Theme Issue Article

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

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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

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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 AB, Huddinge, Sweden); PCR primers (SGS AB, Köping, Sweden); Ready to go PCR beads (Amersham 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-amidinopropane) dihydrochloride], TEP (1,1,3,3-tetraethoxypropane) (Sigma Chemical Co, St Louis, MO, USA); 5,8,11,14-eicosatriynoic acid (ETYA) (Cayman chemical company, Ann Arbor, MI USA); (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) (GF 108203X) (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, KH_2PO_4, 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% CO_2 in 75 cm^2 culture flasks, and then sub cultured in 6-well plates at a density of 0.7 x10^6 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% CO_2. 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 immunofluorescence 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-AGGATTAGATACCCT-3´ and downstream primer MGSO 5´-TGCACCATCTGTCACTCTGTTAACCTC-3´. In short, the DNA of the cells and *C. pneumoniae* culture was extracted by using genomed 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 mM Tris-HCL (pH 9.0 at room temperature), 50 mM KKl, 1.5 mM $MgCl_2$, 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 drawn 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), $MgSO_4$*$7H_2O$ (1.2 mM), KH_2PO_4 (1.7 mM), Na_2HPO_4*$2H_2O$ (8.3 mM), glucose (10 mM); pH 7.3), final cell density $2x10^8$/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 (H_2O_2), 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 10^8/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 H_2O. 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 KBr (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), Na_2HPO_4*2 H_2O (6.7 mM), KH_2PO_4 (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 ultracentrifugation 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.

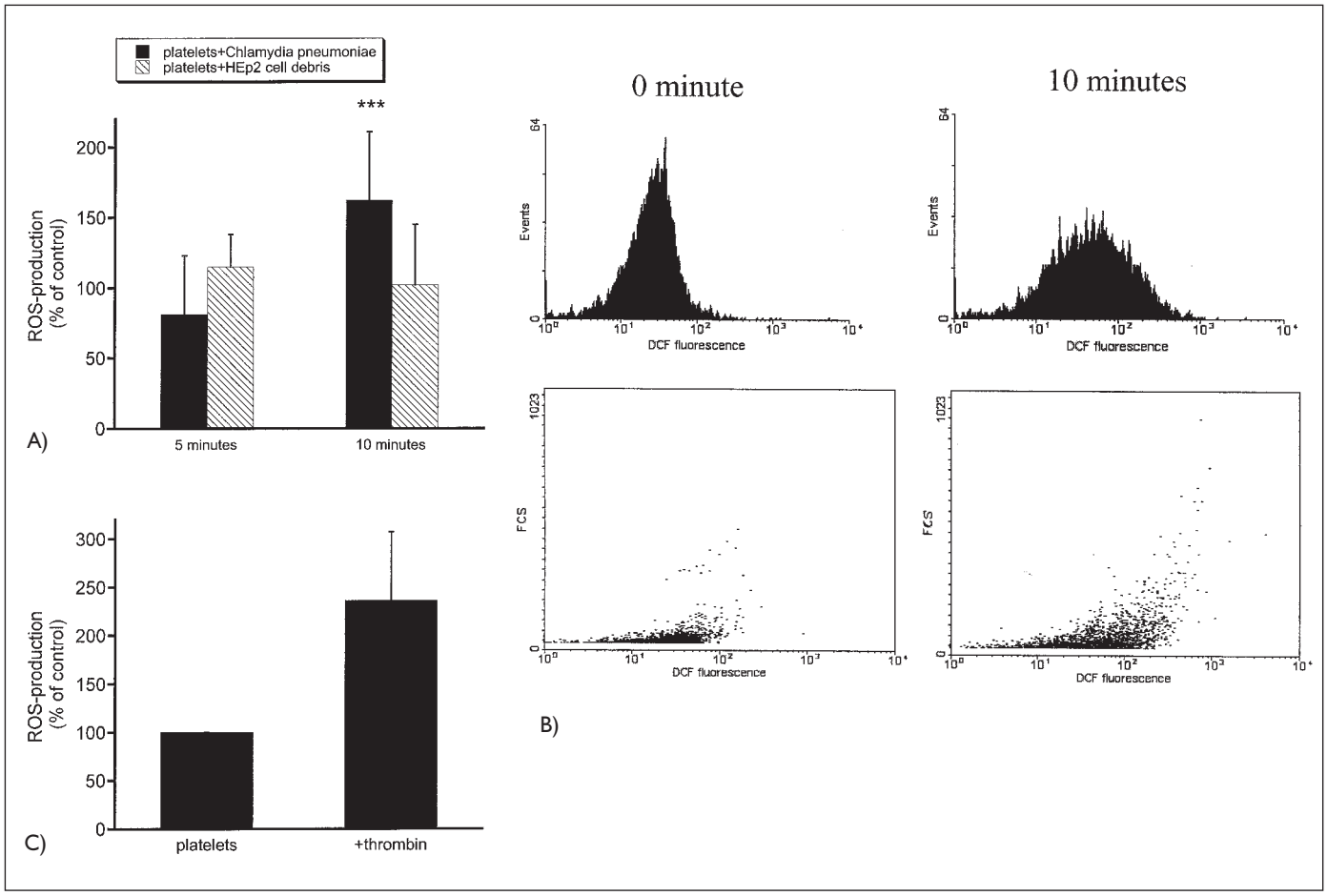

Figure 1: Effects of *C. pneumonaie* on intracellular ROS-production in platelets. Platelets were loaded with DCFH for 15 min at 37°C in dark and then stimulated with *C. pneumoniae* (bacteria:platelet ratio of 1:15) or HEp2 cell debris for 5 or 10 min or thrombin (0.5 units/ml) for 1 min under stirring conditions. Thereafter the cells were fixed with 2.5% formaldehyde and DCF fluorescence in platelets was measured with flow cytometry. The figures show ROS-production of platelets measured as geographical mean fluorescence after stimulation with *C. pneumoniae*, HEp2 cell debris (A) or thrombin (C). B, shows representative flow cytometric analysis of DCF-fluorescence in platelets stimulated with *C. pneumoniae* for 10 min. Data are expressed as mean ± standard deviation in percent of a platelet control of 15 (A) and 3 (C) separate experiments, respectively, run in duplicate. The data are compared to an untreated platelet control by using paired students *t*-test.

LDL (100 µg/ml) was incubated with *C. pneumoniae* and/or platelets (bacteria:platelet ratio of 1:15) or (2-amidinopropane)dihydrochloride (AAPH), 10 mM) in a 24-well plate, for 30 min at 37°C 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 analyzis 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 were 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.

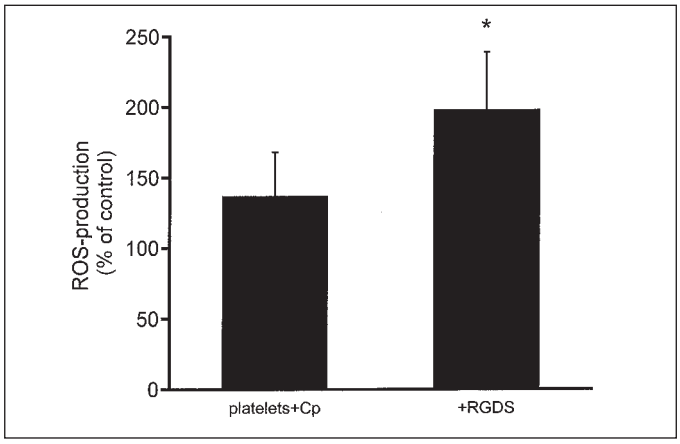

Figure 2: The effects of GpIIb/IIIa inhibition on platelet ROS-production induced by *C. pneumoniae*. Platelets were treated with Arg-Gly-Asp-Ser (RGDS, 1 mg/ml) and DCFH-DA (5 mM) for 15 min and then stimulated with *C. pneumoniae* (bacteria:platelet ratio of 1:15) for 10 min under stirring conditions 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 unstimulated 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.

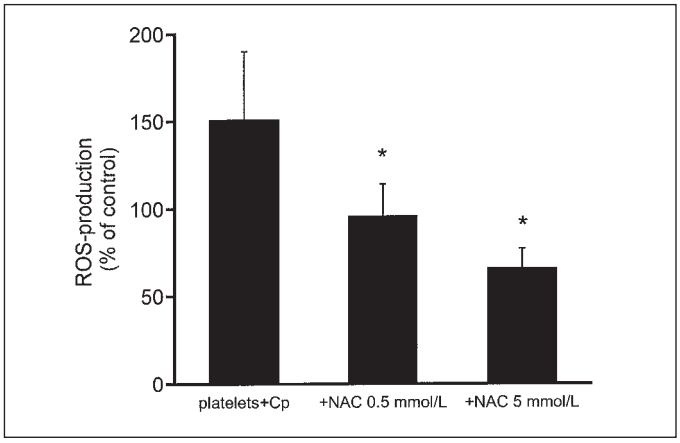

Figure 3: The effects of the ROS-scavenger N-acetyl cysteine (NAC) on platelet ROS-production stimulated by *C. pneumoniae*. Platelets were loaded with DCFH and with or without NAC (0.5 or 5 mM) for 15 minutes at 37°C in dark and then stimulated with *C. pneumoniae* (bacteria:platelet ratio of 1:15) 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.

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 stimulated by *C. pneumoniae* for 10 min (Fig. 2). In comparison 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

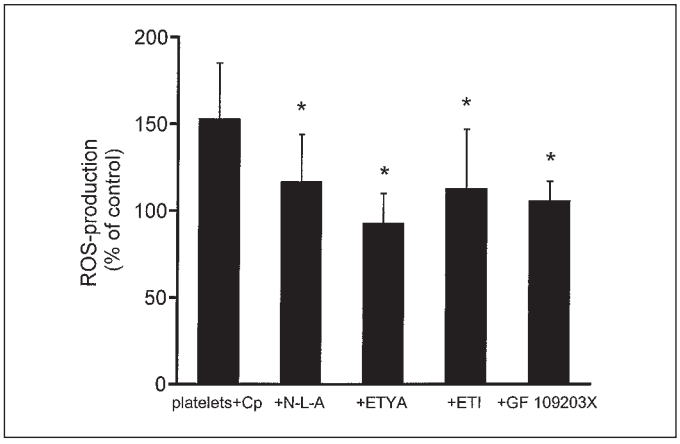

Figure 4: The effects of inhibitors of NOS, COX, LOX and protein kinase C on platelet ROS-production induced by *C. pneumoniae*. Platelets loaded with DCFH, were stimulated with *C. pneumoniae* (bacteria:platelet ratio of 1:15) for 10 min under stirring conditions, in the presence or absence of nitro-L-arginine (N-L-A, 0,1 mM), ETYA (10 μM), ETI (10 μM) or GF 109203X (50 μM). The cells were then fixed 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 nitro-L-arginine, ETYA, ETI or GF 109203X of 5 separate experiments run in duplicate. The data obtained with a specific drug is statistically compared with corresponding control (untreated platelets incubated with *C. pneumoniae*), i.e. the same donor and identical experimental conditions, by using paired students *t*-test.

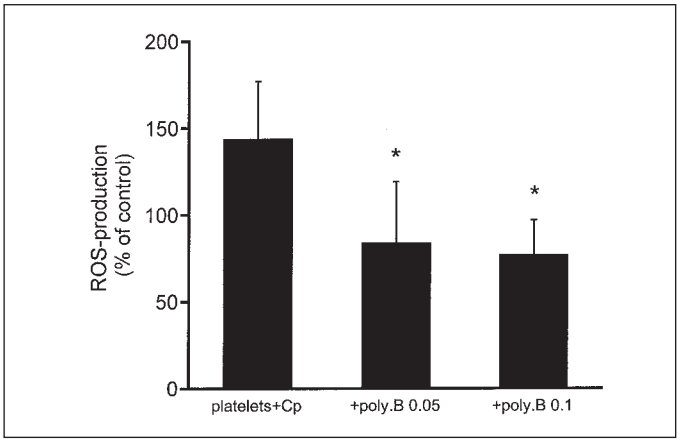

Figure 5: Inhibition of lipopolysaccharides antagonizes chlamydia-induced platelet ROS-production. *C. pneumoniae* was treated with polymyxin B (0.05 or 0.1 mM) for 30 min at room temperature and then mixed with DCFH-loaded platelets for 10 min. The cells were fixed and the fluorescence was thereafter measured with flow cytometry. The first bar shows the ROS production in platelets stimulated with untreated *C. pneumoniae*. Data are expressed as mean ± standard deviation in percent of a platelet control of 5 separate experiments run in duplicate. The ROS-production from platelets stimulated with polymyxin B-treated *C. pneumoniae* was statistically compared with platelets incubated with untreated *C. pneumoniae*. The mean value obtained with a specific concentration of polymyxin B is statistically compared with corresponding control, i.e. the same donor and identical experimental conditions.

kinase C and phosphoinositide 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 phosphoinositide-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 an 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 scavering 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, lipoxygenase (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 ETYA, and the LOX inhibitor 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 $ONOO^-$, H_2O_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_2O_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 GpIIb/IIIa and platelet aggregation increases *C. pneumoniae*-induced DCF-fluorescence correlates with a recent study showing that an antagonist to GpIIb/IIIa enhances platelet NO release (38). Alternatively, an increased enzymatic activity of LOX leads to generation of O_2^-, which reacts with NO and forms $ONOO^-$. Several studies have established that $ONOO^-$ 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 $ONOO^-$ 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

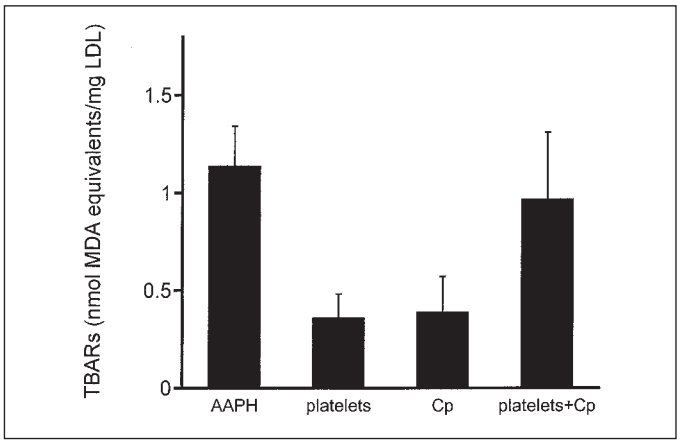

Figure 6: *Chlamydia pneumoniae* and platelets induce oxidation of low-density lipoproteins. Thiobarbituric acid reactive substances (TBARS) was used as an index of lipid peroxidation of LDL treated during 30 min with either AAPH, platelets or *C. pneumoniae* alone or a combination of platelets and *C. pneumoniae*. For further details see *Materials and Methods*. Negative control values obtained with buffer were subtracted from the data. The data are expressed as mean ± standard deviation of 3 different experiments run in triplicate.

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. *P. 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^- 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.

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Abbreviations

AAPH: (2-amidinopropane)dihydrochloride; COX: cyclooxygenase; DCFH-DA: dichlorofluorescin diacetate; DPI: di-phenyleneiodonium; ETI: 5,8,11-eicosatriynoic acid; ETYA: 5,8,11,14-eicosatetraynoic acid; FBS: fetal bovine serum; GF 108203X: (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) ; Gp: glycoprotein; H_2O_2: hydrogen peroxide; IL-1: interleukin 1; LDL: low-density lipoprotein; LOX: lipoxygenase; LPS: lipopolysaccharide; MCP-1: monocyte chemoattractant protein 1; NAC: N-acetyl-L-cysteine; NOS: nitric oxide synthase; ·OH: hydroxyl radical; $ONOO^-$: peroxynitrite; PBS: phosphate-buffered saline; PKC: protein kinase C; RGDS: Arg-Gly-Asp-Ser; ROS: reactive oxygen species; TBARS: thiobarbituric acid reactive substances.

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