Title	Preconditioning with Porphyromonas gingivalis lipopolysaccharide may confer cardioprotection and improve recovery of the electrically induced intracellular calcium transient during ischemia and reperfusion
Author(s)	Fan, MHM; Wong, KL; Wu, S; Leung, WK; Yam, WC; Wong, TM
Citation	Journal Of Periodontal Research, 2010, v. 45 n. 1, p. 100-108
Issued Date	2010
URL	http://hdl.handle.net/10722/67022
Rights	The definitive version is available at www3.interscience.wiley.com

Journal of
PERIODONTAL RESEARCH

Preconditioning with *Porphyromonas gingivalis* lipopolysaccharide may confer cardio-protection and improve recovery of the electrically induced intracellular calcium transient during ischemia and reperfusion

Journal:	*Journal of Periodontal Research*
Manuscript ID:	JRE-09-08-0736.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Fan, Michael; The University of Hong Kong, Department of Physiology, Li Ka Shing Faculty of Medicine Wong, Ka Li; The University of Hong Kong, Department of Physiology, Li Ka Shing Faculty of Medicine Wu, Song; The University of Hong Kong, Department of Physiology, Li Ka Shing Faculty of Medicine Leung, W. Keung; The University of Hong Kong, Faculty of Dentistry; The University of Hong Kong, Department of Physiology, Li Ka Shing Faculty of Medicine Yam, Wing Cheong; The University of Hong Kong, Department of Microbiology, Li Ka Shing Faculty of Medicine Wong, Tak Ming; The University of Hong Kong, Department of Physiology, Li Ka Shing Faculty of Medicine; The University of Hong Kong, College of Life Sciences and Technology, School of Professional and Continuing Education
Keywords:	lipopolysaccharides, Porphyromonas gingivalis, myocardial reperfusion injury, myocytes, cardiac

Preconditioning with *Porphyromonas gingivalis* lipopolysaccharide may confer cardio-protection and improve recovery of the electrically induced intracellular calcium transient during ischemia and reperfusion

M.H.M. Fan[1], K.L. Wong[1], S. Wu[1], W.K. Leung[1,2]*, W.C. Yam[3] and T.M. Wong[1,4]

[1]Department of Physiology, Li Ka Shing Faculty of Medicine, [2]Faculty of Dentistry, [3]Department of Microbiology, Li Ka Shing Faculty of Medicine, and [4]College of Life Sciences and Technology, School of Professional and Continuing Education, The University of Hong Kong, Hong Kong SAR, China.

Key words: lipopolysaccharides; myocardial reperfusion injury; myocytes, cardiac; *Porphyromonas gingivalis*

Running Title: cardio-protection by *P. gingivalis* LPS

Date of resubmission: December 17, 2008.

***Corresponding author**

W. Keung Leung

Faculty of Dentistry, The University of Hong Kong, Room 3B39, Prince Philip Dental Hospital, 34 Hospital Road, Hong Kong SAR, China

Tel: +852-2859-0417

Fax: +852-2858-7874.

E-mail: ewkleung@hkucc.hku.hk

Fan MHM, Wong KL, Wu S, Leung WK, Yam WC, Wong TM. Pre-conditioning with Porphyromonas gingivalis *lipopolysaccharide may confer cardio-protection and improve recovery of the electrically induced intracellular calcium transient during ischemia and reperfusion. J Periodont Res*

Background and Objectives: Sublethal doses of *Escherichia coli* lipopolysaccharide (LPS) induce cardio-protection against ischemic insults. This study investigated if *Porphyromonas gingivalis* LPS might give rise to similar effects. We hypothesized that 1) pre-treatment with *P. gingivalis* LPS at appropriate concentrations would induce cardio-protection against injury induced by ischemia and reperfusion; and 2) *P. gingivalis* LPS pre-treatment at cardio-protective concentrations may reduce Ca^{2+} overload—a precipitating cause of injury—and improve recovery of contractile function.

Material and Methods: Male Sprague-Dawley rats were randomly given intra-peritoneal saline or hot phenol/water-extracted *P. gingivalis* LPS at 0.2, 0.5, 1.0, 2.0. or 4.0 mg/kg 24 h before experiment. The hearts were isolated and subjected to regional ischemia by coronary artery ligation followed by reperfusion. In isolated rat ventricular myocytes, the cytosolic Ca^{2+} level and the electrically induced intracellular calcium ($E[Ca^{2+}]_i$) transient, which reflects contractile function, were determined after pre-treatment with a cardio-protective dose of *P. gingivalis* LPS.

Results: Pre-treatment with 0.5 mg/kg *P. gingivalis* LPS significantly reduced, while pre-treatment with 1.0 to 4.0 mg/kg significantly increased infarct size. The Ca^{2+} overload induced by ischemia/reperfusion was attenuated in myocytes from rats pre-treated with 0.5

mg/kg *P. gingivalis* LPS. Treated myocytes also showed an increased amplitude of the $E[Ca^{2+}]_i$ transient, abolished prolongation of the time to reach the peak $E[Ca^{2+}]_i$ transient, and shorter 50% decay time during reperfusion following LPS pre-treatment.

Conclusion: 0.5 mg/kg *P. gingivalis* LPS confers cardio-protection against ischemia/reperfusion-induced injury and improved intracellular $E[Ca^{2+}]_i$ transient recovery, hence improving myocyte contractile recovery.

Porphyromonas gingivalis is a gram-negative anaerobic bacillus and one of the 500 different species of naturally occurring bacteria in the oral cavity. The bacterium, a known periodontopathogen, has been reported to be involved in cardiovascular diseases in subjects with periodontitis (1, 2). Proposed mechanisms include systemic release of antigen like *P. gingivalis* lipopolysaccharide (LPS) or endotoxin from infected periodontal pockets (3), or local persistent inflammation leading to modulation of systemic cytokine levels (4) and/or ability of the bacterium to invade endothelial cells (5) and blood vessels rendering bacteremia (6). The LPS of *P. gingivalis* is also known to be associated with the development of periodontal diseases (7) and has also been postulated to be a predisposing factor for atherosclerotic diseases (8). Phenol/water-extracted *P. gingivalis* LPS is the ligand for cell surface toll-like receptors (TLR), TLR2 and TLR4 (9), and stimulation of either TLR2 or TLR4 can result in activation of phosphoinositide-3-kinase/Akt-dependent signaling (10), which has been shown to protect myocytes from ischemic reperfusion injury (11). We hypothesized that pre-treatment with *P. gingivalis* LPS at appropriate concentrations would induce cardio-protection against injury induced by ischemia and reperfusion.

Ca^{2+} homeostasis is essential to contractile function of the heart and protection against cardiac injury. When an action potential arrives, the sarcolemmal membrane of cardiac myocyte is depolarized. Depolarization of the sarcolemmal membrane opens the voltage gated Ca^{2+} channel, thus allowing the entry of Ca^{2+} into the myocyte. The Ca^{2+} triggers a massive amount of Ca^{2+} released via the ryanodine receptor from the sarcoplasmic reticulum, the intracellular Ca^{2+} store. The release of a massive amount of Ca^{2+} in a short time results in a sudden increase in cytosolic Ca^{2+}, a Ca^{2+} transient, which triggers contraction. After contraction, Ca^{2+} is removed from the cytosplasm as a result of sequestration of Ca^{2+} back to the sarcoplasmic

reticulum via the Ca^{2+} ATPase and out of the myocyte via the Na^{+}-Ca^{2+} exchanger. Removal of Ca^{2+} from the cytoplasm results in relaxation (12). Alterations in Ca^{2+} homeostasis may therefore lead to alterations in myocyte contractile function and cytosolic Ca^{2+} level. It is well established that myocyte cytosolic Ca^{2+} overload is a precipitating factor of cardiac injury. We reasoned that if *P. gingivalis* LPS had cardio-protective effect, it may affect myocyte Ca^{2+} homeostasis, which may alter its cytosolic Ca^{2+} homeostasis and hence the contractile function.

In the present study we first attempted to test the hypothesis that sublethal dose of *P. gingivalis* LPS pre-treatment may protect the heart against ischemic insult. We studied the effects of hot phenol-water extracted *P. gingivalis* LPS on infarct size of the rat heart subjected to ischemia and reperfusion using the Langendorff isolated perfused heart preparation. In the second series of experiments we attempted to test the hypothesis that *P. gingivalis* LPS pre-treatment at cardio-protective concentrations may alter myocyte Ca^{2+} homeostasis, particularly reducing Ca^{2+} overload, hence improving myocytes contractile recovery upon ischemic insult. We determined the effects of *P. gingivalis* LPS at a concentration, that was shown to reduce the infarct size inducible by ischemic insult in the first series of experiment, on cytosolic Ca^{2+} level and the electrically induced intracellular Ca^{2+} ($E[Ca^{2+}]_i$) transient in single ventricular myocyte according to a procedure routinely used in our laboratory.

Materials and Methods

Study design

To test the first hypothesis the effects of 24 h pretreatment with hot phenol-water extracted *P.*

gingivalis LPS 0.2 - 4 mg/kg body weight on the infarct size of isolated rat heart induced by ischemia/reperfusion were determined. Then the effects of the endotoxin preparation at concentration shown in the first series of experiment that could reduce the infarct size induced by ischemia/reperfusion, on isolated rat myocyte cytosolic Ca^{2+} level and the $E[Ca^{2+}]_i$ transient were followed. The $E[Ca^{2+}]_i$ transient is elicited by an electrical stimulation, which mimicks the arrival of an action potential. An electrical stimulation or action potential depolarizes the sarcolemmal membrane, which opens up the voltage gated L-type Ca^{2+} channel, leading to an influx of Ca^{2+} into the cytoplasm. The Ca^{2+} in turn triggers the release of Ca^{2+} from the intracellular Ca^{2+} store, the sarcoplasmic reticulum, leading to a sudden increase in $[Ca^{2+}]_i$. So the amount, the amplitude, and the rate, time to peak, of Ca^{2+} released determine the contractility. The decay, 50% decay, reflects the removal of Ca^{2+} from the cytosol and determines relaxation. A previous study from our lab showed that the amplitude of the transient is directly correlated to the shortening of the isolated ventricular myocyte (13).

Preparation of *P. gingivalis* LPS

The hot phenol-water extraction method (14) was adopted to prepare *P. gingivalis* LPS. *P. gingivalis* type strain ATCC 33277 was anaerobically cultured in tryptic soy broth supplemented with 1 mg/mL yeast extract, 5 μg/mL of hemin, and 1 μg/mL menadione. At mid-log phase growth (OD at 660 nm = 0.35), the bacteria were harvested by centrifugation (10,000 × *g*), and washed twice in phosphate buffered saline. One gram of cells was suspended in 17.5 mL of water, boiled for 30 min, and disrupted by ultrasonication on ice; 15 mL of this suspension was then mixed with 15 mL of a 50% (v/v) solution of phenol (in the form of 90% phenol crystals) and double-distilled water pre-warmed to 68°C. The mixture was incubated at 68°C with

vigorous shaking for 20 min, incubated in an ice bath for 15 min, and centrifuged at 1,500 × *g* for 45 min at 10°C. The upper aqueous layer containing the LPS was transferred to a new tube on ice, while 15 mL of distilled water at 68°C was added to the phenol layer and the extraction process repeated. The pooled aqueous solution was centrifuged at 12,000 × *g*, 4°C for 10 min and the supernatant was dialyzed against double-distilled water at 4°C for 3 to 5 days. The dialyzed solution was then centrifuged at 146,600 × *g*, 4°C for 4 h. The pellet was washed twice with double-distilled water and centrifuged again, and was finally resuspended in double-distilled water and frozen at -70°C overnight by lyophilization. Every gram wet weight of *P. gingivalis* yielded 61.7 µg of LPS or every *P. gingivalis* bacterium yield 0.037 pg of dry LPS.

A protein assay was performed to confirm the purity of the extracted LPS, and no detectable protein was found in the sample. DNA content of the extracted LPS was analyzed by PCR using *P. gingivalis* specific (15) or universal 16S rDNA primers (16). Detection limits of both protocols were ≥ 0.00001% or 0.0001% of DNA by weight, respectively. No *P. gingivalis* DNA nor any bacterial 16S rDNA was detectable. Lipid A was extracted from the LPS as described previously (17) and subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis at the Genome Research Centre, The University of Hong Kong. The species of lipid A in the preparation was identified as *P. gingivalis* triacylated lipid A, as described in the literature (18). No attempt was made to characterize the level of contaminating cell surface polysaccharides in the preparation.

Animal experiments

The study was approved by the Committee on the Use of Live Animals in Teaching and

Research, The University of Hong Kong. Two series of experiments were performed. The first studied the impact of pre-treating rats with different concentrations of *P. gingivalis* LPS on infarct size induced by ischemia and reperfusion in the isolated perfused rat heart (Fig. 1A). The second series of experiments studied the effects of a pre-conditioning cardio-protective dose of *P. gingivalis* LPS on the resting cytosolic $[Ca^{2+}]_i$ and the electrically induced intracellular Ca^{2+} ($E[Ca^{2+}]_i$) transients in isolated rat ventricular myocyte (Fig. 1B).

P. gingivalis *LPS pre-treatment and rat heart ischemia/reperfusion*

Thirty-six male Sprague-Dawley rats, each about 250 g, were obtained from the Laboratory Animal Unit, Li Ka Shing Faculty of Medicine, The University of Hong Kong, and were randomly divided into control (n = 7) and test groups. The test group was further divided into five subgroups that were given an intra-peritoneal injection of 0.2, 0.5, 1.0, 2.0, or 4.0 mg/kg body weight of *P. gingivalis* LPS in 2 mL normal saline (n = 8, 6, 4, 4, 7, respectively). These doses of *P. gingivalis* LPS were chosen according to earlier publications regarding cardio-protective effects of *Escherichia coli* LPS (19, 20). The control group was injected with 2 mL normal saline only.

Twenty-four hours after the injections, all rats were anesthetized with intra-peritoneal 60 mg/kg sodium pentobarbitone and then injected with 200 IU of heparin intravenously. Hearts were excised rapidly and placed in ice-cold Krebs-Henseleit perfusion buffer (11 mM glucose, 118 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4) before being mounted on a Langendorff apparatus for perfusion at 37°C with perfusion buffer at a constant pressure (100 cm H_2O) and equilibrated with 95% O_2 and 5% CO_2. The hearts were allowed to stabilize for 30 min and then subjected to regional ischemia and

reperfusion as described previously (21). Any hearts exhibiting arrhythmia during stabilization were discarded. A 3-O silk suture was placed around the left anterior descending coronary artery from the area immediately below the left atrial appendage to the right portion of the left ventricle. The ends of the suture were threaded through a propylene tube to form a snare. The left anterior descending coronary artery was occluded by pulling the ends of the suture taut and using a hemostat to clamp the snare onto the pericardial surface for 30 min. Ischemia was verified by regional cyanosis and a substantial decrease in coronary flow. Reperfusion was achieved by releasing the ligature and the heart was reperfused for 120 min to induce myocardial injury (21).

After the reperfusion, the ligature was re-tightened and approximately 0.2 mL of 2.5% Evans blue dye (Sigma Chemical, St. Louis, MO, USA) was infused into the heart through the jugular vein to stain any permeable tissue, leaving non-perfused vascular (occluded and at risk of infarction) tissue uncolored. Excess dye was washed off, and the hearts were frozen and sectioned into slices of 3 to 4 mm and then incubated with sodium phosphate buffer containing 1% 2,3,5-triphenyl-tetrazolium chloride (TTC) (Sigma Chemical, St. Louis, MO, USA) for 15 min at 37°C to visualize the unstained infarcted region. The infarct size (IS, TTC-negative), the area at risk (AAR, TTC-positive) and the size of the left ventricular zone (LV) were determined by planimetry with the software Image J from the National Institutes of Health. The percentage of AAR/LV formed the AAR% while the percentage of IS/AAR formed the IS%.

Electrically induced intracellular Ca^{2+} ($E[Ca^{2+}]_i$) transient

In the second series of experiments, 12 male Sprague-Dawley rats, each about 250 g, were randomly divided into control and test groups. The test group underwent LPS pre-conditioning

by receiving an intra-peritoneal 0.5 mg/kg dose of *P. gingivalis* LPS dissolved in 2 mL saline 24 h before the experiment, whereas the control group received the saline injection only. This dose was chosen as it was shown to confer a cardio-protective effect to ischemia/reperfusion insults in the isolated heart in the first series of experiments.

Twenty-four hours after the injection, the rats were anesthetized with intra-peritoneal 60 mg/kg sodium pentobarbitone and decapitated. Ventricular myocytes were then isolated from the hearts of the rats using the collagenase method as described previously (22). The heart was mounted on an apparatus that allowed retrograde perfusion through the aorta with 0.2% collagenase and 0.03% protease dissolved in Tyrode's solution (5 mM glucose, 143 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 5 mM HEPES) which passed through a water bath at 37^{o}C. After 30 min of digestion, the heart was removed from the apparatus and the ventricle of the heart was cut into pieces in Tyrode's solution and then homogenized by pipetting. The cell suspension was filtered through a mesh screen and the cells were suspended in 20 mL Tyrode's solution at room temperature, with $CaCl_2$ solution gradually added to bring the Ca^{2+} concentration to 1.25 mM over 30 min. The cells were stabilized for 30 min at room temperature and the viability of the myocytes was confirmed using light microscopy.

E$[Ca^{2+}]_i$ transients were measured spectrofluorometrically with fura-2 as the Ca^{2+} indicator. The isolated ventricular myocytes were incubated in the dark for 30 min with fura-2 in Krebs solution (11 mM glucose, 118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4). The suspension was centrifuged and the cells were washed three times with Krebs solution and then resuspended in Krebs solution. A few droplets of the myocyte suspension were added to the chamber on the stage of a microscope (Axiobert S100, Carl Zeiss, Germany), which was connected to a dual-wavelength excitation spectrofluorometer

(Photon Technology International, Princeton, New Jersey, USA). The fluorescence intensities at 340 nm and 380 nm reflect the equilibrium between Ca^{2+}-bound and Ca^{2+}-free fura-2 dye, respectively, and the ratio is a useful indicator of the cytosolic $[Ca^{2+}]_i$ of an isolated myocyte (23).

The microscope chamber had a continuous flow of Krebs solution through an inlet tube and outlet tube connected to a pump, as well as a direct-current electrode that was connected to an electrical stimulation generator that fired every 5 sec (0.2 Hz) to stimulate an action potential. To measure the E$[Ca^{2+}]_i$ transient, we selected rod-shaped myocytes with clear striation and showing a regular, clear rhythm of amplitude during spontaneously beating. We subjected the cells to 600 sec of metabolic inhibition and anoxia (to simulate ischemic conditions) by infusing the chamber with a glucose-free Krebs solution containing 10 mM 2-deoxy-D-glucose and 10 mM sodium dithionite to capture the glucose and oxygen, respectively. The cells then received 1300 sec of reperfusion with normal Krebs solution. The ratio of the change in fluorescence intensities at the two wavelengths were captured by a monitor device and the data were stored for analysis.

The resting Ca^{2+} ($[Ca^{2+}]_i$), and the amplitude, time to peak, and T_{50} (50% decay time) of the E$[Ca^{2+}]_i$ transient were determined. The amplitude and the time to peak represent the amount of Ca^{2+} and rate of Ca^{2+} released from the sarcoplasmic reticulum on electrical stimulation, respectively. The T_{50} indicates the rate of removal of Ca^{2+} from the cytosol.

Statistical analyses

Data were expressed as mean ± standard error of the mean (SEM). Statistical comparisons of the infarct size were performed by one-way analysis of variance followed by Tukey's multiple

comparison tests. The Student *t* test was used to determine the difference between test and control groups for the results obtained from Ca^{2+} transient measurements. The measurements from myocytes of the same rat were averaged and regarded as one sample. A *P* value of less than 0.05 indicated a statistically significant difference.

Results

Effects of *P. gingivalis* LPS pre-conditioning on myocardial injury

Myocardial infarction was induced in all the rat hearts in which the coronary artery had been occluded. Pre-treatment with low doses of *P. gingivalis* LPS resulted in reductions in infarct size and the reduction induced by 0.5 mg/kg *P. gingivalis* LPS was statistically significant. In contrast, pre-conditioning with *P. gingivalis* LPS at higher doses increased the infarct size significantly (Fig. 2).

Effects of *P. gingivalis* LPS pre-conditioning on rat ventricular myocytes

Because 24-h pre-conditioning with 0.5 mg/kg *P. gingivalis* LPS conferred cardio-protection against injury induced by ischemia and reperfusion, we determined the effects of this pre-conditioning protocol on the resting $[Ca^{2+}]_i$ and its transient, which reflects Ca^{2+} handling and contractile functions (Fig. 3). In brief, the control myocyte showed a greater reduction in amplitude in $E[Ca^{2+}]_i$ transient than myocyte from 0.5 mg/kg *P. gingivalis* LPS treated rat during ischemia and at the end of reperfusion. In myocytes of the control animals, the resting $[Ca^{2+}]_i$ was significantly elevated during ischemia and further elevated during reperfusion (Fig.

4). In myocytes of the treatment group, the resting $[Ca^{2+}]_i$ was significantly elevated during ischemia but remained at the same level during reperfusion; the levels during both ischemia and reperfusion were lower than those in the controls but only the difference during reperfusion was statistically significant.

In general, the amplitude of the $E[Ca^{2+}]_i$ transient, which represents the amount of Ca^{2+} released from the sarcoplasmic reticulum, markedly decreased during ischemia and recovered partially during reperfusion (Figs. 3 and 5). In the sample of myocytes from rats treated with 0.5 mg/kg *P. gingivalis* LPS, the reduction in the amplitude of the $E[Ca^{2+}]_i$ transient during the ischemic period was significantly less than in the controls, whereas the recovery in amplitude during reperfusion was significantly greater than in the controls.

The time to reach the peak of the $E[Ca^{2+}]_i$ transient, which reflects the speed of Ca^{2+} release from the sarcoplasmic reticulum, was significantly prolonged during reperfusion in the control group (Figs. 3 and 6). The prolongation was abolished in the treatment group.

The T_{50} of the $E[Ca^{2+}]_i$ transient, which reflects the speed of removal of Ca^{2+} from the cytosol, increased significantly during ischemia and recovered partially during reperfusion in the control group (Figs. 3 and 7). The corresponding value in the treatment group during ischemia was significantly lower than that in the control group.

Discussion

There are two main novel findings in the present study. The first one is that pre-treatment by *P. gingivalis* LPS produced biphasic effects on ischemia/reperfusion-induced injury in the rat heart. At the dose of 0.5 mg/kg administered intra-peritoneally, *P. gingivalis* LPS is beneficial against

ischemia/reperfusion induced injury. At a higher dose range, however, it exacerbates the injury induced by ischemia/reperfusion as has been observed in other gram-negative bacterial endotoxin like *E. coli* LPS (19, 24). The second novel finding is that at the dose of 0.5 mg/kg, which was shown to confer cardio-protection, *P. gingivalis* LPS attenuates the cytosoclic Ca^{2+} overload, in agreement with its cardio-protective action, and more importantly it improves the recovery of E[Ca^{2+}]i transient, which reflects the contractile function. Similar to the current study, reports showed sublethal doses of *E. coli* LPS up to 10 mg/kg protect the rat heart against injury induced by ischemia/reperfusion (19, 25). The beneficial effects of LPS of *P. gingivalis* and *E. coli* support the notion that the endotoxins may prevent infections and damage caused by ischemia/reperfusion (26).

We found for the first time that pre-conditioning with 0.5 mg/kg *P. gingivalis* LPS increased the amplitude and speed of the rising phase and the speed of recovery to the resting level of the E[Ca^{2+}]$_i$ transient, indicating greater Ca^{2+} release at a faster rate and more efficient removal of Ca^{2+} from the cytosol after contraction. These events in turn would increase the contractility and speed up relaxation of myocytes. Hence, the LPS at a dose that confers cardio-protection also improves contractile recovery after ischemic insult.

E. coli and *P. gingivalis* are two different families of the naturally occurring bacteria in the gastrointestinal tract. It is well documented their LPSs possess different biochemical properties and interact selectively on different subtypes of TLRs (27). It is of interest to note that *P. gingivalis* LPS at the dose of 0.5 mg/kg is beneficial whereas *E. coli* LPS up to 10 mg/kg protects the rat heart against injury induced by ischemia/reperfusion (19, 25). The different doses of LPS from two different bacteria that confer cardio-protection may be explained partly by the possible different mechanisms.

Cardio-protection induced by bacterial endotoxins has been linked to the activation of TLR transmembrane receptors (11). The receptors TLR 2, 3, 4, and 6 are expressed on myocytes (28), and LPSs are known to recognize and bind to TLR2 and TLR4 to elicit cellular responses (29, 30). Phenol/water-extracted *P. gingivalis* LPS is the ligand for TLR2 and TLR4 (9), whereas the pure form of the LPS from *E. coli* is the ligand for TLR4 only (9, 31). Stimulation of either TLR2 or TLR4 can result in activation of phosphoinositide-3-kinase/Akt-dependent signaling (10), which has been shown to protect myocytes from ischemic reperfusion injury (11). TLR2 is believed to be cardio-protective against oxidative stress in neonatal rat cardiac myocytes (28). The relative contributions of TLR2 or TLR4 in the host response to *P. gingivalis* LPS challenge are yet to be characterized.

P. gingivalis LPS pre-treatment was administered intra-peritoneally to test animals—an established and convenient administration method. Abdulla and co-workers (32) previously reported on the bioavailability of *E. coli* LPS after intra-peritoneal administration. In brief, the rat serum endotoxin concentration peaked at 6 h to a level of 10 ng/mL after intra-peritoneal injection of 25 µg of *E. coli* LPS, meaning that 0.4% of what was administered entered the systemic circulation. These observations are explained by hepatic first-pass and liver clearance of LPS (33), in which combined actions of Kupffer cells, CD14, and LPS-binding proteins result in highly effective detoxification of the administered LPS (34, 35). We estimate that a peak level of 10 to 50 ng/mL of *P. gingivalis* LPS might be available to the rat systemic circulation approximately 6 h after 0.5 mg/kg LPS intra-peritoneal administration. That amount of LPS administered was estimated to be equivalent to endotoxin extractable from 2.7×10^9 bacterial cells or 27 ml of mid-log phase *P. gingivalis* culture. Further studies are however warranted to quantify the postulated LPS bioavailability. The postulated systemic circulatory

exposure to LPS from periodontopathogens, nevertheless would be highly probable in human with advanced periodontitis in which *P. gingivalis* LPS directly enters the systemic circulation through inflamed ulcerated periodontal pockets (36).

Although this study was performed in rats to closely related models, extrapolation of the deleterious effects of *P. gingivalis* LPS in periodontitis, such as affecting systemic or cardiac health, clinical settings for humans should be made with caution. Furthermore, we used phenol/water-extracted *P. gingivalis* LPS, so further research to study the effects of column-purified *P. gingivalis* LPS on heart tissue as well as the possible mechanisms of host-LPS interactions are needed.

The delayed nature of protection suggests that cardio-protective proteins may be synthesized *de novo*. Three classes of proteins are known to be end-effectors responsible for delayed cardio-protection after pre-conditioning: heat-shock protein families (37-39); intracellular antioxidant enzymes, including catalase (19) and superoxide dismutase (40, 41); and inducible nitric oxide synthase (42, 43). *P. gingivalis* LPS has been shown to be able to enhance expression of the cardio-protective superoxide dismutase in human monocytic cell lines (44). Further study on the contribution of this protein to the cardio-protective action of *P. gingivalis* LPS is warranted.

Paradoxically, when the dose of *P. gingivalis* LPS increased to between 1 and 4 mg/kg, myocardial injury was exacerbated and the infarct size increase significantly. Similarly, high-dose *E. coli* LPS, in contrast to a low dose, can induce TLR4-mediated NF-κB activation, trigger transcription of inflammatory mediators (45), and induce cardiac dysfunction (46, 47). The same phenomena might be in action during challenge with a higher dose of *P. gingivalis* LPS.

Conclusion

We demonstrate for the first time that phenol/water-extracted *P. gingivalis* LPS given intra-peritoneally has biphasic effects on cardiac injury induced by ischemia/reperfusion in the rat heart 24 h after its administration. A sublethal intra-peritoneal dose (0.5 mg/kg) of *P. gingivalis* LPS can reduce the infarct size and $[Ca^{2+}]_i$ overload induced by ischemia and reperfusion, indicating a delayed cardio-protective action. In addition, the same dose of *P. gingivalis* LPS improves recovery of the electrically induced $[Ca^{2+}]_i$ transient, suggesting that LPS may improve contractile recovery. Further experiments are warranted to clarify the exact nature of such bacterial-host cardiac interactions.

Acknowledgments

The study was partly supported by a Cardiovascular Research Fund donated by LCST (Holdings) Ltd. We thank C.P. Mok for technical assistance, Joyce Y.Y. Yau for *P. gingivalis* culture, Becky B.K. Cheung for *P. gingivalis* LPS extraction, and Trevor Lane for technical editing.

References

1. Stelzel M, Conrads G, Pankuweit S, *et al.* Detection of *Porphyromonas gingivalis* DNA in aortic tissue by PCR. *J Periodontol* 2002;**73**:868-870.
2. Bahekar, A.A., Singh, S., Saha, S., Molnar, J., Arora, R. The prevalence and incidence of coronary heart disease is significantly increased in periodontitis: A meta-analysis. *Am Heart J* 2007:**154**:830-837.
3. Geerts, S.O., Nys, M., De Mol, P., *et al.* Systemic release of endotoxins induced by gentle mastication: Association with periodontitis severity. *J Periodontol* 2002;**73**:73-78.
4. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol Mol Biol Rev* 1998;**62**:1244-1263.
5. Deshpande RG, Khan MB, Genco CA. Invasion of aortic and heart endothelial cells by *Porphyromonas gingivalis*. *Infect Immun* 1998;**66**:5337-5343.
6. Hu SW, Huang CH, Huang HC, Lai YY, Lin YY. Transvascular dissemination of *Porphyromonas gingivalis* from a sequestered site is dependent upon activation of the kallikrein/kinin pathway. *J Periodontal Res* 2006;**41**:200-207.
7. Haffajee AD, Socransky SS. Microbial etiologic agents of destructive periodontal disease. *Periodontol 2000* 1994;**5**:78-111.
8. Hajishengallis G, Sharma A, Russell MW, Genco RJ. Interactions of oral pathogens with toll-like receptors: possible role in atherosclerosis. *Ann Periodontol* 2002;**7**:72-78.
9. Ogawa T, Asai Y, Makimura Y, Tamai R. Chemical structure and immunobiological activity of *Porphyromonas gingivalis* lipid A. *Front Biosci* 2007;**12**:3795-3812. Review.
10. Li C, Ha T, Kelley J, *et al.* Modulating Toll-like receptor mediated signaling by

(1→3)-β-D-glucan rapidly induces cardioprotection. *Cardiovasc Res* 2004;**61**:538-547.

11. Ha T, Hua F, Liu X, *et al*. Lipopolysaccharide-induced myocardial protection against ischemia/reperfusion injury is mediated through a PI3K/Akt-dependent mechanism. *Cardiovasc Res* 2008;**78**:546-553.
12. Katz AM. *Physiology of the heart*, 4th edn. Philadelphia: Lippincott Williams & Wilkins, 2006:162-202.
13. Yu XC, Li HY, Wang HX, Wong TM. U-50488H inhibits effects of norepinephrine in rat cardiomyocytes: cross-talk between kappa-opioid and beta-adreneric receptors. *J Mol Cell Cardiol* 1998;**30**: 405-413.
14. Westphal O, Luderitz O, Bister F. Uber die extraction von bacterien mit phenol-wasser. *Z Naturforsch* 1952;**78**:148-155.
15. Ashimoto A, Chen C, Bakker I *et al*. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;**11**:266-273.
16. Aas JA, Barbuto SM, Alpagot T, Olsen I, Dewhirst FE, Paster BJ. Subgingival plaque microbiota in HIV positive patients. *J Clin Periodontol* 2007;**34**:189-195.
17. Qureshi N, Takayama K, Meyer KC, *et al*. Chemical reduction of 3-oxo and unsaturated groups in fatty acids of diphosphoryl lipid A from the lipopolysaccharide of *Rhodopseudomonas sphaeroides*. Comparison of biological properties before and after reduction. *J Biol Chem* 1991;**266**:6532-6538.
18. Al-Qutub MN, Braham PH, Karimi-Naser LM, Liu X, Genco CA, Darveau RP. Hemin-dependent modulation of the lipid A structure of *Porphyromonas gingivalis* lipopolysaccharide. *Infect Immun* 2006;**74**:4474-4485.

19. Brown JM, Grosso MA, Terada LS, *et al.* Endotoxin pretreatment increases endogenous myocardial catalase activity and decreases ischaemic-reperfusion injury of isolated rat hearts. *Proc Nat Acad Sci USA* 1989;**86**:2516-2520.

20. Neviere RR, Li FY, Singh T, Myers ML, Sibbald W. Endotoxin induces a dose-dependent myocardial cross-tolerance to ischemia-reperfusion injury. *Crit Care Med* 2000;**28**:1439-1444.

21. Wang GY, Wu S, Pei JM, Yu XC, Wong TM. Kappa- but not delta-opioid receptors mediate effects of ischemic preconditioning on both infarct and arrhythmia in rats. *Am J Physiol* 2001;**280**:H384-H391.

22. Wu S, Li HY, Wong TM. Cardioprotection of preconditioning by metabolic inhibition in rat ventricular myocyte. Involvement of kappa-opioid receptor. *Circ Res* 1999;**84**:1388-1395.

23. Wang HX, Kwan CY, Wong TM. Electrically induced intracellular Ca^{2+} transient in single ventricular myocytes: A useful parameter for the study of cardiac drugs. *Clin Exp Pharm Physiol* 1999;**26**:835-836.

24. Rowland RT, Meng X, Cleveland JC Jr, Meldrum DR, Harken AH, Brown JM. LPS-induced delayed myocardial adaptation enhances acute preconditioning to optimize postischemic cardiac function. *Am J Physiol* 1997;**272**:H2708-15.

25. Meng X, Ao L, Brown JM, *et al*. LPS induces late cardiac functional protection against ischemia independent of cardiac and circulating TNF-α. *Am. J. Physiol* 1997;**273**:H1894-H1902.

26. Cavaillon JM, Adrie C, Fitting C, Adib-Conquy M. Endotoxin tolerance: is there a clinical relevance? *J Endotoxin Res* 2003;**9**:101-107.

27. Gioannini TL, Weiss JP. Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. *Immunol Res* 2007;**39**:249-260. Review.

28. Frantz S, Kelly RA, Bourcier T. Role of TLR-2 in the activation of nuclear factor-kappa-B by oxidative stress in cardiac myocytes. *J Biol Chem* 2001;**276**:5197-5203.

29. Southerland JH, Taylor GW, Moss K, Beck JD, Wallach D. Commonality in chronic inflammatory diseases: periodontitis, diabetes and coronary heart disease. *Periodontol 2000* 2006;**40**:130-143.

30. Teng YTA. Protective and destructive immunity in the periodontium: Part 1- innate and humoral immunity and the periodontium. *J Dent Res* 2006;**85**:198-208.

31. Tapping RI, Akashi S, Miyake K, Godowski PJ, Tobias PS. Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for *Escherichia* and *Salmonella* lipopolysaccharides. *J Immunol* 2000;**165**:5780-5787.

32. Abdulla D, Goralski KB, Del Busto Cano EG, Renton KW. The signal transduction pathways involved in hepatic cytochrome P450 regulation in the rat during a lipopolysaccharide-induced model of central nervous system inflammation. *Drug Metab Dispos* 2005;**33**:1521-1531.

33. Freudenberg MA, Freudenberg N, Galanos C. Time course of cellular distribution of endotoxin in liver, lungs and kidneys of rats. *Br J Exp Pathol* 1982;**63**:56-65.

34. van Oosten M, van de Bilt E, van Berkel TJC, Kuiper J. New scavenger receptor-like receptors for the binding of lipopolysaccharide to liver endothelial and Kupffer cells. *Infect Immun* 1998;**66**:5107–5112.

35. Vreugdenhil ACE, Rousseau CH, Hartung T, Greve JWM, van't Veer C, Buurman WA. Lipopolysaccharide (LPS)-binding protein mediates LPS detoxification by chylomicrons.

J Immunol 2003;**170**:1399–1405.

36. Pussinen PJ, Tuomisto K, Jousilahti P, Havulinna AS, Sundvall J, Salomaa V. Endotoxemia, immune response to periodontal pathogens, and systemic inflammation associate with incident cardiovascular disease events. *Arterioscler Thromb Vasc Biol* 2007;**27**:1433-1439.
37. Meng X, Brown JM, Ao L, *et al.* Endotoxin induces cardiac HSP70 and resistance to endotoxemic myocardial depression in rats. *Am J Physiol* 1996;**271**:C1316-C1324.
38. Zhou JJ, Pei JM, Wang GY, *et al*. Inducible HSP70 mediates delayed cardioprotection via U-50488H pretreatment in rat ventricular myocytes. *Am J Physiol Heart Circ Physiol* 2001;**281**:H40-H47.
39. Eising GP, Mao L, Schmid-Schonbein GW, Engler RL, Ross J. Effects of induced tolerance to bacterial lipopolysaccharide on myocardial infarct size in rats. *Cardiovasc Res* 1996;**31**:73-81.
40. Hoshida S, Kuzuya T, Fuji H, *et al*. Sublethal ischaemia alters myocardial antioxidant activity in canine heart. *Am J Physiol* 1993;**264**:H33-H39.
41. Kuzuya T, Hoshida S, Yamashita N, *et al*. Delayed effects of sublethal ischaemia on the acquisition of tolerance to ischaemia. *Circ Res* 1993;**72**:1293-9.
42. Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischaemic or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993;**88**:1264-1272.
43. Zingatelli B, Halushka PV, Caputi AP, Cook JA. Increased nitric oxide synthase during the development of endotoxin tolerance. *Shock* 1995;**3**:102-108.
44. Carayol N, Chen J, Yang F, *et al*. A dominant function of IKK/NF-kB signaling in global

lipopolysaccharide-induced gene expression. *J Biol Chem* 2006;**281**:31142-31151.

45. Herbertson M, Werner H, Goddard C, *et al*. Anti-tumor necrosis factor-alpha prevents decreases ventricular contractility in endotoxemic pigs. *Am J Respir Crit Care Med* 1995;**152**:480-488.

46. Haudek SB, Spencer E, Bryant DD, *et al*. Overexpression of cardiac I-kBa prevents endotoxin-induced myocardial dysfunation. *Am J Physiol Heart Circ Physiol* 2001;**280**:H962-H968.

47. Nemoto S, Vellejo JG, Kneufermann P, *et al*. *Escherichia coli* LPS-induced LV dysfunction role of Toll-like receptor-4 in the adult heart. *Am J Physiol Heart Circ Physiol* 2002;**282**:H2316-H2323.

Legends

Fig. 1. Experimental protocol. **A**. *Infarct size experiment.* Rats were treated intra-peritoneally either with normal saline, or 0.2, 0.5, 1.0, 2.0, or 4.0 mg/kg of *P. gingivalis* LPS. 24 hours later, hearts were harvested, mounted on a Langendorff apparatus, stabilized for 30 min, and subjected to 30 min regional ischemia followed by 120 min reperfusion; then the infarct size was measured. **B**. *Ca^{2+} transient experiment*. Rats were treated intra-peritoneally with 0.5 mg/kg of *P. gingivalis* LPS. 24 hours later, hearts were harvested and myocytes were isolated for Ca^{2+} transient measurement with spectrofluorometric method using fura-2 as the Ca^{2+} indicator. Myocytes were stabilized for 30 min before being subjected to 10 min of metabolic inhibition and anoxia (MI/A) with 10 mM 2-deoxy-D-glucose and 10 mM sodium dithionite in glucose-free Krebs solution, followed by 20-min reperfusion.

Fig. 2. Effects of *P. gingivalis* LPS pre-conditioning on ischemia/reperfusion-induced myocardial injury. Rats were pre-conditioned with 24-h saline (control), or 0.2, 0.5, 1.0, 2.0, or 4.0 mg/kg of intra-peritoneally administrated *P. gingivalis* LPS. Isolated perfused rat hearts were subjected to ischemia and reperfusion, and infarct size was determined at the end of reperfusion, expressed as a percentage of the area at risk. Values are mean ± SEM. Control, n = 7; 0.2 mg/kg, n = 8; 0.5 mg/kg, n = 6; 1.0 mg/kg, n = 4; 2.0 mg/kg, n = 4; 4.0 mg/kg, n = 7. ** $P < 0.01$ vs. Control; ### $P < 0.001$ vs. 0.5 mg/kg *P. gingivalis* LPS group.

Fig. 3. Tracings of the electrically induced intracellular Ca^{2+} ($E[Ca^{2+}]_i$) transients in rat ventricular myocytes. The Ca^{2+} transients from the control and test groups 24 hour after intra-peritoneal administration of 0.5 mg/kg of *P. gingivalis* LPS were compared before (stable)

and during ischemia (metabolic inhibition and anoxia; MI/A), and at the end of reperfusion. Note the reduction in amplitude in control group, and to a lesser extent in the test group, during ischemia and at the end of reperfusion. Loss of rhythm can also be observed in the control group during ischemia.

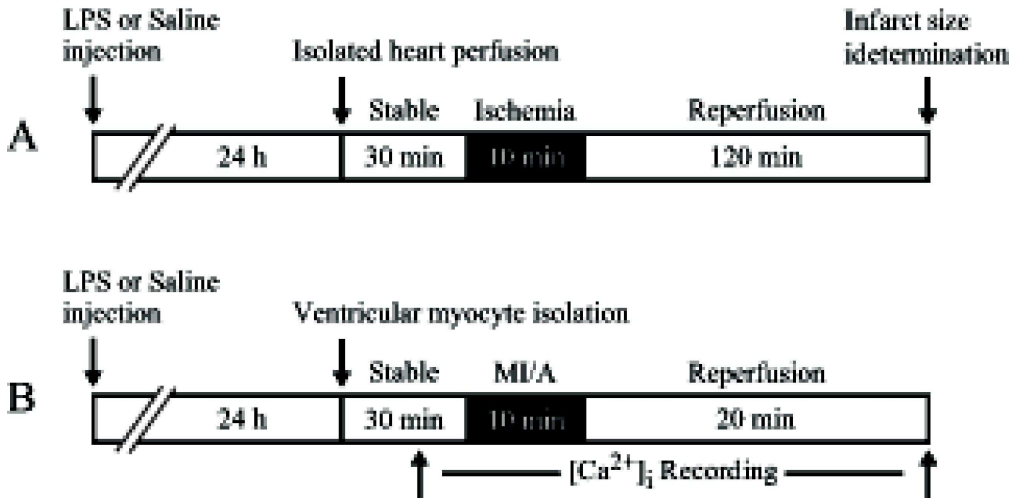
Fig. 4. Effects of 24-h pre-conditioning with 0.5 mg/kg of *P. gingivalis* LPS on resting $[Ca^{2+}]_i$ level in rat ventricular myocytes. Values are expressed as mean ± SEM, n = 6. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. stable period within the group; ◊◊ $P < 0.01$ vs. corresponding control $[Ca^{2+}]_i$ during reperfusion. (MI/A = metabolic inhibition and anoxia.)

Fig. 5. Effects of 24-h preconditioning with 0.5 mg/kg of *P. gingivalis* LPS on amplitude of $E[Ca^{2+}]_i$ of rat ventricular myocytes. Values are expressed as mean ± SEM, n = 6. *** $P < 0.001$ vs. stable period within the group; ### $P < 0.001$ vs. period of metabolic inhibition and anoxia (MI/A) within the group; ◊ $P < 0.05$, ◊◊◊ $P < 0.001$ vs. corresponding control value during the reperfusion and MI/A period, respectively.

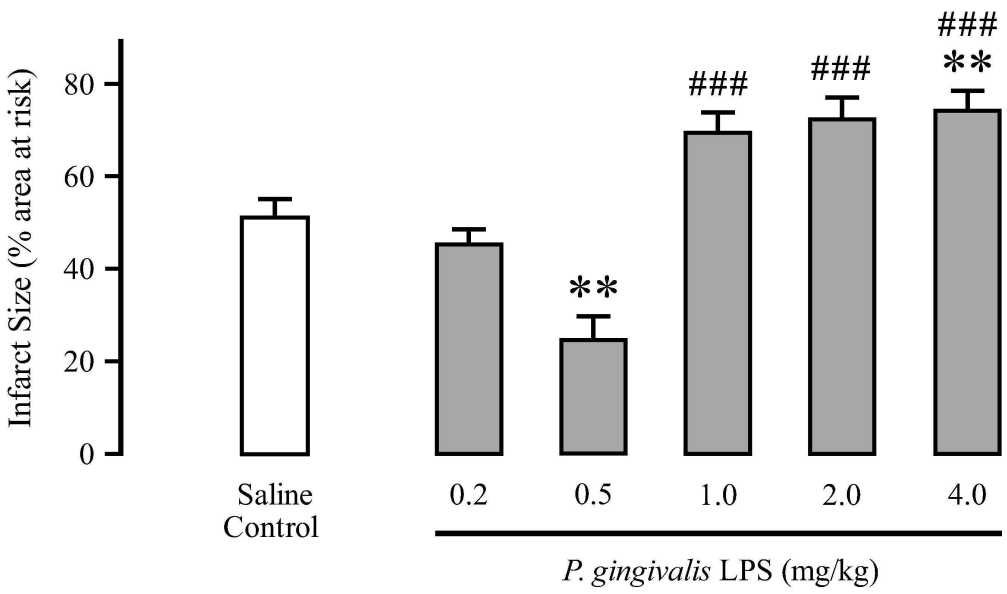
Fig. 6. Effects of 24-h preconditioning with 0.5 mg/kg of *P. gingivalis* LPS on time to peak of $E[Ca^{2+}]_i$ in rat ventricular myocytes. Values are expressed as mean ± SEM, n = 6. * $P < 0.05$ vs. stable period within the group; ◊ $P < 0.05$ vs. the corresponding control value during metabolic inhibition and anoxia (MI/A) or reperfusion period.

Fig. 7. Effects of 24-h preconditioning with 0.5 mg/kg of *P. gingivalis* LPS on T_{50} (50% decay time) of $E[Ca^{2+}]_i$ transient in rat ventricular myocytes. Values are expressed as mean ± SEM, n =

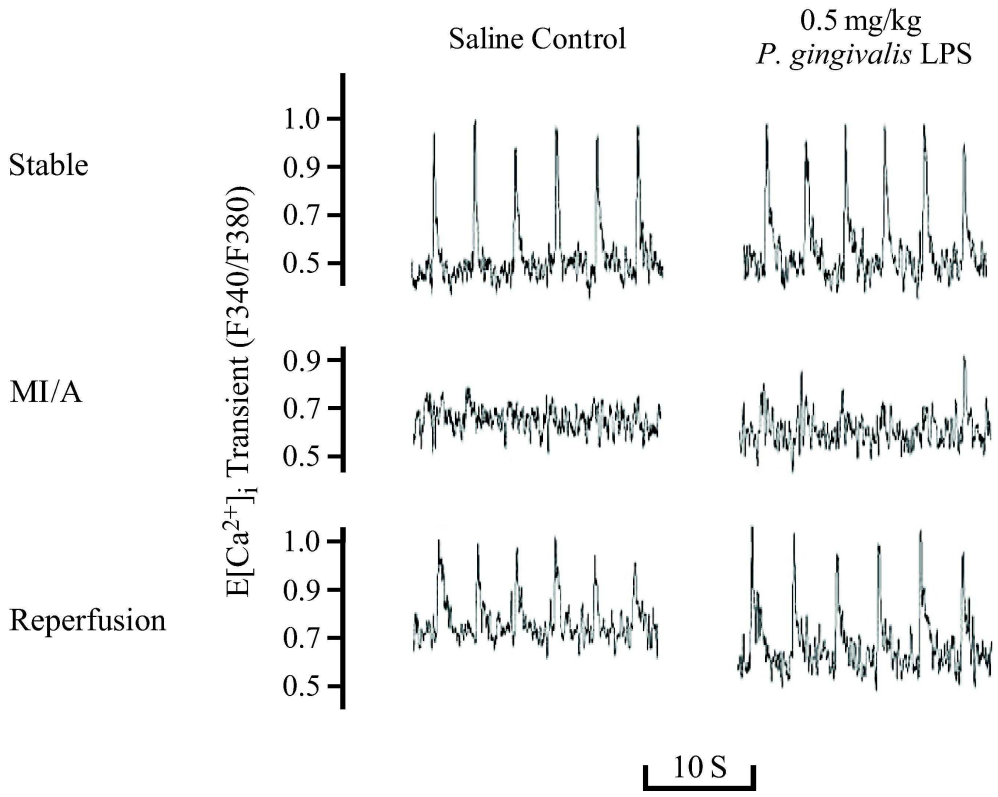
6. * $P < 0.05$, ** $P < 0.01$ vs. stable period within the group; # $P < 0.05$ vs. period of metabolic inhibition and anoxia (MI/A) within the group; ◊ $P < 0.05$ vs. the corresponding control value during the MI/A period.



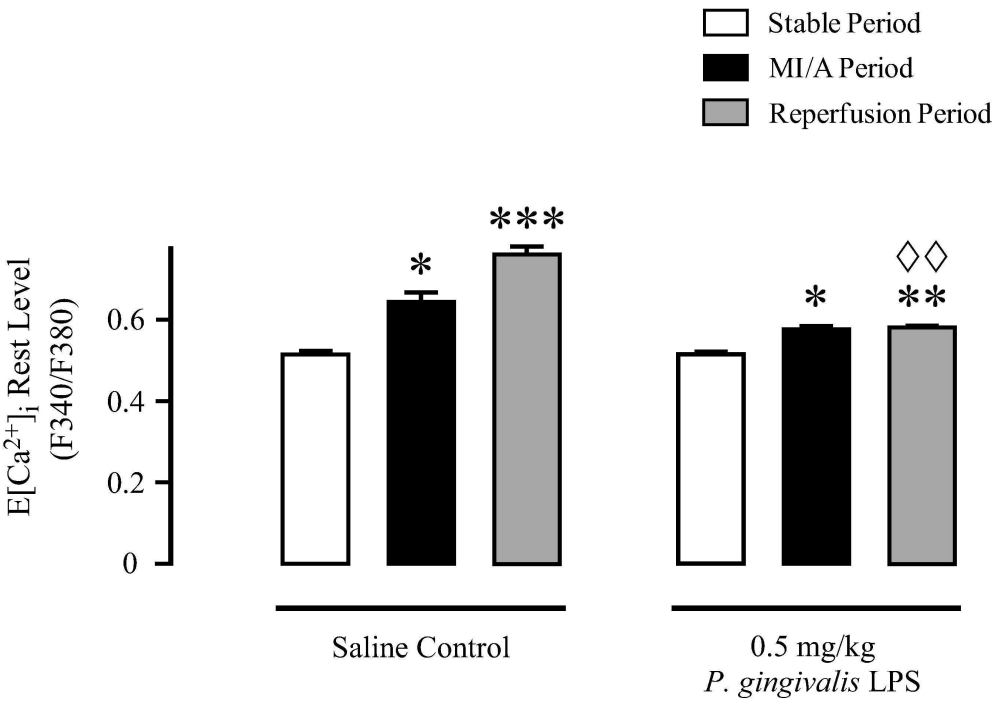
112x55mm (600 x 600 DPI)



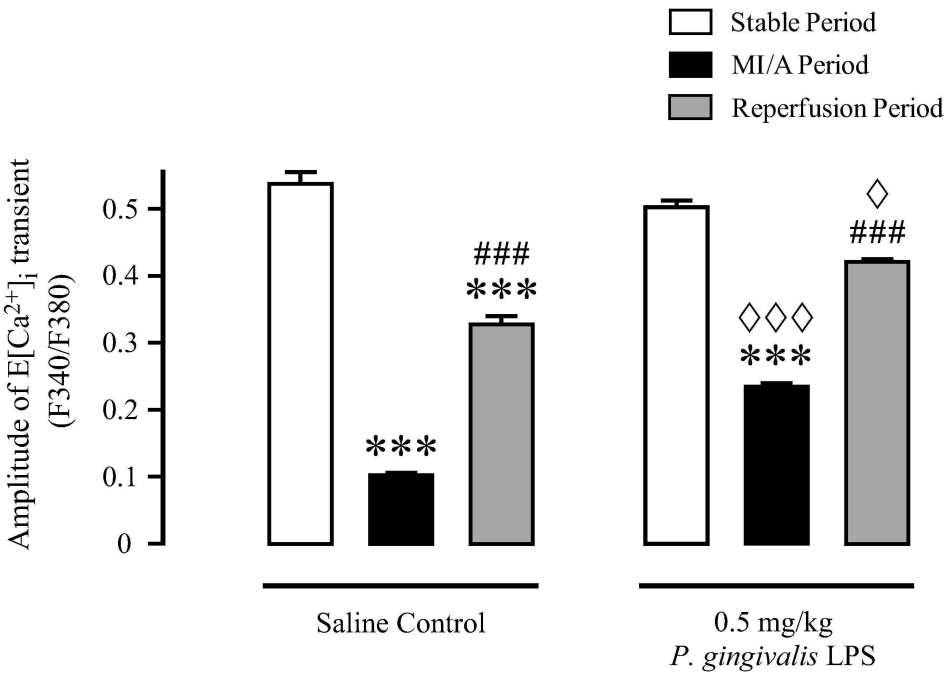
113x84mm (600 x 600 DPI)



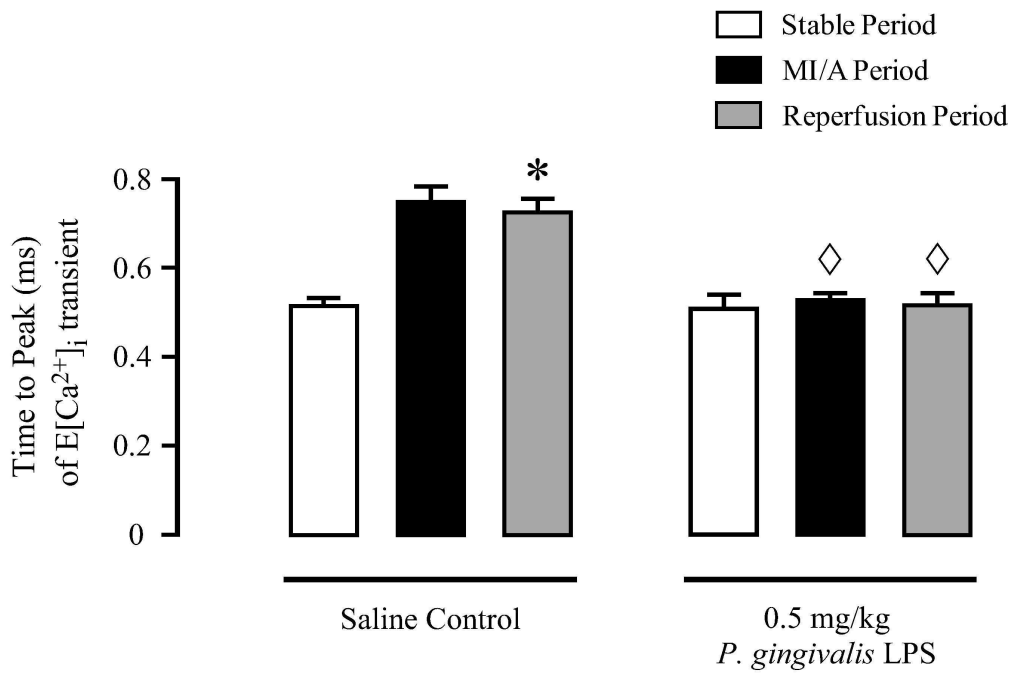
114x90mm (600 x 600 DPI)



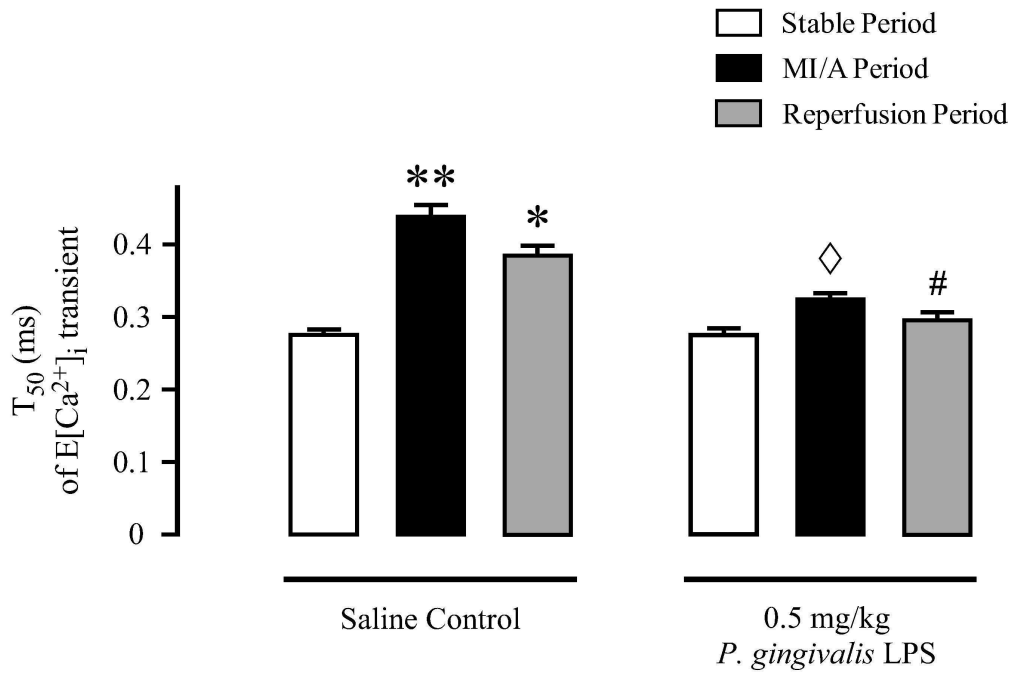
114x79mm (600 x 600 DPI)



120x93mm (600 x 600 DPI)



113x75mm (600 x 600 DPI)



113x75mm (600 x 600 DPI)