Chlamydia pneumoniae induces nitric oxide synthase and lipoxygenase-dependent production of reactive oxygen species in platelets

Effects on oxidation of low density lipoproteins

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Summary

There is increasing evidence that Chlamydia pneumoniae is linked to atherosclerosis and thrombosis. In this regard, we have recently shown that C. pneumoniae stimulates platelet aggregation and secretion, which may play an important role in the progress of atherosclerosis and in thrombotic vascular occlusion. The aims of the present study were to investigate the effects of C. pneumoniae on platelet-mediated formation of reactive oxygen species (ROS) and oxidation of low-density lipoprotein (LDL) in vitro. ROS production was registered as changes in 2’,7’-dichlorofluorescin-fluorescence in platelets with flow cytometry. LDL-oxidation was determined by measuring thiobarbituric acid reactive substances (TBARs). We found that C. pneumoniae stimulated platelet production of ROS. Polymyxin B treatment of C. pneumoniae, but not elevated temperature, abolished the stimulatory effects on platelet ROS-production, which suggests that chlamydial lipopolysaccharide has an important role. Inhibition of nitric oxide synthase with nitro-L-arginine, lipoxygenase with 5,8,11-eicosatriynoic acid and protein kinase C with GF 109203X significantly lowered the production of radicals. In contrast, inhibition of NADPH-oxidase with di-phenyleneiodonium (DPI) did not affect the C. pneumoniae induced ROS-production. These findings suggest that the activities of nitric oxide synthase and lipoxygenase are the sources for ROS and that the generation is dependent of the activity of protein kinase C. The C. pneumoniae-induced ROS-production in platelets was associated with an extensive oxidation of LDL, which was significantly higher compared to the effect obtained by separate exposure of LDL to C. pneumoniae or platelets. In conclusion, C. pneumoniae interaction with platelets leading to aggregation, ROS-production and oxidative damage on LDL, may play a crucial role in the development of atherosclerotic cardiovascular disease.

Keywords

Atherosclerosis, bacteria-cell interaction, oxygen radical, LPS, thrombosis

Introduction

Chlamydia pneumoniae is a common respiratory pathogen with a lifecycle consisting of an intracellular replicating form (reticulate body) and an extracellular non-replicating form (elementary body) (1). In 1988, data were presented indicating for the first time a link between C. pneumoniae infection and acute myocardial infarction (2). Since then a number of seroepidemiological studies have reported a connection between C. pneumoniae seropositivity and cardiovascular disease (3). More evidence derives from the finding that approximately half of all investigated atherosclerotic lesions contains the organism or its proteins or nucleic acids, as demonstrated by PCR, immunohistochemical staining and electron microscopy (4).

C. pneumoniae is thought to spread from the lung tissue to the arteries by infecting monocytes (5). Indeed, in vitro studies have revealed that infected monocytes can transfer the bacteria to coronary endothelial cells (6). Furthermore, C. pneumoniae induces monocytes to oxidize LDL and macrophages to ingest LDL, leading to formation of foam cells (7–9). Oxidation of LDL and foam cell formation are processes involved in the initial phase of atherosclerosis. Endothelial inflammation can further...
be promoted by the ability of *C. pneumoniae* to provoke interleukin 1 (IL-1) and monocyte chemoattractant protein 1 (MCP-1) release from endothelial cells, thereby stimulating transendothelial migration of neutrophils and monocytes (10). Formation of the fibromuscular plaque involves movement of smooth muscle cells into the intima and subsequent proliferation and synthesis of connective tissue. It has been shown that endothelial cells infected by *C. pneumoniae* stimulate smooth muscle cell proliferation (11).

We have recently demonstrated that *C. pneumoniae* stimulates platelet aggregation, secretion and surface expression of P-selectin. Chlamydial lipopolysaccharide (LPS) and platelet glycoprotein (Gp) IIb/IIIa are essential adhesive structures in this interaction (12). An *C. pneumoniae*-induced platelet activation may contribute to both the early and the late stages of atherosclerosis. Secretion of growth factors, cytokines and adhesive proteins from activated platelets may stimulate migration and proliferation of monocytes and smooth muscle cells, which enhances the growth and development of the plaque. During acute coronary events platelet activation causes thrombotic vascular occlusion.

In addition to the above-mentioned release of cytokines and growth factors, platelets also have the ability to produce and release reactive oxygen species (ROS) (13, 14). Accumulating evidence supports participation of ROS in platelet activation and subsequent thrombus formation. Several independent studies have shown that ROS may induce spontaneous aggregation or enhance platelet activation, triggered by different platelet activators (15). Oxidative stress caused by ROS plays a crucial role in the pathogenesis of atherosclerosis (16). ROS impairs cellular function by reacting with lipids, proteins and DNA and are responsible for LDL-oxidation. Oxidized LDL promotes cell injury, smooth muscle cell proliferation, foam cell formation, chemotaxis of leukocytes, cellular secretion of inflammatory mediators, and other events that modulate atheroma biology (17, 18).

The enzymatic sources of ROS in platelets are not completely clarified. The suggested origins are arachidonic acid metabolism by cyclooxygenase and lipoxygenase (19, 20), the NADH/NADPH-oxidase (21) and the nitric oxide synthase (22, 23). *In vitro*, platelet ROS-production could be triggered by, e.g. collagen, thrombin and lipopolysaccharides (LPS) (13, 24). The findings that staurosporine and wortmannin inhibit LPS-induced platelet ROS-production suggest that protein kinase C and phosphoinositide 3-kinase are important in the intracellular signalling cascade (13).

The aims of this study were to investigate the ability of *C. pneumoniae* to induce platelet ROS-production and clarify the involved intracellular radical generating systems and signalling pathways. Moreover, we studied the effect of chlamydia-platelet interaction on LDL-oxidation. We found that *C. pneumoniae* stimulates nitric oxide synthase and lipoxygenase dependent ROS-production in platelets and that this effect was associated with a significant oxidation of LDL. These findings further support an active role of *C. pneumoniae*-platelet interaction in the development of atherosclerosis.

**Methods**

**Chemicals and materials**

The materials and their origin were as follows: RPMI 1640 medium, fetal bovine serum, gentamicin, L-glutamine (Gibco, BRL, Life Technologies, Paisley, Scotland); cell culture plastics (Nunc, Roskilde Denmark); cyclohexamide (ICN Biomedicals Inc, Aurora, OH, USA); anti-chlamydia LPS FITC conjugated antibodies (Boule Nordic A B, Huddinge, Sweden); PCR primers (SGS AB, Köping, Sweden); Ready to go PCR beads (Amer sham Biosciences, Uppsala, Sweden); kit for DNA extraction (Genomed, Löhne, Germany); 2',7'-dichlorofluorescin acetate (DCFH-DA) (Molecular probes, Eugene, OR, USA); Optilyse (Immunotech, Marseille, France); polymyxin B, N-acetyl-L-cysteine (NAC), Nitro-L-arginine, di-phenyleneiodonium (DPI), wortmannin, butylated hydroxytoluene (BHT), AAPH [2,2'-azo-bis-(2-aminopropane) dihydrochloride], TEP (1,1,3,3-tetraethoxypropane) (Sigma Chemical Co, St Louis, MO, USA); 5,8,11,14-eicosatetraynoic acid (ETYA) (Cayman chemical company, Ann Arbor, MI USA); 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF 108023X) (Tocris, Ellisville, MO, USA); 5,8,11-eicosatriynoic acid (ETI) (a kind gift from Professor S. Hammarström, Department of Cell Biology, Linköping University, Linköping, Sweden); NaCl, KH₂PO₄, KBr, thiobarbituric acid (Merck, Darmstadt, Germany); KCl, Na₂HPO₄, sucrose, disodium EDTA, trichloroacetic acid (Riedel-De Haën, Seelze, Germany).

**Cell culture**

HEp2 cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mg/l gentamicin, and 2 mM L-glutamine. The cells were incubated at 37°C and 5% CO₂ in 75 cm² culture flasks, and then sub cultured in 6-well plates at a density of 0.7 x10⁶ cells/well prior of infection with chlamydiae.

**Chlamydia pneumoniae propagation**

Chlamydia pneumoniae (strain T45) was cultured in HEP2 cells, grown and maintained in RPMI 1640, essentially as described by Redecke et al. (25). The bacteria were added to sub confluent monolayers of HEP2 cells in 6-well plates. The plates were centrifuged at 480 x g for 45 minutes at 25°C, and incubated for 2 h at 37°C and 5% CO₂. Nonadherent bacteria were removed and infected cells were incubated in fresh RPMI 1640, supplemented with 1 µg/ml cyclohexamide. Infected cells were incubated for 72 h, as mentioned above, to allow development of characteristic chlamydial inclusions. The chlamydiae were harvested by disrupting HEP2 cells with glass beads followed by centrifugation at 900 x g for 10 minutes at 4°C to remove cellular debris. Supernatants were centrifuged at 12 000 x g for 30 minutes at 4°C, and the bacteria were suspended in sucrose-phosphate buffer (sp-2-buffer), supplemented with FBS (10 %), counted by immuno-fluorescence staining and then stored at –70°C until use. The chlamydiae are expressed as inclusion forming units (IFU) throughout the study. Uninfected HEP2 cells (HEP2 cell debris) were handled exactly as chlamydia-infected cells and used as a control. To study the involvement of heat-labile structures, *C. pneumoniae* was incubated at 70°C for 30 minutes.
**Mycoplasma PCR**

The bacteria and cells were tested for mycoplasma contamination by using mycoplasma specific PCR essentially according to van Kuppeveld et al. (26). The nucleotide sequences of primers used in the Mycoplasma group-specific PCR assay were as follows: upstream primer GPO-3 5’-GGGAGCAAAC-AGGATTAGATACCC-3’ and downstream primer MGSO 5’-TGCAACCATTCTGACTCTGTAACCTC-3’. In short, the DNA of the cells and *C. pneumoniae* culture was extracted by using genomic jetquick tissue DNA spin kit. The DNA (1 µl) was added to 25 µl of the following PCR mixture: 1.5 units of Taq DNA polymerase, 10 µM Tris-HCL (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP and stabilizers, including BSA, 0.8 µM upstream primer GPO-3, and 0.8 µM downstream primer MGSO. The PCR protocol used in a PTC-100™ (SDS, Falkenberg, Sweden) was as follows: denaturation at 94°C for 40 seconds, annealing temperature at 55°C for 40 seconds, extension at 72°C for 1 minute at 25 cycles.

**Preparation of platelets**

Platelets were isolated from freshly heparinized human peripheral blood, donated by apparently healthy and drug free adult volunteers at the blood bank at Linköping University Hospital, Linköping, as previously described (27). Five parts of blood were mixed with one part of an acid citrate/dextrose solution (85 µM trisodium citrate dihydrate, 71 µM citric acid hydrate and 111 mM D-glucose), followed by centrifugation at room temperature for 20 minutes at 220 x g to obtain platelet rich plasma (PRP). The PRP was centrifuged at room temperature for 20 minutes at 480 x g, and the platelets were then gently washed and resuspended in Krebs-Ringer-glucose buffer (NaCl (120 mM), KCl (4.9 mM), MgSO₄·7H₂O (1.2 mM), KH₂PO₄ (1.7 mM), NaH₂PO₄·2H₂O (8.3 mM), glucose (10 mM); pH 7.3), final cell density 2x10⁹/ml and stored in plastic tubes at room temperature before use. To obtain functional but non-activated platelets, the isolation was performed without any specific platelet inhibitors, and, due to this, extra care was taken when handling the cells. Morphological studies showed discoid, solitary platelets displaying no signs of activation due to the preparation procedure. No contaminating leukocytes or erythrocytes were found in the washed platelet suspension controlled by light microscopy and flow cytometry. The extracellular calcium concentration was adjusted to 1 mM immediately before each experiment.

**Platelet ROS-production induced by *C. pneumoniae* measured by flow cytometry**

Production of ROS by platelets, stimulated by *C. pneumoniae*, was measured by using a fluorescent dye, 2’,7’-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA is a nonpolar compound that is converted into a nonfluorescent polar derivative (DCFH) by cellular esterases after incorporation into cells. DCFH is membrane-impermeable and rapidly oxidizes into the highly fluorescent 2’,7’-dichlorofluorescin (DCF) in the presence of intracellular hydrogen peroxide (H₂O₂), peroxynitrite (ONOO⁻), peroxidases and hydroxyl radicals (·OH) (28). The fluorescence intensity of the platelet population was determined with flow cytometry (Becton Dickinson, FACs Calibur) with excitation and emission settings of 488 and 530–565 nm, respectively.

Platelets (2 x 10¹⁰/ml) were preincubated with 5 mM DCFH-DA and with or without NAC (0.5 or 5 mM), RGDS (1 mg/ml), nitro-L-arginine (0.1 mM), ETYA (10 mM), ETI (10 µM), DPI (10 or 100 µM), wortmannin (100 nM) or GF 109203X (50 mM) for 15 minutes at 37°C in a 24 well plate before being mixed with *C. pneumoniae* to a concentration containing bacteria and platelets in a ratio of 1:15. Samples were taken immediately before, and 5, 10 and 20 min after adding *C. pneumoniae* to the platelet suspension. In some experiments, viable *C. pneumoniae* was replaced with heat inactivated (70°C, 30 min) *C. pneumoniae*, HEP2– debris or thrombin (0.5 units/ml). The involvement of chlamydial lipopolysaccharide (LPS) was evaluated by treating *C. pneumoniae* with polymyxin B (50–100 µg/ml) for 30 min at room temperature. The samples were then fixed with Optilyse (with 2.5 % formaldehyde) at room temperature for 10 minutes in the dark and diluted in distilled H₂O. The platelet population was identified by means of its light-scatter characteristics, and by confirming that more than 99% of analysed particles in each sample were GpIb-positive. The mean fluorescence value of each sample was determined from 7000 cells.

**Preparation of LDL**

LDL was isolated from fresh human plasma by sequential density-gradient ultracentrifugation according to da Silva et al. (29). Whole blood was drawn from nonfasted healthy adult volunteers and collected in vacuum tubes containing EDTA. After centrifugation at 1200 x g for 10 min at room temperature, the plasma was pooled and mixed with EDTA (final concentration 1 mg/ml) and sucrose (final concentration 0.5%) to prevent LDL oxidation and aggregation, respectively. Five ml of plasma was then adjusted to a density of 1.22 g/ml by addition of KB (0.3264 g/ml) under stirring and transferred to a centrifuge tube (Beckman polycarbonate tube, 16 x 76 mm). The tube was carefully filled with ice-cold phosphate buffered saline (NaCl (137 mM), KCl (2.7 mM), Na₂HPO₄·2H₂O (6.7 mM), KH₂PO₄ (1.5 mM); pH 7.3), without mixing the two phases, and finally heat-sealed. Samples were centrifuged at 290 000 g for 2 h at 4°C in a Beckman XL-90 ultracentrifuge (Beckman Instruments, USA) equipped with a Beckman Type 70.1 Ti fixed-angle rotor. LDL was collected by careful aspiration of the yellow band in the middle of the tube using a 1x60 mm 19G needle on a 2 ml plastic syringe. The LDL was then transferred to a new ultracentrifuge tube and carefully covered with a KBr solution with a density of 1.10 g/ml (0.133 g KBr/ml, 1 mg EDTA/ml). The filled tube was heat-sealed and centrifuged in the same way as described above. After centrifugation, the albumin free LDL in the top of the tube was aspirated with a needle on a syringe as described above. The LDL fraction was desalted with a PD10 desalting column (Amersham Pharmacia Biotech, USA) with phosphate-buffered saline (PBS, 154 mM, pH 7.3) as desalting buffer.

Protein concentration was determined by a protein assay (Bio-Rad no. 500–0006, Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin as standard. Isolated LDL was used within two weeks.
Chlamydia pneumoniae induces platelet ROS-production

LDL (100 µg/ml) was incubated with C. pneumoniae and/or platelets (bacteria:platelet ratio of 1:15) or HEp2 cell debris for 5 or 10 min or thrombin (0.5 units/ml) for 1 min under stirring conditions. Thereafter the cells and bacteria were pelleted by centrifugation at 12000 x g for 30 min at room temperature. The supernatant containing LDL was used for analysis of degree of lipid peroxidation using a thiobarbituric acid reactive substances assay.

**Determination of thiobarbituric acid reactive substances (TBARS)**

The TBARs assay was performed according to a method previously described by Folcik et al. (30). Samples (50 µl of reaction mixture containing LDL treated as described above) were transferred to the wells of a 96-well microplate (Corning, no. 3599) and supplemented with 5 µl butylated hydroxytoluene (1 µM) and 5 µl EDTA (10 mg/ml) to prevent oxidation during the assay. Then, 50 µl trichloroacetic acid (50%, w/v) and 75 µl 1% thiobarbituric acid in 0.3% NaOH were added. Standards were prepared of 0.5–5 µM 1,1,3,3,-tetraethoxypropane (Sigma T-9889) which upon heating transforms into one molecule of malondialdehyde. Samples and standards were analysed on the same plate and in duplicate. The plate was sealed with a heat-resistant adhesive PCR film (Abgene, AB-0558) and incubated at 90°C for 40 min. The plate was then chilled on ice and the fluorescence at 538 nm was read on a FLUOStar fluorescence plate reader (BMG Labtechnologies, Offenburg, Germany) with an excitation wavelength of 485 nm. Quantification of the samples was made from the malondialdehyde standard curve and TBARS was expressed as malondialdehyde equivalents.

**Statistics**

Data are expressed as the mean ± standard deviation. Statistical differences between means were assessed by the paired, two tailed students t-test. P<0.05 was considered to be statistical significant. The data obtained with a specific drug is statistically compared with corresponding control, i.e. the same donor and identical experimental conditions.
Results

Platelet ROS-production stimulated by Chlamydia pneumoniae

Platelet ROS-production was determined with flow cytometry by measuring the fluorescence obtained when the intracellular probe DCFH reacts with ROS. We found a slight decrease in intracellular ROS-production of platelets incubated with Chlamydia pneumoniae for 5 min at 37°C (Fig. 1A). However, after 10 min of incubation with the bacteria the platelet ROS-production was significantly increased (162% of control; Fig. 1A, B). After additional 10 min of incubation a complete platelet aggregation was obtained, which made it impossible to analyse the samples. However, inhibition of platelet aggregation with Arg-Gly-Asp-Ser (RGDS, 1 mg/ml), which blocks fibrinogen binding to Gp IIb/IIIa, further increased the ROS-production in platelets (Fig. 2). In contrast to the effects of C. pneumoniae, stimulation of platelets with thrombin (0.5 units/ml) for 1 minute increased the ROS-production approximately 2 fold (Fig. 1C). Control samples from uninfected Hep2 cells (see Methods) did not cause ROS-production in platelets (Fig. 1A). This excludes an effect of an eventual contamination of debris from Hep2 cells in the chlamydia population. Preincubation of platelets with the intracellular ROS-scavenger N-acetyl-L-cysteine (NAC, 0.5–5 mM) for 15 minutes at 37°C significantly inhibited the C. pneumoniae-induced ROS-production in platelets (Fig. 3). To investigate the influence of plasma on the C. pneumoniae-stimulated ROS-production in platelets, experiments with platelet rich plasma (PRP) were performed. We found that C. pneumoniae increased the ROS-production in PRP to the same extent as in washed platelet suspensions (about 60% increase, not shown). However, the basal DCF fluorescence in PRP was very low (more than 10 times lower than in washed platelets), which implies that this method is uncertain to use in registering ROS-production in PRP.

The enzymatic source of ROS

Suggested enzymatic sources of ROS in platelets are arachidonic acid metabolism by cyclooxygenase and lipoxygenase (COX and LOX), NADH/NADPH-oxidase and nitric oxide synthase (NOS). Inhibitors of these enzymes were tested for evaluating the effects on the C. pneumoniae-induced ROS-production in platelets. Nitro-L-arginine, which competitively inhibits NOS, significantly reduced the platelet ROS-production triggered by C. pneumoniae (Fig. 4). In contrary, addition of 10 or 100 μM of the NADPH-oxidase inhibitor DPI did not affect the platelet ROS-production (not shown). Arachidonic acid is released from the plasma membrane, predominantly by the action of phospholipase A2, and then converted to eicosanoids by the enzymes COX and LOX. It has been shown that during this enzymatic reaction ROS can be formed (19, 20). In the present study, ETYA (10 μM), an inhibitor of both COX and LOX, and ETI (10 μM), a selective inhibitor of LOX, significantly lowered the C. pneumoniae-induced platelet ROS-production (Fig. 4).

Chlamydia-induced ROS production is dependent on protein kinase C

The intracellular signalling pathway activated in platelets during the interaction with C. pneumoniae and its role in ROS-production was studied by using inhibitors directed against protein

Figure 2: The effects of Gpllb/IIIa inhibition on platelet ROS-production induced by C. pneumoniae. Platelets were treated with Arg-Gly-Asp-Ser (RGDS, 1 mg/ml) and DCNH-DA (5 mM) for 15 min and then stimulated with C. pneumoniae (bacteria:platelet ratio of 1:15) for 10 min under stirring conditions at 37°C. The samples were thereafter fixed with formaldehyde (2.5%) and the DCF-fluorescence was measured with flow cytometry. The data are expressed as percent of a control with untreated platelets, with or without RGDS, and represent 3 different experiments run in duplicate. The data from the RGDS-treated platelets are statistically compared with data from untreated platelets, by using paired students t-test.

Figure 3: The effects of the ROS-scavenger N-acetyl cysteine (NAC) on platelet ROS-production stimulated by C. pneumoniae. Platelets were loaded with DCFH and with or without NAC (0.5 or 5 mM) for 15 minutes at 37°C in dark and then stimulated with C. pneumoniae (bacteria:platelet ratio of 1:15) for 10 minutes under stirring conditions. Thereafter the cells were fixed with formaldehyde and the DCF-fluorescence of platelets was determined with flow cytometry. The data are expressed as mean ± standard deviation in percent of control of untreated platelets or platelets pretreated with NAC of 5 separate experiments run in duplicate. The data from the NAC-treated platelets are statistically compared with data from untreated platelets, by using paired students t-test. The mean value obtained with a specific concentration of NAC is statistically compared with corresponding control, i.e. the same donor and identical experimental conditions.
kinase C and phosphoinosside 3-kinase. Inhibition of protein kinase C by GF 109203X (50 µM) significantly reduced the ROS-production (Fig. 4), whereas wortmannin (100 nM), an inhibitor of phosphoinosside-3-kinase, was ineffective (not shown). These findings suggest a role for protein kinase C in chlamydia-induced ROS-production.

The role of chlamydial lipopolysaccharide

To determine whether platelet activation required viable bacteria, an active release of chlamydial cell components, and/or binding to heat-labile chlamydial surface structures, experiments using heat-inactivated *C. pneumoniae* were performed. We found that heat-treatment of *C. pneumoniae* at 70°C for 30 min did not change the ability of the bacteria to induce platelet ROS-production (not shown). To study the role of lipopolysaccharide (LPS) in the interaction between *C. pneumoniae* and platelets, the bacteria were preincubated with polymyxin B (50 or 100 µg/ ml) for 30 min at room temperature. Polymyxin B is a polycation and binds directly to the anionic lipid A portion of LPS, and thereby inhibits the functional ability of LPS (25). Polymyxin B-treated *C. pneumoniae* was unable to induce platelet ROS-production (Fig. 5).

### LDL-oxidation induced by platelets and *Chlamydia pneumoniae*

Oxidation of LDL is considered to be an important process in the initiation and progression of atherosclerosis. We studied whether the *C. pneumoniae*-stimulated ROS-production in platelets causes LDL- oxidation. Indeed, significant increase in LDL-oxidation, measured as TBARS, was observed after incubation of purified human plasma LDL with platelets and *C. pneumoniae* for 30 minutes at 37°C, compared to a buffer control (Fig. 6). Platelets and *C. pneumoniae* separately caused an oxidation of LDL, though not to the same extent as the bacteria-platelet mixture. (2-amidinopropane)dihydrochloride (AAPH) generates peroxyl radicals and was used as a positive control of LDL-oxidation.

### Discussion

Platelets play an important role in the pathogenesis of cardiovascular diseases. During recent years much interest has been focused on the platelet as an inflammatory cell by its ability to release inflammatory mediators and ROS, modulate leukocyte function and interact with bacteria (31). An association between *Chlamydia pneumoniae* and atherosclerosis has been demonstrated in a number of epidemiological, serological, immunohistochemical, and molecular biological investigations (4). We have previously shown that *C. pneumoniae* stimulates platelet aggregation and secretion, which are processes that can promote both atherosclerosis and thrombosis. In this study, we found that *C. pneumoniae* changes the redox state in platelets, revealed by an increased DCF-fluorescence.

Addition of *C. pneumoniae* to DCF-loaded platelets caused a decrease in DCF-fluorescence after 5 min, followed by a significant increase after 10 min. Inhibition of platelet aggregation by RGDS further elevated the increase in DCF-fluorescence, thus demonstrating that the chlamydia-induced ROS-production in platelets is independent of aggregation and activation of GpIIb/IIIa. Earlier studies have reported that some bacteria re-
lease radical scavengers in order to protect themselves from the host defence (32, 33). Thus, the initial decrease in ROS-production may be due to a radical scavenging system in chlamydia. Several studies have demonstrated an ability of platelets to generate ROS (34). Furthermore, platelet aggregation is associated with a burst of hydrogen peroxide that, in turn, contributes to the activation of platelet function (35).

The mechanisms by which platelets generate ROS are not fully understood. ROS may be generated due to the enzymatic activity of cyclooxygenase, lipooxygenase (19, 20), and NADH/ NADPH-oxidase (21). Moreover, nitric oxide synthase (NOS) has been purified from human platelet cytosolic fractions (23). In the current study, the NOS inhibitor nitro-L-arginine significantly lowered the platelet ROS-production induced by C. pneumoniae, which suggests an important role of NOS. In addition, the combined COX and LOX inhibitor ETV4, and the LOX inhibi-tor ETI, completely inhibited the chlamydia-triggered ROS-production in platelets. However, the NADH/NADPH oxidase inhibitor DPI had no effect. These results indicate that activation of both NOS and LOX is crucial for the ROS-production in chlamydia-stimulated platelets.

The fluorogenic compound DCFH-DA has been utilized extensively as a marker for oxidative stress, and is suggested to reflect the overall oxidative status of the cell (28). A recent study showed that DCFH is sensitive towards oxidation by ONOO\(^2^-\), H\(_2\)O\(_2\), and -OH, while it is not suitable for registering NO, HOCl, or O\(_2^-\) in biological systems (28). Lufrano & Balazy (2003) suggested that the fluorescence from DCF-loaded platelets is specific for ONOO\(^-\). In correlation to our results, they found that a NOS inhibitor considerably decreased the generation of ONOO\(^-\) (36). In this study, we suggest that the DCF-fluorescence in platelets stimulated by C. pneumoniae at least partly reflects formation of ONOO\(^-\). The formation of ONOO\(^-\) requires a simultaneous production of O\(_2^-\) and NO. Besides generating NO, NOS is a potential source of O\(_2^-\)/H\(_2\)O\(_2\) (37). We suggest that the C. pneumoniae-induced increase in DCF-fluorescence registers production of ONOO\(^-\), which is formed from O\(_2^-\) and NO generated by NOS. Our finding that inhibition of GPiB/IIia and platelet aggregation increases C. pneumoniae-induced DCF-fluorescence correlates with a recent study showing that an antagonist to GPiB/IIia enhances platelet NO release (38). Alternatively, an increased enzymatic activity of LOX leads to generation of O\(_2^\cdot\), which reacts with NO and forms ONOO\(^\cdot\). Several studies have established that ONOO\(^\cdot\) can pass the lipid part of membranes (39–41). In addition, ONOO\(^-\) may be transported across the platelet membrane by an anion transporter (the HCO\(_3^-\)/Cl\(^-\) transporter) (36). Release of ONOO\(^\cdot\) from platelets may support oxidative processes including oxidation of LDL and tissue damage, and thereby contribute to the development of atherosclerosis (42–44). Furthermore, conversion of O\(_2^-\) and NO to ONOO\(^-\) decrease the bioavailability of NO as a potent inhibitor of platelet activation, thus promoting vascular thrombosis.

We have previously shown that inhibition of LPS by polymyxin B abolishes the effects of C. pneumoniae on platelet aggregation and secretion (12). In correlation, the current study shows that treatment of C. pneumoniae with polymyxin B significantly antagonizes the effects on platelet ROS-production, which indicates an important role for LPS. Chlamydial LPS contains a unique lipid A, lacks an O-chain and exposes a genus-specific highly immunogenic epitope on the polysaccharide core (45). Similar LPS has also been identified in Porphyromonas gingivalis (46). Bacteria with modified LPS, e.g. F. gingivalis and C. pneumoniae, are much more potent activators of platelets, than classical gram-negative bacteria (47). Interestingly, different forms of LPS from Proteus mirabilis stimulate O\(_2^\cdot\) generation in platelets, which is dependent on activation of protein kinase C (PKC) and phosphoinositide 3-kinase (13). We found that inhibition of phosphoinositide 3-kinase had no effect on the ROS-generation in platelets stimulated by C. pneumoniae. On the other hand, inhibition of PKC significantly reduced the bacteria-induced ROS-production in platelets. PKC plays a key role in platelet activation by phosphorylating proteins involved in shape change, aggregation and secretion. We propose that C. pneumoniae, via LPS, interacts with platelet surface structures, resulting in activation of PKC. In correlation, other studies have shown that platelets are directly stimulated by lipid A through an activation of PKC (48).

ROS have a crucial role in the atherosclerotic process by oxidizing LDL and function as a signalling molecule in cell proliferation (16). Oxidized LDL exerts several proatherogenic effects, which include increased synthesis and secretion of adhesion molecules, monocyte chemotaxis and adhesion, cytotoxicity to endothelial cells, enhanced foam cell formation and increased smooth muscle cell proliferation (49, 50). In this study, we found that C. pneumoniae and platelets, separately, caused a slight LDL-oxidation. However, coincubation of platelets and chlamydia resulted in a significantly higher degree of LDL-oxidation. In support to our observations, C. pneumoniae has previously been shown to induce a low degree of LDL oxidation and stimulate monocytes to oxidize LDL (8).

Previous findings suggest that chlamydial infection, platelet activation and LDL-oxidation separately contribute to the pathogenesis of atherosclerosis. We have recently demonstrated that
C. pneumoniae induces platelet aggregation and secretion proposing a mechanism for bacteria-induced thrombotic vascular occlusion. In this study, we show that C. pneumoniae increases the production of oxidative radicals in platelets leading to oxidation of LDL. In conclusion, the causal role of chlamydial infection in atherosclerosis and coronary artery disease may be a chlamydia-induced oxygen radical release from platelets with an associated damage of LDL.

Acknowledgement

We thank Olle Stendahl at the department of Medical Microbiology for advice and support.

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