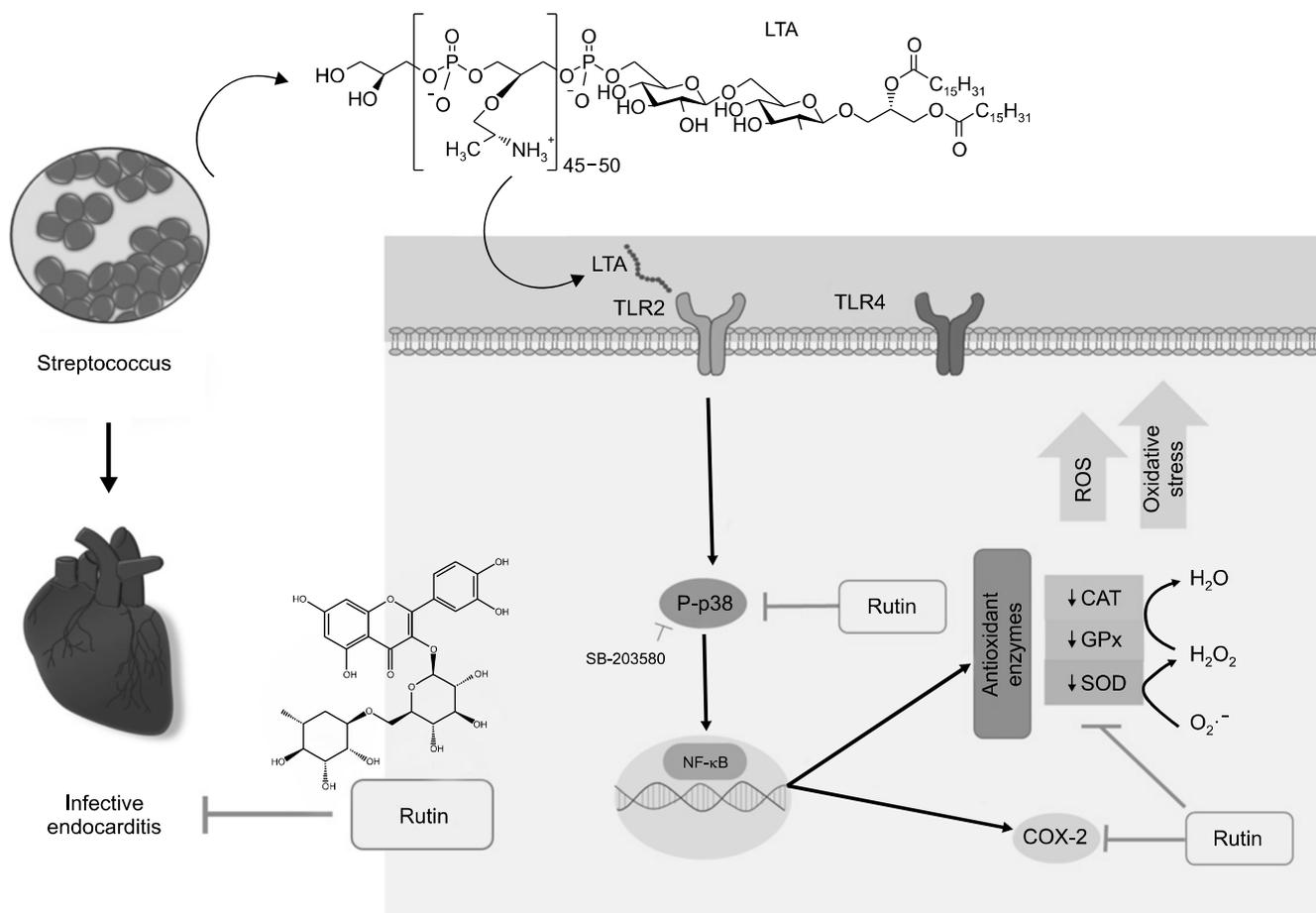


Fig. 7. Model of potential rutin interaction with lipoteichoic acid (LTA) effects in infective endocarditis. LTA treatment induces production of cell-damaging reactive oxygen species (ROS), which are reduced by superoxide dismutase (SOD)-1, catalase (CAT), and glutathione peroxidase (GPx). LTA suppression of these antioxidant enzymes may be a consequence to LTA-induced activation of the mitogen-activated protein kinases p38, which may also underlie LTA induction of the pro-inflammatory enzyme COX-2. TLR, Toll-like receptor; NF- κ B, nuclear factor- κ B.

prior study with Gram-positive bacteria (Gutiérrez-Venegas and Cardoso-Jiménez, 2011), LTA induced expression of the pro-inflammatory enzyme COX-2. We further found in this study that this effect on COX-2 could be blocked with rutin, and that rutin can salvage CAT and GPx activities, consistent with a prior study showing that rutin can restore CAT and GPx activities in amylin-treated BV-2 cells (Yu et al., 2015).

The plant-derived flavonoid rutin has been reported to have clinically relevant benefits, including antioxidant, antihypertensive, and anti-inflammatory activities, which are thought to relate to its antioxidant property (Kurisawa et al., 2003; Al-Dhabi et al., 2015). The ability of rutin to scavenge several ROS including 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid, 2,2'-diphenyl-1-picrylhydrazyl, $O_2^{\cdot-}$, hydroxyl radical (OH^{\cdot}), peroxy radical, and peroxynitrite may prevent lipid peroxidation and low-density lipoprotein oxidation (Heijnen et al., 2001; Kesler et al., 2003; Kurisawa et al., 2003; Al-Dhabi et al., 2015; Lee et al., 2015).

We found that rutin pre-treatment prevented LTA induced reductions in SOD, GPx, and CAT levels. The SOD

data suggest that LTA exposure, unabated, is likely to lead to production of $O_2^{\cdot-}$. Importantly, $O_2^{\cdot-}$ is converted to H_2O_2 , which crosses cell membranes, leading to increased intracellular production of OH^{\cdot} . Hence in addition to rutin having antioxidant properties itself, rutin facilitated re-establishment of antioxidant enzymes would be expected to limit ROS production, and thus protect cells from LTA-associated oxidative damage.

The ability of flavonoids to alleviate LTA effects (Gutiérrez-Venegas and Bando-Campos, 2010; Gutiérrez-Venegas et al., 2013; Gutiérrez-Venegas et al., 2014a; Gutiérrez-Venegas et al., 2014b; Gutiérrez-Venegas and González-Rosas, 2017) are consistent with the notion that oxidative stress is likely a major mechanism mediating LTA-induced cellular damage and IE. The presently demonstrated efficacy of rutin in this regard further supports this notion.

Little is known about the regulatory mechanisms by which LTA affects ROS, but our data provide some hints to be pursued in future research. Our MAPK data may also provide hints regarding the mechanisms of LTA-flavonoid interactions. A model of plausible TLR- and MAPK-

mediated interactions of LTA's oxidative effects with the protective effects of rutin is shown in Fig. 7. In conclusion, this study demonstrates for the first time that the flavonoid rutin can prevent LTA-induced reductions in the levels of major antioxidant enzymes, block LTA activation of the expression of the pro-inflammatory enzyme COX-2, and block LTA-induced increases in the phosphorylation of the MAPKs (p38 and JNK). These data provide hints for future studies aimed at resolving the mechanisms of LTA-related pathogenesis and inflammatory processes in diseases such as IE. The use of rutin in contexts where oxidative stress is induced is encouraged.

ACKNOWLEDGEMENTS

We would like to thank to Programa de Becas Posdoctorales de la UNAM, Dirección General de Asuntos del Personal Académico (DGAPA). PAPIIT IN201816.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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