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

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Background and Objective: Porphyromonas gingivalis lipopolysaccharide (LPS) is a ligand for cell surface toll-like receptors (TLR), TLR2 and TLR4 while stimulation of either leads to cardioprotection. We hypothesized that: (1) pretreatment with *P. gingivalis* LPS at appropriate concentrations would induce cardioprotection against injury induced by ischemia and reperfusion; and (2) *P. gingivalis* LPS pretreatment at cardioprotective concentrations may reduce Ca^{2+} overload, which is a precipitating cause of injury, and improve recovery of contractile function.

Material and Methods: Male Sprague–Dawley rats were randomly selected to receive intraperitoneal 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 the 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 pretreatment with a cardioprotective dose of *P. gingivalis* LPS.

Results: Pretreatment with 0.5 mg/kg *P. gingivalis* LPS significantly reduced, while pretreatment with 1.0–4.0 mg/kg significantly increased infarct size. The Ca^{2+} overload induced by ischemia–reperfusion was attenuated in myocytes from rats pretreated with 0.5 mg/kg *P. gingivalis* LPS. Pretreated myocytes also showed

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an increased amplitude of the $E[Ca^{2+}]_i$ transient, no prolongation of the time to reach the peak $E[Ca^{2+}]_i$ transient and shorter 50% decay time during reperfusion.

Conclusion: At a dosage of 0.5 mg/kg, *P. gingivalis* LPS confers cardioprotection 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 such as *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, leading to 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 ischemia–reperfusion injury (11). We hypothesized that pretreatment with *P. gingivalis* LPS at appropriate concentrations would induce cardioprotection against injury induced by ischemia and reperfusion.

Calcium homeostasis is essential to contractile function of the heart and protection against cardiac injury. When an action potential arrives, the sarcolemmal membrane of the cardiac myocyte is depolarized. Depolarization of the sarcolemmal membrane opens voltage-gated Ca^{2+} channels, thus allowing the entry of Ca^{2+} into the myocyte. The Ca^{2+} triggers a massive amount of Ca^{2+} release, via ryanodine receptors, from the sarcoplasmic reticulum, which is 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 into 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 were to have a cardioprotective effect, it might affect myocyte Ca^{2+} homeostasis, which might alter cytosolic Ca^{2+} homeostasis, hence the contractile function.

In the present study, we first attempted to test the hypothesis that pretreatment with a sublethal dose of *P. gingivalis* LPS 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 pretreatment at cardioprotective concentrations may alter myocyte Ca^{2+} homeostasis, particularly reducing Ca^{2+} overload, hence improving the contractile recovery of myocytes 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 experiments, on cytosolic Ca^{2+} level and the electrically induced intracellular Ca^{2+} ($E[Ca^{2+}]_i$) transient in single ventricular myocytes according to a procedure routinely used in our laboratory.

Material 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 size of infarcts induced by ischemia–reperfusion in isolated rat hearts were determined. Then the effects were investigated of the endotoxin preparation, at concentration shown in the first series of experiments to reduce the infarct size induced by ischemia–reperfusion, on isolated rat myocyte cytosolic Ca^{2+} levels and the $E[Ca^{2+}]_i$ transient. 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+} channels, 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 $[Ca^{2+}]_i$. Therefore, contractility is determined by the amount, amplitude and rate (time to peak) of the Ca^{2+} released. The 50% decay reflects the removal of Ca^{2+} from the cytosol and determines relaxation. A previous study from our laboratory showed that the amplitude of the transient is directly correlated with 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. *Porphyromonas 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 (o.d. 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 prewarmed 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 1500*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 dialysed against double-distilled water at 4°C for 3–5 days. The dialysed 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 on average 61.7 μg of LPS or every *P. gingivalis* bacterium yield on average 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. The DNA content of the extracted LPS was analysed 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 pretreating 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 preconditioning cardioprotective 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 myocytes (Fig. 1B).

P. gingivalis LPS pretreatment and rat heart ischemia–reperfusion Thirty-six male Sprague–Dawley rats, each weighing 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 and 7, respectively). These doses of *P. gingivalis* LPS were chosen according to earlier publications regarding cardioprotective effects of *E. 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 intraperitoneal 60 mg/kg sodium pentobarbitone and then injected intravenously with 200 IU of heparin. 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 $NaHCO_3$ and 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 cmH_2O) and equilibrated with 95% O_2 and 5% CO_2. The hearts were allowed to

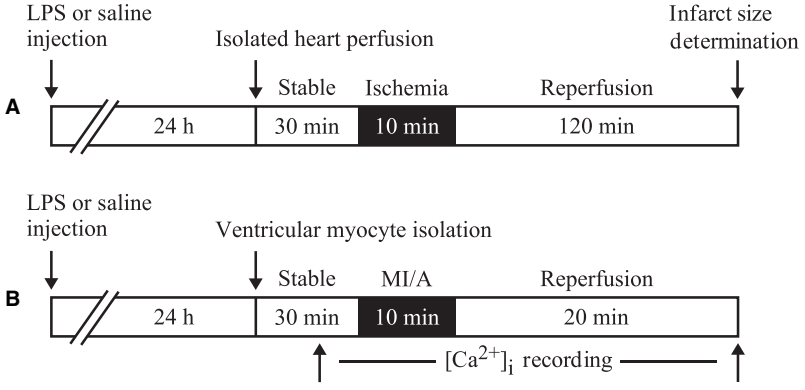

Fig. 1. Experimental protocol. (A) Infarct size experiment. Rats were treated intraperitoneally either with normal saline or with 0.2, 0.5, 1.0, 2.0 or 4.0 mg/kg of *P. gingivalis* LPS. Twenty-four hours later, hearts were harvested, mounted on a Langendorff apparatus, stabilized for 30 min and then subjected to 30 min regional ischemia followed by 120 min reperfusion; then the infarct size was measured. (B) Calcium transient experiment. Rats were treated intraperitoneally with 0.5 mg/kg of *P. gingivalis* LPS. Twenty-four hours later, hearts were harvested and myocytes were isolated for Ca^{2+} transient measurement with a 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.

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 3–0 (2.0 metric) 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 retightened, and approximately 0.2 mL of 2.5% Evans Blue dye (Sigma Chemical Co., 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 3–4 mm and then incubated with sodium phosphate buffer containing 1% 2,3,5-triphenyl-tetrazolium chloride (TTC; Sigma Chemical Co.) 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 ImageJ from the National Institutes of Health (http://rsbweb.nih.gov/ij/). The AAR as a percentage of LV (AAR%) and the IS as a percentage of AAR (IS%) were assessed.

Electrically induced intracellular Ca^{2+} *(*$E[Ca^{2+}]_i$*) transient* In the second series of experiments, 12 male Sprague–Dawley rats, each weighing about 250 g, were randomly divided into control and test groups of 6 rats each. The test group underwent LPS preconditioning by receiving an intraperitoneal 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 because it was shown to confer a cardioprotective 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 an intraperitoneal dose of 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 solution (5 mM glucose, 143 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.3 mM NaH_2PO_4 and 5 mM Hepes), which passed through a water bath at 37°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 solution and then homogenized by pipetting. The cell suspension was filtered through a mesh screen, and the cells were suspended in 20 mL Tyrode 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.

The $E[Ca^{2+}]_i$ 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 $NaHCO_3$ and 1.2 mM KH_2PO_4). The suspension was centrifuged at 1000 *g* for 3 min at 25°C, 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, Jena, Germany), which was connected to a dual-wavelength excitation spectrofluorometer (Photon Technology International, Princeton, NJ, USA). The fluorescence intensities at 340 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 s (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 spontaneously beating with a regular, clear rhythm of amplitude. We subjected the cells to 600 s 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 s of reperfusion with Krebs solution. The ratio of the change in fluorescence intensities at the two wavelengths was 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 are expressed as means ± SEM. Statistical comparisons of the infarct size were performed by one-way analysis of variance followed by Tukey's multiple comparison tests. Student's unpaired *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 < 0.05 indicated a statistically significant difference.

Results

Effects of *P. gingivalis* LPS preconditioning on myocardial injury

Myocardial infarction was induced in all the rat hearts in which the coronary artery had been occluded. Pretreatment 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, preconditioning with *P. gingivalis* LPS at higher doses increased the infarct size significantly (Fig. 2).

Effects of *P. gingivalis* LPS preconditioning on rat ventricular myocytes

Since 24 h preconditioning with 0.5 mg/kg *P. gingivalis* LPS conferred cardioprotection against injury induced by ischemia and reperfusion, we determined the effects of this preconditioning protocol on the resting $[Ca^{2+}]_i$ and its transient, which reflects Ca^{2+} handling and contractile functions (Fig. 3). In brief, the control myocytes showed a greater reduction in amplitude in $E[Ca^{2+}]_i$ transient than myocytes from rats treated with 0.5 mg/kg *P. gingivalis* LPS, both during simulated ischemia and at the end of reperfusion. In myocytes of the control animals, the resting $[Ca^{2+}]_i$ was significantly elevated during simulated ischemia and further elevated during reperfusion (Fig. 4). In myocytes of the LPS-treated group, the resting $[Ca^{2+}]_i$ was significantly elevated during the simulated ischemia but remained at the same level during reperfusion; the levels during both simulated ischemia and reperfusion were lower than those in the control cells, but only the difference during reperfusion was statistically significant.

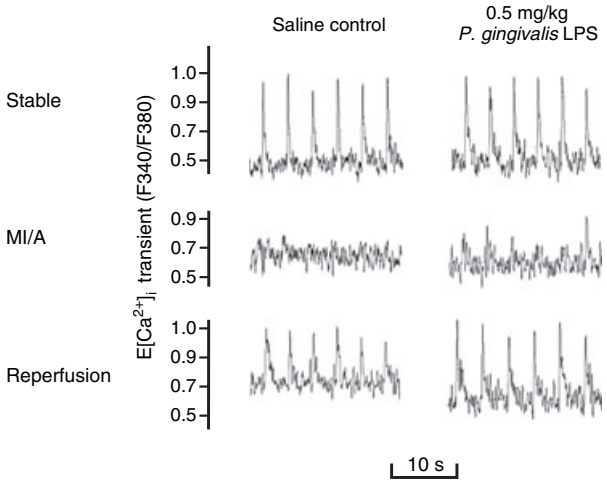

Fig. 3. Traces of the electrically induced intracellular Ca^{2+} ($E[Ca^{2+}]_i$) transients in rat ventricular myocytes. The Ca^{2+} transients from the control and test groups 24 h after intraperitoneal administration of 0.5 mg/kg of *P. gingivalis* LPS were compared before (stable), during simulated ischemia (metabolic inhibition and anoxia; MI/A) and at the end of reperfusion. Note the reduction in amplitude in the control group, and to a lesser extent in the test group, during simulated ischemia and at the end of reperfusion. Loss of rhythm can also be observed in the control group during simulated ischemia.

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 simulated ischemia and partly recovered 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 simulated ischemic period was significantly less than in the control cells, whereas the recovery in amplitude during reperfusion was significantly greater than in the control myocytes.

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 LPS-treated group.

The t_{50} of the $E[Ca^{2+}]_i$ transient, which reflects the speed of removal of

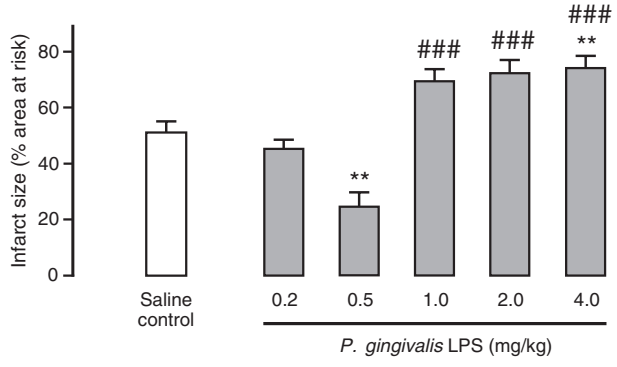

Fig. 2. Effects of 24 h preconditioning with *P. gingivalis* LPS on ischemia–reperfusion-induced myocardial injury. Rats were preconditioned with saline (control) or with 0.2, 0.5, 1.0, 2.0 or 4.0 mg/kg of intraperitoneally administrated *P. gingivalis* LPS. Isolated perfused rat hearts were subjected 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 means + 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, $n = 4$; and 4.0 mg/kg, $n = 7$. $^{**}p < 0.01$ vs. control group; and $\#\#\#p < 0.001$ vs. 0.5 mg/kg *P. gingivalis* LPS group.

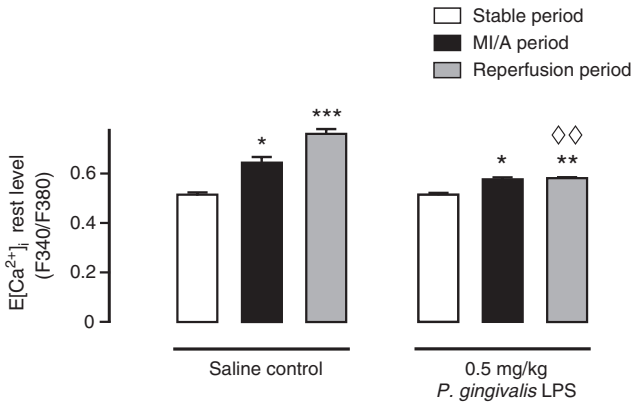

Fig. 4. Effects of 24 h preconditioning with 0.5 mg/kg of *P. gingivalis* LPS on resting $[Ca^{2+}]_i$ level in rat ventricular myocytes. Values are expressed as means + SEM, $n = 6$ per group. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs. stable period within the group; and $\diamond\diamond p <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

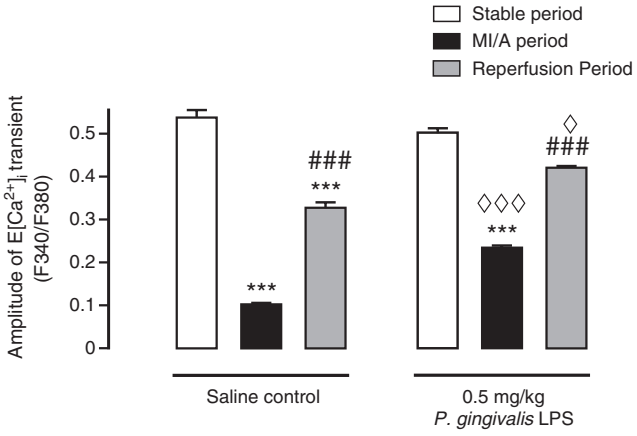

Fig. 5. Effects of 24 h preconditioning with 0.5 mg/kg of *P. gingivalis* LPS on amplitude of $E[Ca^{2+}]_i$ of rat ventricular myocytes. Values are expressed as means + SEM, $n = 6$ per group. $***p < 0.001$ vs. stable period within the group; $\#\#\#p < 0.001$ vs. period of metabolic inhibition and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

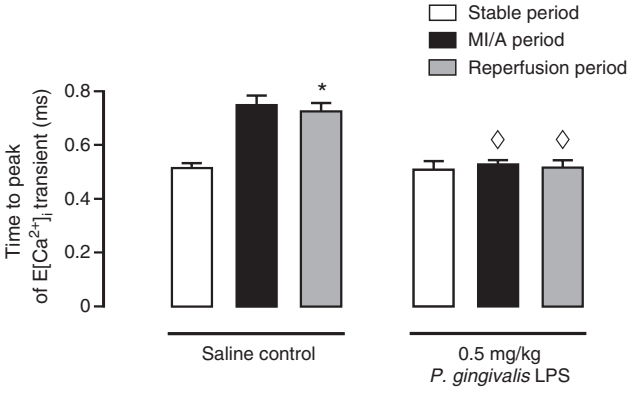

Fig. 6. Effects of 24 h preconditioning with 0.5 mg/kg of *P. gingivalis* LPS on time to peak of $E[Ca^{2+}]_i$ in rat ventricular myocytes. Values are expressed as means + SEM, $n = 6$ per group. $*p < 0.05$ vs. stable period within the group; and $\diamond p < 0.05$ vs. the corresponding control value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

Ca^{2+} from the cytosol, increased significantly during the simulated ischemia and partly recovered during reperfusion in the control group (Figs 3 and 7). The corresponding value in the LPS-treated group during simulated 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 pretreatment with *P. gingivalis* LPS produced biphasic effects on ischemia–reperfusion-induced injury in the rat heart. At a dose of 0.5 mg/kg administered intraperitoneally, *P. gingivalis* LPS is beneficial against ischemia–reperfusion-induced injury. At a higher dose range, however, it exacerbates the injury induced by ischemia–reperfusion, which has been observed with other gram-negative bacterial endotoxins, such as *E. coli* LPS (19,24). The second novel finding is that at the dose of 0.5 mg/kg, which was shown to confer cardioprotection, *P. gingivalis* LPS attenuates the cytosoclic Ca^{2+} overload, in agreement with its cardioprotective action and, more importantly, it improves the recovery of the $E[Ca^{2+}]_i$ transient, which reflects contractile function. Similar to the present study, other reports have shown that 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 from *P. gingivalis* and *E. coli* support the notion that the endotoxins may prevent damage caused by ischemia–reperfusion (26).

We found, for the first time, that preconditioning 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, LPS at a dose that confers cardioprotection also improves contractile recovery after ischemic insult.

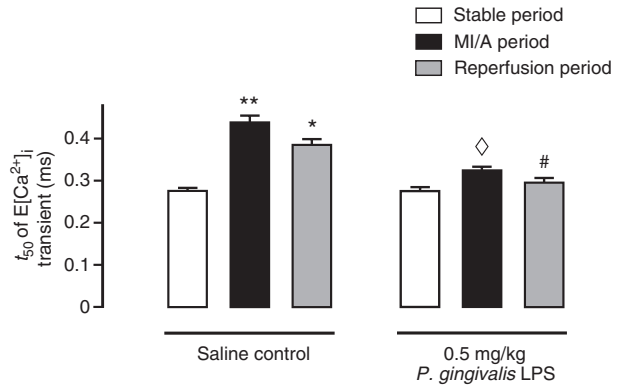

Fig. 7. Effects of 24 h preconditioning with 0.5 mg/kg of *P. gingivalis* LPS on t_{50} (50% decay time) of E[Ca^{2+}]$_i$ transient in rat ventricular myocytes. Values are expressed as means + SEM, $n = 6$ per group. *$p < 0.05$, **$p < 0.01$ vs. stable period within the group; $p < 0.05$ vs. period of metabolic inhibition and anoxia (MI/A) within the group; ◇$p < 0.05$ vs. the corresponding control value during the MI/A period.

E. coli and *P. gingivalis* represent two different families of the naturally occurring bacteria in the gastrointestinal tract. It is well documented that 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 a 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 cardioprotection may be explained partly by their possible different mechanisms of action.

Cardioprotection induced by bacterial endotoxins has been linked to the activation of TLR transmembrane receptors (11). The receptors TLR2, TLR3, TLR4 and TLR6 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 a ligand for TLR2 and TLR4 (9), whereas the pure form of the LPS from *E. coli* is a 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 ischemia–reperfusion injury (11). Toll-like receptor 2 is believed to be cardioprotective against oxidative stress in neonatal rat cardiac myocytes (28). The relative contributions of TLR2 and TLR4 in the host response to challenge with *P. gingivalis* LPS are yet to be characterized.

P. gingivalis LPS pretreatment was administered intraperitoneally to test animals, which is an established and convenient method of administration. Abdulla *et al.* (32) previously reported on the bioavailability of *E. coli* LPS after intraperitoneal administration. In brief, the rat serum endotoxin concentration peaked at 6 h to a level of 10 ng/mL after intraperitoneal 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 10–50 ng/mL of *P. gingivalis* LPS might be available to the rat systemic circulation approximately 6 h after intraperitoneal administration of 0.5 mg/kg LPS. 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 warranted, however, to quantify the postulated LPS bioavailability. The postulated systemic circulatory exposure to LPS from periodontopathogens would nevertheless be highly probable in humans 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 in two *in vitro* models, extrapolation of the deleterious effects of *P. gingivalis* LPS in human periodontitis, towards systemic or cardiac health should be made with caution. Furthermore, we used phenol–water-extracted *P. gingivalis* LPS, so further research is needed to study the effects of column-purified *P. gingivalis* LPS on heart tissue, as well as the possible mechanisms of host–LPS interactions.

The delayed nature of protection suggests that cardioprotective proteins may be synthesized *de novo*. Three classes of proteins are known to be end-effectors responsible for delayed cardioprotection after preconditioning: 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 cardioprotective superoxide dismutase in human monocytic cell lines (44). Further study on the contribution of this protein to the cardioprotective action of *P. gingivalis* LPS is warranted.

Paradoxically, when the dose of *P. gingivalis* LPS was increased to between 1 and 4 mg/kg, myocardial injury was exacerbated and the infarct size significantly increased. Similarly, high-dose *E. coli* LPS, in contrast to a low dose, can induce TLR4-mediaated nuclear factor-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 intraperitoneally has biphasic effects on cardiac injury induced by ischemia–reperfusion in the rat heart 24 h after its administration. A sublethal intraperitoneal 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 cardioprotective 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 bacteria–host cardiac interactions.

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