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Porphyromonas gingivalis and its lipopolysaccharide differentially regulate the expression of cathepsin B in endothelial cells

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SUMMARY

***Porphyromonas gingivalis* infection and cathepsins protease upregulation are independently implicated in atherosclerosis worsening. In this study, we evaluated the effects of *P. gingivalis* infection and *P. gingivalis* -purified lipopolysaccharide (*Pg*-LPS) stimulation on the expression of cathepsin B (CATB) in endothelial cells (ECs). Analysis of the enzymatic activity and expression of CATB were investigated at the messenger RNA, protein and protein-phosphorylation levels. Effects of Toll-like receptors 2 and 4 blocking on CATB activity were also analysed. Our results showed that *P. gingivalis* and *Pg*-LPS significantly increased the activity of CATB but with different kinetics. The peak of CATB activity was observed 3 h after *P. gingivalis* infection but it appeared 48 h after *Pg*-LPS stimulation. The increase of CATB activity was related to its rapid tyrosine-dephosphorylation during *P. gingivalis* infection, whereas the levels of CATB messenger RNAs and proteins did not vary after *P. gingivalis* infection or *Pg*-LPS stimulation. Inhibition of Toll-like-receptors 2 and 4 differentially decreased *P. gingivalis* and *Pg*-LPS CATB activations. These results showed for the first time that *P. gingivalis* infection rapidly affects ECs and modulates CATB activity, whereas *Pg*-LPS effects appear to be delayed. This study suggests that direct infection of ECs by *P. gingivalis* may worsen atherosclerotic plaque formation via activation of the CATB pathway.**

INTRODUCTION

For many years, epidemiological studies have shown that periodontitis is an important risk factor for cardiovascular disease, especially atherosclerosis (Kebschull *et al.*, 2010). Atherosclerosis is a pathological thickening of the arterial wall in response to various forms of injuries (Packard & Libby, 2008). Many cell types are implicated in this process, such as smooth muscle cells, endothelial cells (ECs), lymphocytes and macrophages (Packard & Libby, 2008; Sima *et al.*, 2009). Regarding their location between blood and tissues, ECs play several roles including the transport of plasma molecules, lipid homeostasis and immune recognition (Sima *et al.*, 2009). In response to injury or aggression, these cells can either produce cytokines (Kocgozlu *et al.*, 2009) or enter apoptosis (Sima *et al.*, 2009). Apoptosis of ECs appears to play an important role in the atherosclerotic plaque rupture and the thrombotic complications of atherosclerosis (Sima *et al.*, 2009).

Atherosclerotic plaque infection by various pathogens, such as *Chlamydia pneumoniae* (Al-Ghamdi

et al., 2011) and *Porphyromonas gingivalis* (Lalla *et al.*, 2003; Amar *et al.*, 2009), is one of the main pathways of atherosclerosis worsening. *Porphyromonas gingivalis* is an anaerobic gram-negative bacterium classically associated with severe inflammation and destruction of periodontal tissues (Kebschull & Papapanou, 2011). It can also be detected in the bloodstream of patients suffering from severe periodontitis and atherosclerosis, and in atherosclerotic plaques (Elkaim *et al.*, 2008a; Marcelino *et al.*, 2010). *In vivo* studies on apolipoprotein E-deficient mutant mice show that oral infection or injection of *P. gingivalis* worsened atherosclerotic plaque formation (Lalla *et al.*, 2003; Amar *et al.*, 2009; Pereira *et al.*, 2011). Various theories have been proposed to explain this link. Plaque formation may be an indirect effect of *P. gingivalis* periodontal tissue infection by increasing circulating proinflammatory cytokines, by immune mimicking or through a direct effect by vascular wall infection (Wada & Kamisaki, 2010). However, most of the pathogenic mechanisms involving *P. gingivalis* in the initiation and development of atherosclerosis remain unknown (Wada & Kamisaki, 2010).

Porphyromonas gingivalis is considered one of the most virulent perio-pathogens (Kebschull & Papapanou, 2011). Classically, *P. gingivalis* exacerbates proinflammatory cytokines (Kocgozlu *et al.*, 2009; Taylor, 2010) and extracellular matrix protease production (Potempa *et al.*, 2000). This pathogen is also able to invade gingival epithelial cells (Tribble & Lamont, 2010) and avoid the humoral immune response using cell-to-cell dissemination (Yilmaz *et al.*, 2006). Furthermore, *P. gingivalis* has been shown to control cell apoptosis in various cell types, such as gingival epithelial cells and macrophages (Yilmaz, 2008; Stathopoulou *et al.*, 2009). Action of *P. gingivalis* is mediated through many virulence factors, such as gingipains, hemagglutinins, fimbriae and lipopolysaccharides (*Pg*-LPS) (Hajishengallis, 2009). The *Pg*-LPS is considered the major virulence factor of *P. gingivalis* (Jain & Darveau, 2010) and elicits specific cell responses in gingival epithelial cells (Kocgozlu *et al.*, 2009), fibroblasts (Wang & Ohura, 2002), and ECs, notably through the activation of Toll-like receptors (TLR) (Kocgozlu *et al.*, 2009).

The TLRs are members of a large family of innate immune recognition receptors that detect conserved microbial patterns and endogenous ligands and play a key role in innate immune responses (Hayashi *et al.*, 2010). Recognition of bacteria's virulence factor by TLRs activates nuclear factor-κB and the production of proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1 (IL-1), IL-6 and IL-12 (Hans & Hans, 2011). TLR-mediated inflammatory pathways are implicated both in periodontal diseases and in atherosclerosis (Hayashi *et al.*, 2010; Hans & Hans, 2011). In atherosclerosis, ECs respond via TLR2 and TLR4 receptors to several pathogenic species such as LPS from *P. gingivalis* and *Escherichia coli* (Erridge *et al.*, 2008). Furthermore, previous study showed LPS-induced cell death through a TLR4-mediated pathway leading to activation of cathepsin B (CATB) in ECs (Li *et al.*, 2009).

Cathepsins are lysosomal cysteine proteases expressed in various tissues and cells (Lutgens *et al.*, 2007). They are involved in tissue remodeling, turnover of the extracellular matrix, and apoptosis, as well as in antigen and pro-protein processing (Reiser *et al.*, 2010). CATB is overexpressed in atherosclerotic plaques and implicated in the progression of atherosclerosis (Lutgens *et al.*, 2007; Lafarge *et al.*, 2010). CATB regulates the production of proinflammatory cytokines like IL-1β, and it also regulates cell apoptosis via inflammasome activation, especially in ECs (Duewell *et al.*, 2010; Niemi *et al.*, 2011). CATB activity is regulated by factors such as *P. gingivalis* in gingival epithelial cells (Elkaim *et al.*, 2008b), *C. pneumoniae* in macrophages (He *et al.*, 2010) and *E. coli* LPS in ECs (Li *et al.*, 2009). The cellular mechanisms of cathepsin regulation occur at both the transcriptional and post-translational levels (Reiser *et al.*, 2010) and with the expression of the cathepsin inhibitor Cystatin C (Elkaim *et al.*, 2008b).

The aim of this study was to investigate the cellular and molecular mechanisms involved in direct/indirect *P. gingivalis* action on atherosclerotic plaque formation. More specifically, we aimed to investigate the effects of *P. gingivalis* infection and *Pg*-LPS stimulation on CATB activity in EC cultures and their mechanisms of regulation.

METHODS

Bacterial strains

The *P. gingivalis* strain 381 was a gift from Dr. Christine Roques (Montpellier, France) and was cultivated under strict anaerobic conditions at 37°C

in a brain–heart infusion medium (Sigma, St. Louis, MO, USA) supplemented with hemin (5 μg ml^{-1}) (Sigma) and menadione (1 μg ml^{-1}) (Sigma). On the day of infection, bacteria were centrifuged, washed twice with phosphate-buffered saline (PBS) and counted as previously described (Elkaim *et al.*, 2008b).

The *Pg*-LPS used was a commercial ultrapure preparation (InvivoGen, San Diego, CA, USA) extracted from *P. gingivalis* strain ATCC 33277 using successive enzymatic hydrolysis steps and purified using an extraction protocol that removed contaminating lipopeptides.

Culture of human umbilical vein ECs

Human umbilical vein ECs (HUVECs; PromoCell, Heildelberg, Germany) were cultured in EGM2 medium (PromoCell) containing antibiotics (100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) and an EC growth supplementation mix (PromoCell) at 37°C in a humidified atmosphere with 5% CO_2.

Infection of HUVECs with *P. gingivalis*

At 24 h before the infection, 2×10^5 cells were plated in each well of a 24-well plate. On the day of infection, cells were washed twice with PBS and infected with *P. gingivalis* at the indicated multiplicity of infection (MOI) per cell in cell growth medium as described previously (Elkaim *et al.*, 2008b). Cells were infected for 1 to 8 h and cytosolic extracts were prepared from samples collected at each point of the time–course infection. Cells incubated without *P. gingivalis* served as controls. When indicated, 2 h before the start of the infection, cells were preincubated with 1 μg ml^{-1} of the neutralizing antibodies (anti-human TLR4-IgA2 or anti-human TLR2-IgA2) or with 1 μg ml^{-1} of the human-IgA2 isotype as a control antibody (InvivoGen, San Diego, CA, USA). The same concentrations of antibodies were kept in the medium during all the time–course infection of HUVECs.

Stimulation of HUVECs by *Pg*-LPS

At 24 h before activation, HUVECs were seeded at a concentration of 2×10^5 cells per well of a 24-well plate. On the day of activation, cells were washed twice with PBS and cell growth medium was added, containing purified *Pg*-LPS at final concentrations ranging from 10 ng ml^{-1} to 1 μg ml^{-1}. After 24, 48 and 72 h, cytosolic extracts were harvested as previously described (Elkaim *et al.*, 2008b) to measure the enzymatic activity of CATB. Cells incubated without *Pg*-LPS served as controls. A commercial preparation of *E. coli*-LPS (strain O127:B8) (Sigma) at final concentrations ranging from 10 ng ml^{-1} to 1 μg ml^{-1} was also used to determine the specificity of the effects observed with *Pg*-LPS. For the use of neutralizing antibodies, cells were pretreated for 2 h before the beginning of the stimulation in the same conditions as described above for *P. gingivalis* infection.

Reverse transcription quantitative polymerase chain reaction

Total RNA was extracted using the RNeasy® kit (Qiagen, Les Ulis, France) according to the manufacturer's instructions. Reverse transcription was performed on 100 ng of the purified RNA using the iScript cDNA Synthesis kit as described by the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). Real-time polymerase chain reaction (PCR) was performed using the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics, Meylan, France) in a 20-μl reaction mixture containing 2 μl template cDNA and according to the manufacturer's instructions. Validated PCR primer pairs for the human genes CATB, cystatin C, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were purchased from Qiagen (Quantitect Primer Assay, Qiagen) and were used at the final concentration recommended by the manufacturer. Amplification was performed under the following conditions: activation of hot-start *Taq* polymerase at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 10 s, and elongation at 72°C for 15 s. The specificity of the reaction was controlled using melting curve analysis. The housekeeping genes GAPDH and β-actin were amplified as controls, and the specificity of the amplification products was assessed using melting curve analysis. Samples were processed in triplicate according to the manufacturer's guidelines. Relative gene expression was calculated using the comparative threshold (Ct) method ($2^{-\Delta\Delta Ct}$).

Evaluation of the enzymatic activity of CATB

Cells were harvested with ice-cold lysis buffer (65 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.5% NP-40), were immediately collected and centrifuged at 10,000 ***g*** for 10 min at 4°C, and the supernatants (cytosolic extracts) were immediately frozen. The enzymatic activity of CATB was determined using the specific fluorogenic substrate Z-Arg-Arg-AMC (Bachem, Voisins le Bretonneux, France) in previously described conditions (Ellis *et al.*, 2005). The specific activity of CATB was expressed as μmol AMC produced in 1 h by 1 μg protein from the cytosolic extract. The stimulation of the enzymatic activity of CATB was expressed as a ratio obtained by dividing the specific activity measured in the tested cells by the specific activity measured in the control cells. Concerning experiments involving TLR2 and TLR4 in the CATB enzymatic activity, the results were expressed relative to the one obtained with the control antibody human-IgA2 isotype.

SDS–PAGE and immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), immunoblotting and band quantification were performed as previously described (Elkaim *et al.*, 2006). Cells collected from the stimulation of HUVECs by *Pg*-LPS or infection with *P. gingivalis* were lysed for 5 min on ice in 200 μl ice-cold RIPA buffer (65 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.5% sodium deoxycholate) supplemented with phosphatase inhibitor cocktails I and II and protease inhibitor cocktail (Sigma), which causes a selective permeabilization of the plasma membrane without perturbing lysosomes. Lysates were centrifuged at 10,000 ***g*** at 4°C for 10 min and the supernatants (cytosolic extracts) were quantified to perform either SDS–PAGE followed by immunoblotting or immunoprecipitation. The antibodies against human CATB (sc-6493), cystatin C (sc-16989) and GAPDH (sc-31915) and the secondary antibodies conjugated to horseradish peroxidase were all purchased from Tebu-Bio (Le Perray en Yvelines, France). The antibody against phosphotyrosine (clone PY20) was obtained from Interchim (Montluçon, France). All antibodies were used at the dilutions recommended by the manufacturer. The protein concentrations of the cytosolic extracts were quantified using the Bio-Rad DC assay (Bio-Rad Laboratories).

Immunoprecipitation and evaluation of CATB phosphorylation

A total of 10 μg protein from the cytosolic extracts obtained using RIPA buffer was immunoprecipitated overnight at 4°C in 1 ml ice-cold Tris-Buffered Saline (TBS) buffer supplemented with phosphatase inhibitor cocktails I and II, protease inhibitor cocktail and the anti-human CATB diluted at 1 : 200 or the anti-phosphotyrosine antibody PY20 at 1 μg ml^{-1} (Sasajima *et al.*, 2006). A final concentration of 0.1 mg ml^{-1} of protein G agarose was added, and the tubes were agitated for 2 h at room temperature. Tubes were centrifuged for 1 min at 500 ***g***, and the bead pellets were washed three times with 1 ml TBS containing 0.05% Tween-20. Bound proteins were eluted with 30 μl of the two-fold concentrated loading buffer used for SDS–PAGE, denatured for 5 min by boiling and analysed using SDS–PAGE and immunoblotting. Western blotting and detection with anti-phosphotyrosine PY20 or anti-human CATB were performed as previously described above.

Statistical analysis

Statistical analyses were performed using the non-parametric Mann–Whitney rank sum test. Differences between two numbers were considered significant when the confidence interval exceeded 95% ($P < 0.05$). The reported data are the means of at least three separate experiments performed under similar conditions.

RESULTS

Infection with *P. gingivalis* and stimulation by *Pg*-LPS activate the enzymatic activities of CATB with different kinetics

After infection of ECs for 8 h with the whole bacterium *P. gingivalis* ranging from 2 MOI to 200 MOI, we observed an increase in the enzymatic activity of CATB which was dependent on the MOI used (Fig. 1 A). Significant increases of the enzymatic activity of CATB were obtained at 20 MOI (2.2-fold) and 200 MOI (2.7-fold) whereas a weak increase observed at

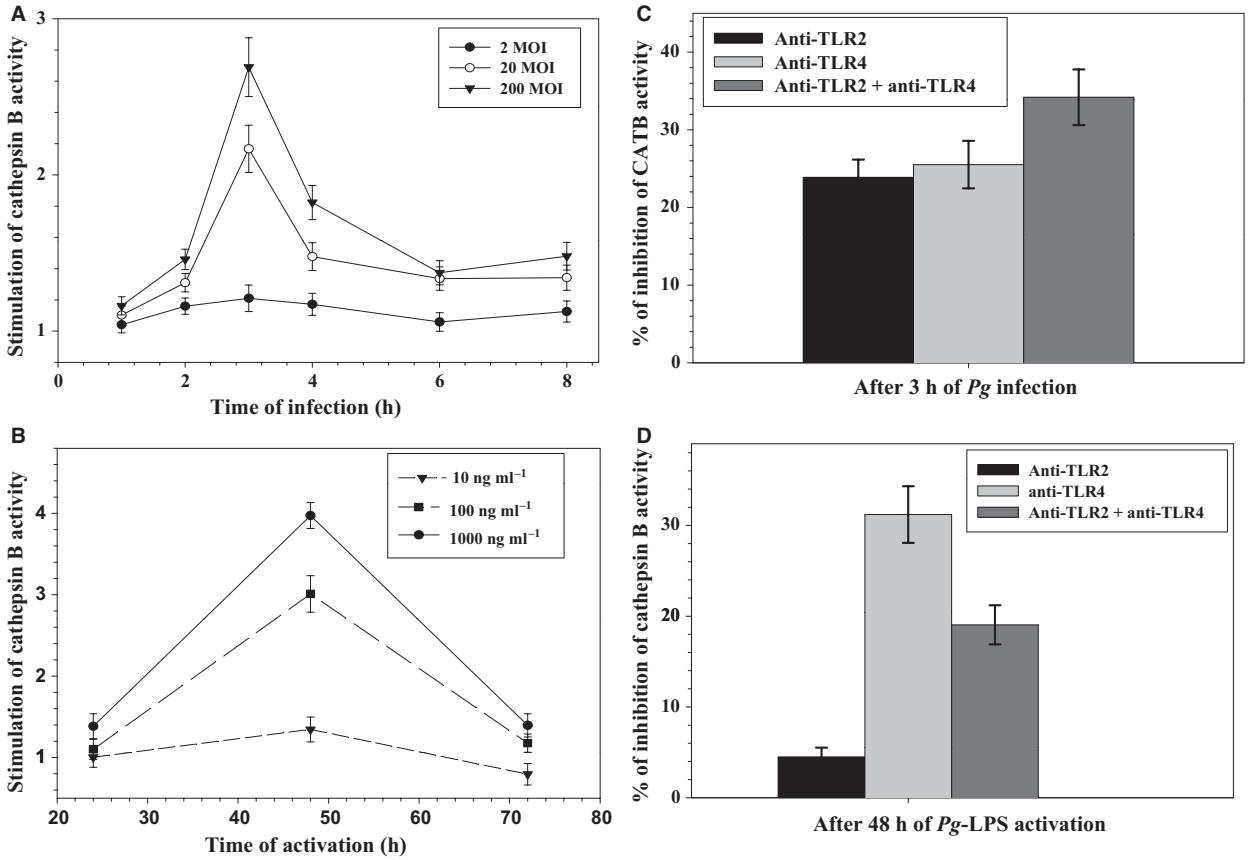

Figure 1 Activation of cathepsin B (CATB) in endothelial cells (ECs) infected with *Porphyromonas gingivalis* or stimulated by *P. gingivalis* lipopolysaccharide (*Pg*-LPS). ECs were infected with the whole *P. gingivalis* with doses ranging from 2 MOI to 200 MOI (A) or stimulated with increasing doses of *Pg*-LPS ranging from 10 ng ml^{-1} to 1 μg ml^{-1} (B). Cytosolic extracts were prepared at the indicated time-points to measure the enzymatic activity of CATB. At each indicated time-point, stimulation of the enzymatic activity was expressed as a ratio obtained by dividing the specific activity of the cathepsin measured in the assay by the specific activity measured in the control extract. Neutralizing antibodies directed against Toll-like receptor 4 (TLR4), TLR2 or human-IgA2 isotype used as control were all used at 1 μg ml^{-1} for 2 h before the beginning of the infection (C) or *Pg*-LPS stimulation (D). The CATB specific activity was expressed relative to the one measured with the control antibody. Results indicate the percentages of inhibition relative to a normal infection or to *Pg*-LPS stimulation. All assays were performed in triplicate and values represent the mean of the triplicate samples. Standard errors were calculated from the triplicate experiments.

2 MOI was not significant compared with the control cells. At each MOI, the maximum levels were obtained after 3 h of infection.

After a 3-day time–course stimulation of ECs by *Pg*-LPS at concentrations ranging from 10 ng ml^{-1} to 1 μg ml^{-1}, we observed an increase of the enzymatic activity of CATB (Fig. 1B) Maximum and significant increase was observed after 48 h using 1 μg ml^{-1} of *Pg*-LPS (a four-fold increase for CATB). At the same time-point, a significant increase (three-fold) was also observed after using 100 ng ml^{-1} *Pg*-LPS, indicating a dose-dependent effect. Before 24 h, no significant increase of CATB activity was quantified. LPS purified from other strains of *P. gingivalis* (i.e. ATCC 33277 or W381) gave similar results as well as LPS purified from *E. coli* (strain O127:B8), suggesting that CATB activation is not restricted to *Pg*-LPS (data not shown). Polymyxin B, a specific *Pg*-LPS inhibitor, used at 1 μg ml^{-1} gave a complete inhibition in the increase of CATB activity compared with untreated cells, indicating that CATB stimulation does not result from potential contaminating materials present within the *Pg*-LPS preparation (data not shown).

TLR2 and TLR4 are involved in the CATB activation pathway

To investigate the role of TLR4 and TLR2 pathways in CATB activation we used a neutralizing antibody specific for each receptor. Three hours after *P. gingi-*

valis infection, 26% and 24% reductions of the CATB peak activity were observed in cells pre-incubated with the anti-human TLR4-IgA2 and TLR2-IgA2 neutralizing antibodies, respectively, compared with control (Fig. 1C).

Concerning CATB activation by *Pg*-LPS, a 31% inhibition of the enzymatic activity was observed after treatment with the anti-human TLR4-IgA2 antibody whereas no significant effect of the human TLR2-IgA2 antibody was observed (Fig. 1D).

These results showed that CATB activation by *P. gingivalis* infection and *Pg*-LPS stimulation involved differentially TLR2 and TLR4 receptor pathways.

The increase in CATB enzymatic activity is not associated with mRNA expression or protein concentration

To determine if the increase in the enzymatic activity of CATB after infection with *P. gingivalis* and stimulation by *Pg*-LPS resulted from an increase in transcription, we performed reverse transcription quantitative PCR to quantify the levels of the corresponding messenger RNA (mRNA). When ECs were either infected with *P. gingivalis* or stimulated by *Pg*-LPS, we found no significant differences in the expression rates of the mRNAs encoding for CATB (Fig. 2A,B). The mRNA expression of cystatin C was also evaluated and revealed no difference between the expression rates of stimulated or infected cells to controls.

Furthermore, the amounts of CATB protein in cytosolic extracts from stimulated or infected ECs were also evaluated by Western blotting. No significant differences were observed between treated cells and controls (Fig. 3).

The kinetics of the tyrosine-dephosphorylation of CATB in ECs infected with *P. gingivalis* correlates with the kinetics of CATB activation

Structural analysis of CATB revealed many phosphorylation sites for protein kinase C or casein kinase II and one tyrosine kinase phosphorylation site. Tyrosine phosphorylation is a post-translational mechanism that controls a number of cellular events and modulates the activity of enzymes, such as endothelial nitric oxide synthase (eNOs) (Elkaim *et al.*, 2006; Paniagua *et al.*, 2010; Tigno-Aranjuez *et al.*, 2010; Yang *et al.*, 2010; Ben *et al.*, 2011). Therefore, we tested whether the tyrosine-phosphorylated or dephosphorylated form of CATB correlated with the increase of its enzymatic activity.

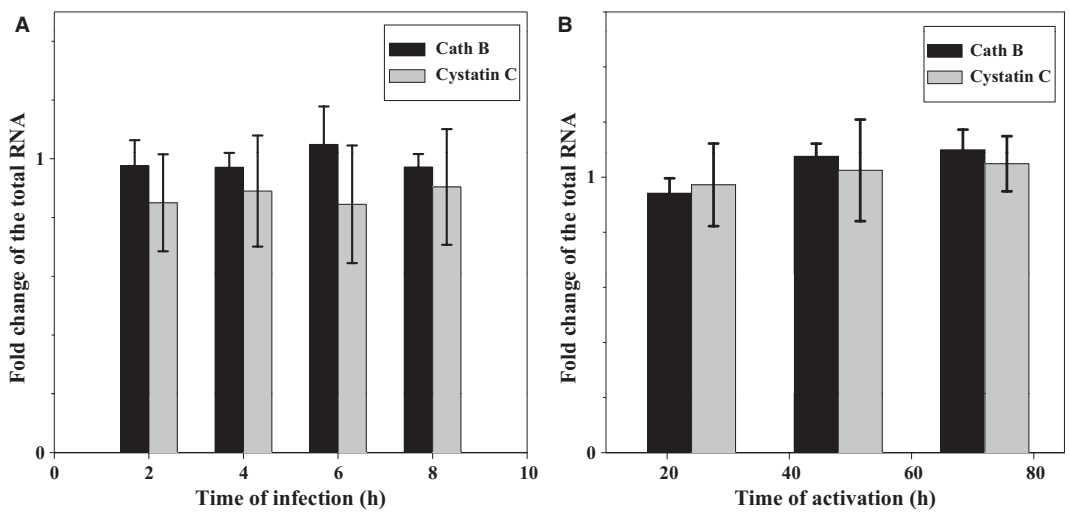

Figure 2 Reverse transcription quantitative polymerase chain reaction (RT-qPCR) of cathepsin B (CATB) and cystatin C. Endothelial cells (ECs) were infected with *Porphyromonas gingivalis* at 200 MOI (A) or stimulated by 1 μg ml^{-1} of *P. gingivalis* lipopolysaccharide (*Pg*-LPS) (B). Total RNA was extracted and analysed using RT-qPCR to quantify the fold change of the mRNA encoding CATB and cystatin C. GAPDH and β-actin were amplified as housekeeping genes and the relative gene expression was calculated using the comparative threshold (Ct) method ($2^{-\Delta\Delta Ct}$). All assays were performed in triplicate, and values represent the mean of the triplicate samples. Standard errors were calculated from the triplicate experiments.

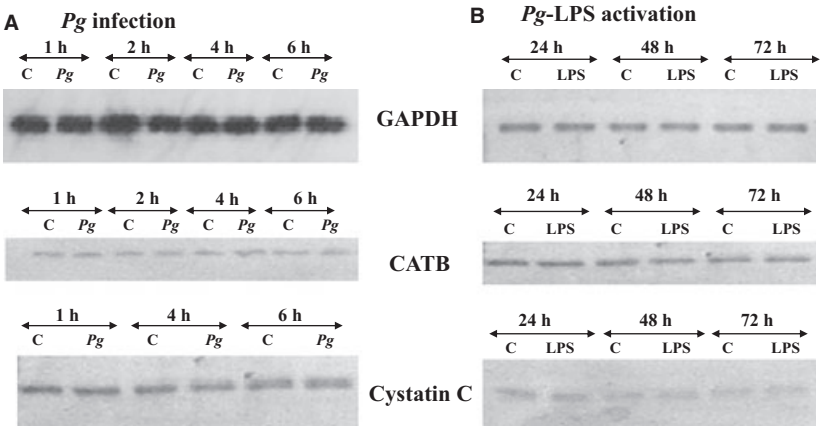

Figure 3 Protein expression levels of cathepsin B (CATB) and cystatin C in endothelial cells (ECs). ECs were infected with *Porphyromonas gingivalis* at 200 MOI (A) or stimulated by 1 μg ml^{-1} of *P. gingivalis* lipopolysaccharide (*Pg*-LPS) (B). The cytosolic extracts were prepared at each indicated time-point and 10 μg protein was separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted using the indicated antibodies for the revelation of the membranes. GAPDH was used to verify the equal loading of proteins in each well.

Using the PY-20 antibody for immunoprecipitation and the anti-CATB antibody for Western blotting revelation, a decrease in the tyrosine-phosphorylated form of CATB was observed in cytosolic extracts of ECs after *P. gingivalis* infection. Maximum dephosphorylation (40%) was reached after 3 h, whereas after 6 h the level of tyrosine-phosphorylated CATB was similar to its initial level (Fig. 4A). The same kinetic pattern was observed for the enzymatic activation of CATB in ECs infected with *P. gingivalis*, suggesting that the enzymatic activity of CATB may correlate in part with its tyrosine-phosphorylated state. In ECs stimulated by *Pg*-LPS the levels of tyrosine-phosphorylated CATB in cytosolic extracts immuno-precipitated with anti-CATB were similar at any time-point of the experiment (Fig. 4B). The same results were obtained by using cytosolic extracts immunoprecipitated with PY20 (data not shown).

DISCUSSION

This study showed that EC infection with *P. gingivalis* and stimulation by *Pg*-LPS affects the expression of CATB. Previous investigations have demonstrated that *Pg*-LPS stimulation of ECs also induced a pro-inflammatory response (Kocgozlu *et al.*, 2009). *Porphyromonas gingivalis* has been frequently detected in atherosclerotic plaques in humans and in experimental models, but its target cell type has not been precisely determined (Lalla *et al.*, 2003; Elkaim *et al.*, 2008a). Endothelial cells are the first type of vascular cells encountered by circulating *P. gingivalis*, and this pathogen has the capacity to invade ECs, as shown by *in vitro* studies (Walter *et al.*, 2004; Li *et al.*, 2008). Furthermore, *P. gingivalis* has been detected *in situ* close to the endothelium in atherosclerotic plaques (Kozarov *et al.*, 2005). Infection or invasion of ECs has been suggested to worsen atherosclerosis and has been described for different pathogens, such as *Legionella pneumophila* (Morinaga *et al.*, 2010) and *C. pneumoniae* (Watson & Alp, 2008; He *et al.*, 2010). Bacteria have developed several strategies to affect vessel cells (Erridge, 2008). For instance, phagocytosed *C. pneumoniae* in circulating and thereafter transmigrating monocytes in vessel walls could promote monocyte diapedesis by inducing synthesis of cytokines in ECs, such as vascular cell adhesion molecule-1 or monocyte chemoattractant protein-1 (Erridge, 2008). This mechanism may be transposed to *P. gingivalis* according to the fact that *P. gingivalis* could survive inside monocytes and use this cell type to translocate (Suwatanapongched *et al.*, 2010). A second potential mechanism of EC infection and activation is related to transient bacteremia occurring during mastication or tooth brushing (Forner *et al.*, 2006; Elkaim *et al.*, 2008a). In the last case, bacteria will directly adhere to ECs and have deleterious effects (Bélanger *et al.*, 2006). Our data reinforces the hypothesis that *P. gingivalis* infection could directly affect atherosclerotic plaques during periodontitis (Kebschull *et al.*, 2010).

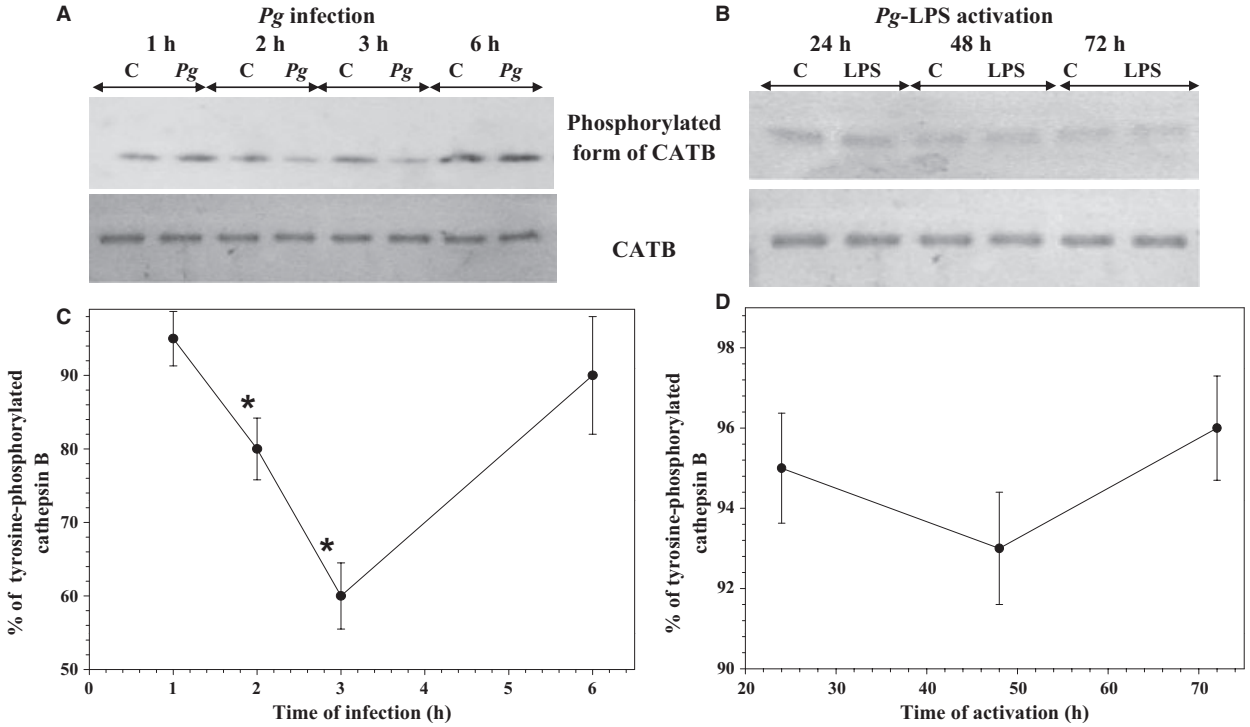

Figure 4 Tyrosine dephosphorylation of cathepsin B (CATB). Endothelial cells (ECs) were infected with *Porphyromonas gingivalis* at 200 MOI (A) or stimulated by 1 μg ml^{-1} *P. gingivalis* lipopolysaccharide (*Pg*-LPS) (B). Ten micrograms protein in the cytosolic extracts were immunoprecipitated using the antibody directed against CATB. Half the total of immunoprecipitated proteins was resolved using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted using PY20. The second half was similarly treated and revealed using the antibody against CATB to check for equal amount of immunoprecipitated CATB in each sample. At each time-point, the percentage of phosphorylated CATB in ECs infected with *P. gingivalis* (C) or stimulated by *Pg*-LPS (D) was calculated by quantification of the band corresponding to tyrosine-phosphorylated CATB, as revealed with the antibody PY20, divided by quantification of the band revealed with the anti-CATB antibody recognizing both tyrosine phosphorylated and dephosphorylated forms. Standard errors were calculated from the triplicate experiments. *$P < 0.05$ compared with percentage obtained at the beginning of the experiment.

In our experiments, both *P. gingivalis* and *Pg*-LPS significantly increased the enzymatic activity of CATB but with different kinetics. In *P. gingivalis* -infected ECs, a peak of CATB activity was observed within 3 h after infection, whereas this peak appeared to be delayed (48 h) in *Pg*-LPS-stimulated cells. Similar kinetic trends for CATB activities have previously been shown for gingival epithelial cells after *P. gingivalis* infection and *Pg*-LPS stimulation (Elkaim *et al.*, 2008b). Our results showed that TLR4 was involved in CATB activity regulation by *P. gingivalis* and its LPS, as shown previously for *Pg*-LPS-induced cytokine synthesis in endothelial cells (Kocgozlu *et al.*, 2009). TLR2 was only involved in *P. gingivalis* -induced CATB activation, suggesting that it recognized other virulence factors of *P. gingivalis* such as fimbriae, as shown for cathepsin E in macrophage in a Cathepsin E-deficient mouse model (Tsukuba *et al.*, 2006). This synergy between TLR4 and TLR2 could increase TLR-dependent response to *P. gingivalis* infection. Contrary to gingival epithelial cells (Elkaim *et al.*, 2008b), the increase of CATB activity in ECs was not associated with an up-regulation of CATB gene expression or protein synthesis, or a decrease of cathepsin inhibitor expression. Similar data have been described in a model of macrophage cells stimulated by *E. coli*-LPS, in which the increase of enzymatic activity has been explained by modification of acidity and size of endocytic compartments (Creasy & McCoy, 2011). This cell-specific response had already been observed in a previous study, which showed different effects of *Pg*-LPS in the cytokine production of ECs vs. gingival epithelial cells (Kocgozlu *et al.*, 2009). In our study, the increase of CATB activity in *P. gingivalis* -infected

cells was associated with a post-translational tyrosine-dephosphorylation process. The role of CATB phosphorylation has already been suggested as a mode of protein activation in another model of infection by *Plasmodium* in monocytes (Shio *et al.*, 2009). Furthermore, in atherosclerosis and especially in ECs, the control of protein phosphorylation, notably by mitogen-activated protein kinases such as extracellular signal-regulated kniase 1/2, Jun N-terminal kinase or p38, has an important role in endothelial activation and so in cytokine production (Hoefen & Berk, 2002). Our results evidenced a novel molecular mechanism of *P. gingivalis* virulence potentially associated with atherosclerosis worsening by *P. gingivalis* infection.

The patterns and molecular pathways of CATB regulation by whole *P. gingivalis* bacteria suggest that the cell invasion or internalization process by *P. gingivalis* could favour cathepsin activation. In human coronary arterial ECs, *P. gingivalis* traffics into cells quickly via the autophagic pathway, which takes between 15 and 120 min. It has been shown that the contact with lysosomal proteases triggers activation of cathepsins (Bélanger *et al.*, 2006). Consecutive activation of the inflammasome pathway leads to cell death (Niemi *et al.*, 2011). Interestingly, in our experiment, EC cultures did not survive when subjected to more than 8 h of *P. gingivalis* infection, whereas they could be maintained at least for 3 days after LPS stimulation (data not shown). *In vivo*, the major role of *P. gingivalis* cell invasion is reinforced by the fact that infection with a non-invasive strain of *P. gingivalis* did not accelerate atherosclerosis in apolipoprotein E-deficient mutant mice (Gibson *et al.*, 2004; Amar *et al.*, 2009) or stimulate prothrombotic activity of arterial smooth muscular cells (Roth *et al.*, 2009). Clinically, *P. gingivalis* infection of periodontal tissues is a continuous process according to subgingival biofilm properties. The presence of *P. gingivalis* in blood circulation could be considered transient according to the presence of numerous killing and neutralizing compounds such as *P. gingivalis* antibodies. Interestingly, *P. gingivalis* immunization suppresses the worsening effect of *P. gingivalis* oral infection on atherosclerosis plaque formation (Gibson *et al.*, 2004) but not on experimental periodontitis (Polak *et al.*, 2010). The rapidity of the endothelial response, as shown here for CATB activation after *P. gingivalis* infection and for cytokine synthesis after *Pg*-LPS stimulation (Kocgozlu *et al.*, 2009), appeared to be a major element of *P. gingivalis* virulence in cardiovascular diseases (Bélanger *et al.*, 2006; Erridge, 2008).

Our study confirmed that *Pg*-LPS can stimulate ECs. In a previous study, *Pg*-LPS stimulation was shown to increase proinflammatory cytokine synthesis of ECs via TLR4 activation within 3 h after stimulation (Kocgozlu *et al.*, 2009). The delayed increase of CATB observed in the present study has also been described in gingival epithelial cells (Elkaim *et al.*, 2008b). These data suggest another molecular regulation pathway of *Pg*-LPS effects in which TLRs and MyD88 dependent or independent pathways are involved (Stoll *et al.*,2004; Mahanonda & Pichyangkul, 2007; Elkaim *et al.*, 2008b; Kocgozlu *et al.*, 2009). High serum levels of LPS observed in patients suffering from severe periodontitis have been considered as a risk factor for atherosclerosis (Stoll *et al.*, 2004; Erridge, 2008). The LPS may trigger or accelerate atherosclerosis through multiple mechanisms, including increases in reactive oxygen species, chemotactic and proinflammatory cytokines and adhesion molecules in vascular cells (Stoll *et al.*, 2004; Kocgozlu *et al.*, 2009). Our data on CATB also suggests that *Pg*-LPS endotoxemia may be involved in EC apoptosis. However, while the demonstration of specific *Pg*-LPS endotoxemia during periodontitis is suspected, it has not yet been confirmed (Kallio *et al.*, 2008).

Finally, this study shows that ECs are the first line of defence against *P. gingivalis* infection as is proposed for gingival epithelial cells (Elkaim *et al.*, 2008b). The control of their cellular responses to *P. gingivalis* infection, such as cathepsin activation and cytokine production (Kocgozlu *et al.*, 2009), appears to be a major element of atherosclerosis and periodontitis pathogenesis (Amar *et al.*, 2009). This work reinforces the need to suppress or limit the amount of aggressive periodontal pathogens in patients at risk for cardiovascular disease (Huck *et al.*, 2011; Ying Ouyang *et al.*, 2011).

CONFLICT OF INTEREST AND SOURCE OF FUNDING

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