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Platelet activation triggered by *Chlamydia pneumoniae* is antagonized by 12-lipoxygenase inhibitors but not cyclooxygenase inhibitors

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Abstract

Chlamydia pneumoniae is a respiratory pathogen that has been linked to cardiovascular disease. We have recently shown that *C. pneumoniae* activates platelets, leading to oxidation of low-density lipoproteins. The aim of the present study was to evaluate the inhibitory effects of different pharmacological agents on platelet aggregation and secretion induced by *C. pneumoniae*. Platelet interaction with *C. pneumoniae* was studied by analyzing platelet aggregation and ATP-secretion with Lumi-aggregometry. Platelet aggregation and ATP-secretion induced by *C. pneumoniae* was markedly inhibited by the NO-donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), an effect that was counteracted by the guanylyl cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). Pre-treatment of platelets with the 12-lipoxygenase (12-LOX) inhibitors cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) and 5,6,7-trikydroxyflavone (baicalein) completely blocked the activation, whereas the cyclooxygenase (COX) inhibitors 2-acetyloxybenzoic acid (aspirin) and (8E)-8-[hydroxy-(pyridin-2-ylamino)methylidene]-9-methyl-10,10-dioxo-10$l^(6)thia-9-azabicyclo[4.4.0]deca-1,3,5-trien-7-one (piroxicam) had no inhibitory effects. Opposite to *C. pneumoniae*-induced activation, platelets stimulated by collagen were inhibited by the COX-inhibitors but were unaffected by the 12-LOX-inhibitors. The platelet activating factor (PAF) antagonist Ginkgolide B blocked the *C. pneumoniae*-induced platelet activation, whereas the responses to collagen were unaffected. Furthermore, the $P2Y_1$ and $P2Y_{12}$ purinergic receptor antagonists 2'-deoxy-N^6-methyladenosine 3',5'-bisphosphate (MRS2179) and *N*(6)-(2-methyl-thioethyl)-2-(3,3,3-trifluoropropylthio)-beta,gamma-dichloromethylene-ATP (cangrelor) inhibited the aggregation and secretion caused by *C. pneumoniae*. It is well-known that the efficacy of COX inhibitors in the prevention and treatment of cardiovascular disease varies between different patients, and that patients with low responses to aspirin have a higher risk to encounter cardiovascular events. The findings in this study showing that platelets stimulated by *C. pneumoniae* are unaffected by COX inhibitors but sensitive to 12-LOX inhibitors, may thus be of importance in future management of atherosclerosis and thrombosis.

Keywords: *Chlamydia pneumoniae*; Platelet; Aggregation; Lipoxygenase; Cyclooxygenase

1. Introduction

Chlamydia pneumoniae is a common respiratory pathogen with a lifecycle consisting of an intracellular replicating phase (reticulate body) and an extracellular non replicating phase (elementary body). Approximately 70% of adults worldwide have antibody evidence of prior infection by this bacterium (Krull et al., 2005). In 1988, the first study was published showing an association between *C. pneumoniae* infection and acute myocardial infarction (Saikku et al., 1988), and since then a number of studies have reported a connection between *C. pneumoniae* and cardiovascular disease (Mussa et al., 2006).

C. pneumoniae (or its components) is frequently found in atherosclerotic plaques, which has been demonstrated by polymerase chain reaction (PCR) methods, immunohistochemical staining and electron microscopy (Gutierrez et al., 2001). The pathogen has been isolated from atheromas and propagated *in vitro*. Furthermore, several studies in animals have shown that *C. pneumoniae* promotes lesion initiation and progression, and that antibiotic treatment of infected animals prevented the

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development of arterial lesions (Hauer et al., 2006). Numerous *in vitro* experimental investigations have indicated that *C. pneumoniae* modulates the function of atheroma-associated cell-types in ways that are consistent with a contribution to atherogenesis (Hirono et al., 2003; Kalayoglu and Byrne, 1998; Molestina et al., 1999).

We have recently shown that *C. pneumoniae* binds to platelets and stimulates aggregation, secretion and surface up-regulation of P-selectin (Kalvegren et al., 2003). Furthermore, we found that platelets incubated with *C. pneumoniae* produce reactive oxygen species (ROS), leading to oxidation of low-density lipoproteins (LDL) (Kalvegren et al., 2005). This means that *C. pneumoniae*-induced platelet activation may be involved in both the initiation and progression of atherosclerosis and in the acute phase of thrombus formation.

The mechanisms involved in platelet interaction with *C. pneumoniae* are uncompletely understood. Our previous findings suggest that chlamydial lipopolysaccharide (LPS) is responsible for platelet activation. Furthermore, platelet glycoprotein IIb/IIIa has an important role in the bacteria-induced aggregatory response, whereas nitric oxide synthase (NOS), lipoxygenase (LOX) and activation of protein kinase C are involved in the ROS production (Kalvegren et al., 2005; Kalvegren et al., 2003).

The growing amount of evidence for an active role of *C. pneumoniae* and platelets in atherosclerosis argues for development of pharmacological strategies to reduce *C. pneumoniae*-induced platelet activation. The aim of this study was to evaluate the effects of a wide range of pharmacological agents on *C. pneumoniae*-induced platelet activation. The results obtained indicate that *C. pneumoniae* stimulates platelet activation by mechanisms different from collagen and thrombin by involving platelet activating factor (PAF) and activation of 12-LOX, rather than cyclooxygenase (COX).

2. Materials and methods

2.1. Materials

The following buffers and chemicals were used: acid citrate/dextrose solution (ACD): 85 mmol/l trisodium citrate dihydrate, 71 mmol/l citric acid hydrate and 111 mmol/l D-glucose; Krebs–Ringer Phosphate buffer (KRG): 0.1 mol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgSO_4$, 2 mmol/l KH_2PO_4, 8 mmol/l Na_2HPO_4 and 10 mmol/l glucose; PBS pH 7.3: 0.1 mmol/l NaCl, 3 mmol/l KCl, 7 mmol/l Na_2HPO_4 and 2 mmol/l KH_2PO_4; sucrose–phosphate buffer: 0.2 mol/l sucrose, 12.1 mmol/l K_2HPO_4 and 8.09 mmol/l KH_2PO_4; *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) (Alexis Biochemicals, San Diego, CA, USA); 5,8,11,14-eicosatetraynoic acid (ETYA) (Cayman Chemical Company, Ann Arbor, MI, USA); 5′-*N*-ethylcarboxamidoadenosine (NECA), thrombin, 3-hydroxy-2-(3-hydroxy-1-octenyl)-5-oxocyclopentaneheptanoic acid (prostaglandin E_1, PGE_1), 1*H*-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ), 2-acetyloxybenzoic acid (aspirin) (Sigma Chemical Corporation, St Louis, MO, USA); 5,6,7-trikydroxyflavone (baicalein), cinnamyl-3,4-dihydroxy-α-cyano-cinnamate (CDC) and (8E)-8-[hydroxy-(pyridin-2-ylamino) methylidene]-9-methyl-10,10-dioxo-10$l^(6)-thia-9-azabicyclo [4.4.0]deca-1,3,5-trien-7-one (piroxicam), Ginkgolide B (Biomol, Plymouth Meeting, PA, USA); collagen, luciferin–luciferase (Chrono-log Corporation, Haverton, PA; USA); 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate (MRS2179) and N^6-(2-methyl-tioethyl)-2-(3,3,3-trifluoro propylthio)-β, γ-dichloromethylene ATP tetrasodium salt) (cangrelor, formerly AR-C69931MX) was kindly provided by AstraZeneca and the Medicines Company; 12-hydroxyeicosatetranoic acid (12-HETE) was a kind gift from Professor S. Hammarström, Department of Cell Biology, Linköping University, Linköping, Sweden).

2.2. 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 mmol/l L-glutamine. The cells were incubated at 37 °C and 5% CO_2 in 75 cm^2 culture flasks, and then subcultured in 6-well plates at a density of 0.7×10^6 cells/well prior of infection with chlamydiae.

2.3. C. pneumoniae propagation

C. pneumoniae (strain T45) was cultured in HEp2 cells, grown and maintained in RPMI 1640, essentially as described by Redecke et al. (Redecke et al., 1998). The bacteria were added to subconfluent monolayers of HEp2 cells in 6-well plates. The plates were centrifuged at 480 ×*g* for 45 min 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 ± g/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 ×*g* for 10 min at 4 °C to remove cellular debris. Supernatants were centrifuged at 12 000 ×*g* for 30 min at 4 °C, and the bacteria were suspended in sucrose–phosphate buffer, supplemented with FBS (10%, heat inactivated), 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. The bacteria and cells were tested for mycoplasma contamination by using mycoplasma specific PCR essentially according to van Kuppeveld et al. (Van Kuppeveld et al., 1992).

2.4. 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, Sweden as previously described (Bengtsson et al., 1996). Five parts of blood were mixed with one part of ACD solution, followed by centrifugation at room temperature for 20 min at 220 ×*g* to obtain platelet rich plasma. The platelet rich plasma was centrifuged for 20 min at 480 ×*g*, and the platelets were then gently washed and resuspended in

KRG (final cell density 2×10^8/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. The contamination of other blood cells was negligible. The extracellular calcium concentration was adjusted to 1 mmol/l immediately before each experiment.

2.5. Platelet aggregation and ATP-secretion

Platelet aggregation and ATP-secretion were analyzed under stirring conditions at 37 °C using a two-sample, Lumi-Aggregometer model 560 (ChronoLog Corpration, Haverton, PA, USA). Aggregation was measured as the change in light transmission using KRG buffer as a reference. The unstimulated platelet suspension was set to 0% light transmission and the KRG-buffer to 100% light transmission. In parallel ATP secretion was measured as a change in bioluminescence. In the presence of ATP the firefly extract luciferin interacts with the enzyme luciferase and the emitted light can be detected and quantified. By adding a known amount of ATP at the end of each measurement, the ATP-release in each sample was calculated.

Before measurement, 1 mmol/l $CaCl_2$ and a mixture of luciferin–luciferase (1.6 μg/ml luciferin and 176 U/ml luciferase) were added to the platelet sample. Samples were then incubated with or without different inhibitors: 10 μmol/l SNAP (IC_{50} 7.1 μmol/l (Zembowicz et al., 1990)), 2 μmol/l ODQ (IC_{50} 0.72 μmol/l (Schrammel et al., 1996)), 10 μmol/l NECA (IC_{50} 86 nmol/l (Barrington et al., 1989)), 10 μmol/l PGE_1 (IC_{50} 21 nmol/l), 10 μmol/l ETYA (inhibits platelet 12-LOX and COX-1 with IC_{50} values of 4 μmol/l and 8 μmol/l (Hammarstrom, 1977)), 10 μmol/l piroxicam (IC_{50} 17.7 μmol/l (Carty et al., 1980)), 1 μmol/l CDC (IC_{50} 0.063 μmol/l (Cho et al., 1991)), 1 μmol/l baicalein (IC_{50} 0.12 μmol/l (Sekiya and Okuda, 1982)), 10 nmol/l cangrelor (IC_{50} 4 nmol/l (Chattaraj, 2001)), 10 μmol/l MRS2179 (IC_{50} 5 μmol/l (Nylander et al., 2003)) and/or 1 μmol/l Ginkgolide B (IC_{50} 2.22 μmol/l (Nunez et al., 1986)) for 5 min and 100 μmol/l aspirin for 30 min followed by stimulation with *C. pneumoniae* (*C. pneumoniae*:platelet ratio of 1/15), 0.1 U/ml thrombin or 1 μg/ml collagen. Control measurements with cell debris from uninfected HEp2 cells were also accomplished. CDC and baicalein are selective inhibitors of 12-LOX. As mentioned above the IC_{50} value for baicalein is 0.12 μmol/l and approximately 7000 times lower than the IC_{50} value of this drug for COX (Sekiya and Okuda, 1982). Furthermore, baicalein also inhibits macrophage leukotriene biosynthesis with an IC_{50} value of 9.5 μmol/l (Butenko et al., 1993). The IC_{50} values for CDC are 0.063 μmol/l, 3.33 μmol/l and 1.89 μmol/l for 12-, 15- and 5-LOX respectively in isolate enzyme preparations (Cho et al., 1991).

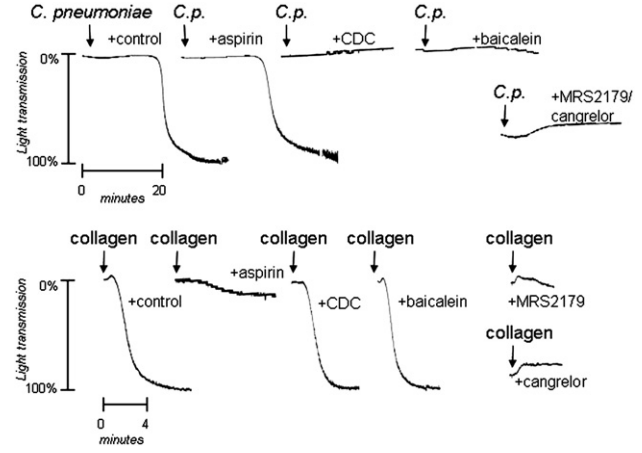

Fig. 1. Representative traces of platelet aggregation induced by *Chlamydia pneumoniae* and collagen. Platelets were monitored for aggregation during interaction with A) *C. pneumoniae* (Cp: platelet ratio of 1:15) and B) collagen (1 μg/ml). The figure also shows the traces when platelets were pre-incubated with aspirin (100 μmol/l), CDC (1 μmol/l), baicalein (1 μmol/l), cangrelor (10 nmol/l) and MRS2179 (10 μmol/l) before stimulation.

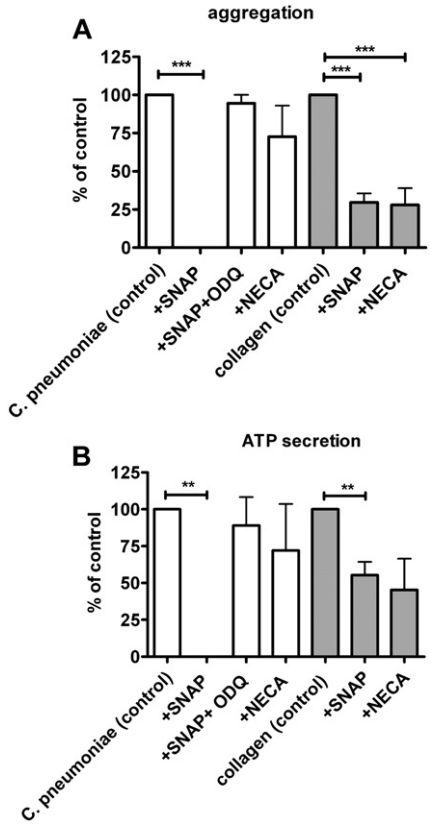

Fig. 2. Modulation of the cGMP and cAMP system in collagen and *Chlamydia pneumoniae* stimulated platelets. Platelets were preincubated in 37 °C for 5 min with SNAP (10 μmol/l), SNAP (10 μmol/l)/ODQ (2 μmol/l) and NECA (10 μmol/l), stimulated with *C. pneumoniae* (Cp:platelet ratio 1:15) or collagen (1 μg/ml) and then monitored for aggregation and ATP secretion. The data are expressed as % of control of platelets incubated without inhibitors and then stimulated with *C. pneumoniae* and collagen, and represent the mean ± S.E.M. from 3–4 different experiments.

2.6. Statistical analysis

All results are presented as mean ± S.E.M. One-way ANOVA (Newman–Keuls Multiple Comparison Test) was used for statistical analysis. A P-value of <0.05 was considered to be statistically significant.

3. Results

The addition of *C. pneumoniae* to a suspension of platelets (2×10^8 platelets/ml) in a *C. pneumoniae*:platelet ratio of 1:15 triggered an extensive aggregation and ATP-secretion after a lag period of approximately 10–20 min (Fig. 1). Collagen (1 μg/ml; Fig. 1) and thrombin (0.1 units/ml) induced platelet activation to similar magnitude, however, considerably faster. In order to characterize the *C. pneumoniae*-induced platelet activation we investigated the effect of a number of anti-platelet drugs.

3.1. Manipulation of the cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) signalling systems

Many platelet activators are effectively antagonized by NO/cGMP- and cAMP-elevating agents. We found that the NO-donor SNAP (10 μmol/l) completely inhibited the *C. pneumoniae*-induced aggregation and ATP-secretion. This effect was reversed by the gyanylyl cyclase inhibitor ODQ (2 μmol/l; Fig. 2A and B), indicating a pivotal role of cGMP. Furthermore, PGE_1 (10 μmol/l) completely inhibited the bacteria-induced stimulation (data not shown), whereas the A_2 adenosine receptor agonist NECA (10 μmol/l) partly antagonized the effect (Fig. 2A and B). In comparison, the collagen-induced activation was significantly reduced by both SNAP and NECA (Fig. 2A and B).

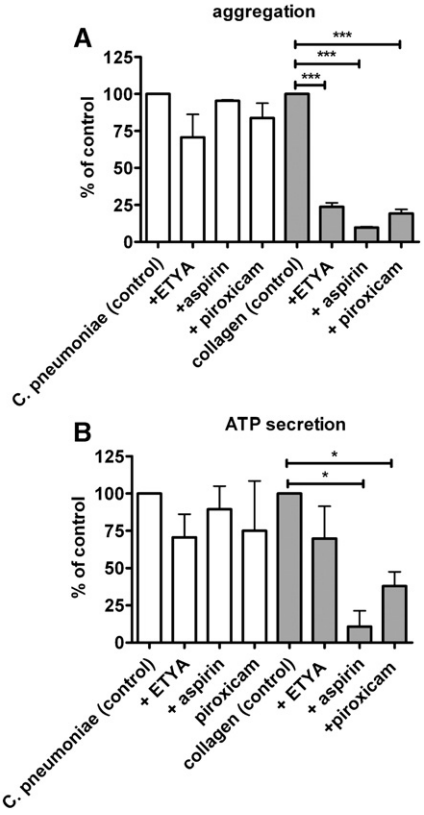

Fig. 3. Inhibition of the cyclooxygenase in *Chlamydia pneumoniae* and collagen stimulated platelets. After preincubation at 37 °C for 30 min with aspirin (100 μmol/l) and 5 min with ETYA (10 μmol/l) and piroxicam (10 μmol/l), platelets were stimulated with *C. pneumoniae* (Cp:platelet ratio 1:15) or collagen (1 μg/ml) and then monitored for aggregation and ATP secretion. The data are expressed as % of control of platelets incubated without inhibitors and then stimulated with *C. pneumoniae* and collagen, and represent the mean±S.E.M. from 3–6 different experiments.

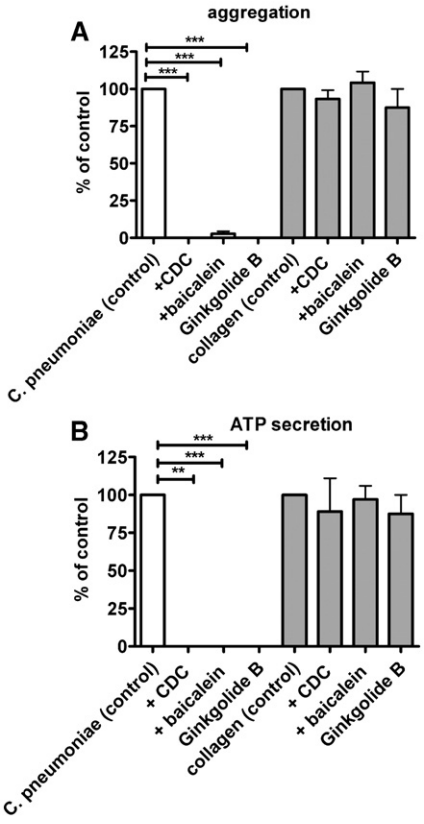

Fig. 4. Inhibition of the lipoxygenase in *Chlamydia pneumoniae* and collagen stimulated platelets. Platelets were incubated with CDC (1 μmol/l), baicalein (1 μmol/l) and Ginkgolide B (1 μmol/l) for 5 min and then stimulated with *C. pneumoniae* (Cp:platelet ratio 1:15) or collagen (1 μg/ml). The platelet aggregation and ATP secretion were thereafter registered. The data are expressed as % of control of platelets incubated without inhibitors and then stimulated with *C. pneumoniae* and collagen, and represent the mean±S.E.M. from 3–4 different experiments.

3.2. Inhibition of the LOX and COX pathways

Arachidonic acid metabolites, especially thromboxane A_2 (TxA_2), play an important role in platelet activation. The combined LOX and COX inhibitor ETYA (10 μmol/l) only slightly reduced *C. pneumoniae*-induced platelet activation (Fig. 3A and B). However, this inhibitor significantly prevented the collagen-induced aggregation, and partly diminished the associated ATP-secretion (Fig. 3A and B).

When separately manipulating the COX pathway of the arachidonic acid metabolism we found that the COX-inhibitors aspirin (100 μmol/l) and piroxicam (10 μmol/l) had no effects on *C. pneumoniae*-induced aggregation and ATP secretion (Fig. 3A and B). In contrast collagen responses were markedly antagonized by the COX inhibitors (Fig. 3A and B).

The influence of the 12-LOX pathway was further characterized by pre-treating platelet with the specific 12-LOX inhibitors baicalein (1 μmol/l) and CDC (1 μmol/l). We found that these inhibitors entirely blocked the platelet responses

induced by *C. pneumoniae* (Fig. 4A and B), whereas the responses to collagen were unaffected. 12-LOX in platelets metabolizes arachidonic acid to 12-hydroperoxyeicosatetraenoic acid (12-HPETE), which is unstable and either directly reduced to 12-HETE or metabolized to lipoxins. We investigated the effects of 12-HETE (1.2 μmol/l) and 12-HPETE (1 μmol/l) on platelet aggregation and secretion, but found no stimulatory effects of these 12-LOX products. Furthermore, 12-HETE did not reverse the inhibitory effects of CDC or baicalein on *C. pneumoniae*-induced platelet activation (data not shown).

3.3. Inhibition of PAF

Platelets synthesize from arachidonic acid the lipid mediator PAF, which stimulates platelets by binding to specific receptors. In the present study, we used Ginkgolide B, which accelerates PAF hydrolysis by promoting PAF-acetylhydrolase activity (Bonin et al., 2004). Ginkgolide B totally blocked the *C. pneumoniae*-induced platelet aggregation and secretion (Fig. 4A and B). In contrast, PAF-inhibition had no inhibitory effects on collagen-induced platelet activation (Fig. 4A and B).

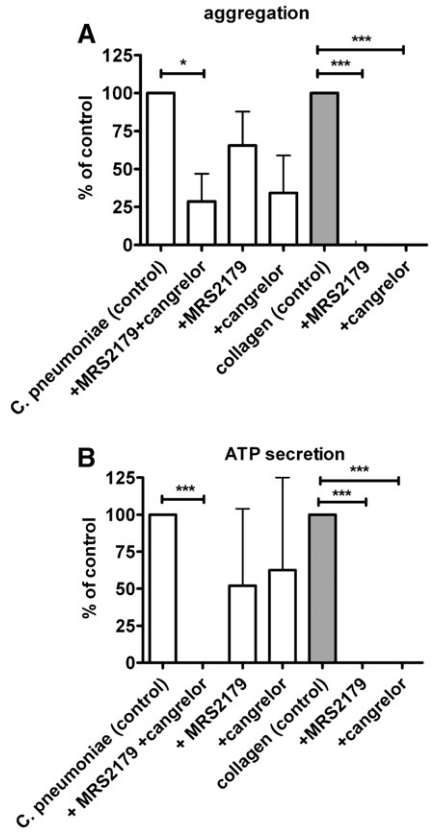

Fig. 5. Effects of ADP-receptor antagonists on *Chlamydia pneumoniae* and collagen induced platelet activation. Platelets were treated with the $P2Y_1$ and $P2Y_{12}$ receptor antagonists MRS2179 (10 μmol/l) and cangrelor (10 nmol/l) for 5 min, followed by stimulation with *C. pneumoniae* (Cp:platelet ratio of 1:15) or collagen (1 μg/ml). Thereafter the aggregation and ATP secretion were calculated. The data are expressed as % of control of platelets incubated without inhibitors and then stimulated with the bacteria, and represent the mean ± S.E.M. from 3–6 different experiments.

3.4. The role of $P2Y_1$ and $P2Y_{12}$ purinergic-receptors

ADP has been shown to augment platelet activation. When studying the importance of the ADP-receptors in *C. pneumoniae*-induced platelet activation we found that incubation with both the $P2Y_1$ receptor antagonist MRS 2179 (10 μmol/l) and the $P2Y_{12}$ receptor antagonist cangrelor (10 nmol/l) significantly inhibited the aggregatory and secretory response (Fig. 5A and B). When used separately, MRS2189 and cangrelor also showed reducing effects, however not in a significant way. In contrast, MRS2179 and cangrelor significantly inhibited collagen-induced platelet activation when used separately (Fig. 5).

3.5. Modulation of thrombin-induced platelet activation

Thrombin is considered to be the most effective activator of human blood platelets. None of the drugs used in this study affected thrombin (0.1 U/ml)-stimulated platelets (data not shown).

4. Discussion

There is a growing amount of evidence for a role of *C. pneumoniae* in the initiation and progression of atherosclerosis (Campbell and Kuo, 2004). We have recently shown that *C. pneumoniae* stimulates platelet aggregation, granule secretion and ROS production (Kalvegren et al., 2005; Kalvegren et al., 2003). These events may directly participate in the progression of atherosclerosis and formation of thrombus. When exploring new strategies to prevent atherosclerosis and thrombosis it may thus be of importance to inhibit platelet activation caused by *C. pneumoniae* infection. In this study, we therefore evaluated the effect of a wide range of platelet-inhibitors on *C. pneumoniae*-induced platelet activation.

In consistent with our earlier findings (Kalvegren et al., 2003), we found that *C. pneumoniae* triggers platelet aggregation and secretion. The maximal magnitude of the platelet aggregatory response induced by the bacteria was comparable with that observed when adding high doses of collagen or thrombin. This indicates that *C. pneumoniae* has pronounced effects on human blood platelet function. However, opposite to collagen and thrombin, *C. pneumoniae*-induced platelet aggregation was preceded by a surprisingly long lag-phase. This lag-phase is probably related to the low *C. pneumoniae*:platelet ratio (1:15). More specifically, we propose that elementary bodies of *C. pneumoniae* initially stimulate a few platelets, which then activate neighbouring platelets by an autocrine-signalling mechanism. Ultimately, this leads to a cascade reaction, finally resulting in a powerful platelet aggregation and secretion.

We found that the NO-donor SNAP abolished platelet aggregation and ATP-secretion induced by *C. pneumoniae*. The inhibitory effects of SNAP are considered to be due to an NO-mediated activation of guanylyl cyclase and a subsequent increase in platelet cGMP content. The increase in cGMP lowers the cytosolic levels of calcium (Geiger et al., 1992). However, several cGMP-independent actions of NO have also been described (Davis et al., 2001). In our study, the inhibitory

effects of SNAP were fully antagonized by the guanylyl cyclase inhibitor ODQ, which strongly suggests that cGMP mediates the inhibition of *C. pneumoniae*-stimulated platelets.

The purine nucleoside adenosine is an anti-aggregatory and anti-inflammatory agent and has been considered as an inhibitor of the atherosclerotic process (Sands and Palmer, 2005). The effects of adenosine on platelets are mediated by an A_2-receptor-induced activation of adenylyl cyclase leading to increased formation of cAMP (Feoktistov et al., 1992). We found that the adenosine analogue NECA only slightly reduced the *C. pneumoniae*-induced aggregation, whereas the drug significantly suppressed platelet activation triggered by collagen. However PGE_1, which acts on its receptor protein and thereby activates adenylyl cyclase, strongly inhibited platelet activation induced by *C. pneumoniae*. Taken together, we propose that elevation of either cGMP or cAMP antagonizes *C. pneumoniae*-induced platelet activation. In accordance, it is well-known that cyclic nucleotide-elevating agents suppress the effects of a wide range of platelet activators (Paul et al., 1990). It is to be noted that thrombin-evoked platelet responses were unaffected by pretreatment with SNAP or NECA. This is partially consistent with our earlier findings, investigating the effect of the NO-donor GEA 3175 and adenosine on thrombin-induced platelet aggregation and secretion (Grenegard et al., 1996; Asplund Persson et al., 2005). These drugs could not inhibit thrombin-stimulated aggregation separately, but when administrated together they markedly reduced the response. This means that thrombin easily could overcome the inhibitory actions of the NO/cGMP or the cAMP signalling pathway.

The purine nucleotide ADP, stored in platelet dense granule, has a crucial role as an autocrine activator of platelets. Two G-protein coupled purinergic receptors, termed $P2Y_1$ and $P2Y_{12}$, are expressed on the surface of human platelets (Gachet, 2006). The $P2Y_1$ receptor is considered to initiate the aggregatory response to collagen and ADP (Gachet, 2006; Mangin et al., 2004). The other purinergic-receptor, $P2Y_{12}$, has an important role in the completion of ADP-induced platelet aggregation, and in contrast to $P2Y_1$, this receptor amplifies the aggregation triggered by all known platelet agonists (Conley and Delaney, 2003). In the present study, both the $P2Y_1$ and the $P2Y_{12}$ purinergic-receptor antagonists MRS2179 and cangrelor, inhibited the collagen-induced aggregation and secretion of platelets. The aggregation and secretion induced by *C. pneumoniae*, however, was inhibited by a combination of MRS2179 and cangrelor. Mono treatment of platelets with either MRS2179 or cangrelor was less effective in this regard. This points out for an important role of both $P2Y_1$ and $P2Y_{12}$ receptor signalling in mediating aggregation and secretion of *C. pneumoniae*-stimulated platelets.

A large number of studies have implicated a pivotal role for TxA_2 biosynthesis and release in platelet activation. In similarity with earlier studies (Gaynor and Constantine, 1979), the COX-inhibitors aspirin and piroxicam gave a pronounced suppressing effect on collagen-stimulated platelets. This is explained by the fact that activation of platelets by collagen, as well as by many other activators, is considered to require the synthesis of TxA_2 (Roberts et al., 2004). Most interestingly, we found that the COX inhibitors aspirin and piroxicam had no inhibitory influence on platelet activation induced by *C. pneumoniae*, which indicates that the COX pathway and its biological active end products (*i.e.* TxA_2) have a minor role. On the contrary, both 12-LOX inhibitors (baicalein and CDC) displayed complete antagonizing effects on *C. pneumoniae*-induced platelet activation, whereas the inhibitors had no effects on collagen-stimulated platelets. Thrombin is the most powerful endogenous platelet activator. This probably explains why the COX and LOX inhibitors did not affect thrombin-induced platelet aggregation and secretion.

Our results thus suggest that eicosanoids metabolized by the 12-LOX pathway have a pivotal role in platelet activation induced by *C. pneumoniae*. In platelets, 12-LOX metabolizes arachidonic acid to 12-HPETE, which is unstable and either directly reduced by cellular peroxidases to 12-HETE, or metabolized to lipoxins (Samuelsson, 1983; Setty et al., 1992). This probably means that the *C. pneumoniae*-induced platelet activation is dependent on 12-HETE and/or lipoxins produced by 12-LOX. Interestingly, it has been found that a reduction of intraplatelet 12-HETE levels in dogs results in inhibition of coronary thrombosis (Katoh et al., 1998), thus indicating a role of 12-LOX activity in the pathogenesis of thrombosis. Further support for a specific role of the 12-LOX pathway in *C. pneumoniae*-induced platelet activation is the recent study by Olivera-Severo et al. (2006) showing that *Bacillus pasteurii* urease-induced platelet aggregation is mediated by 12-LOX and secreted ADP, but independent of COX activation (Olivera-Severo et al., 2006). Our findings showing that addition of 12-HETE did neither by itself stimulate platelet aggregation nor reverse the effects of 12-LOX-inhibitors on *C. pneumoniae*-induced platelet aggregation may have several explanations: i) failure in mimicking the intracellular generation and activity of 12-HETE by an exogenous administration of this short-lived metabolite ii) other 12-LOX metabolites and reactive intermediates may have a crucial role, by themselves or together with 12-HETE iii) other intracellular mediators than eicosanoids may be important in a joint action with 12-LOX metabolites. PAF and the eicosanoids are usually synthesized in parallel and may act synergistically to elicit cellular responses (Wykle, 2004). Interestingly, platelet aggregation in response to PAF has recently been shown to be activated mainly by the 12-LOX pathway (Michibayashi, 2005). In correlation, we found that the PAF antagonist Ginkgolide B totally prevented the *C. pneumoniae*-induced platelet aggregation. Consequently, it is possible that the platelet activation triggered by *C. pneumoniae* is dependent on a synergistic action of both PAF and 12-LOX metabolites. In neutrophils, the sensitivity to PAF as a trigger of degranulation is increased 100-fold in the presence of the eicosanoid 5-hydroxyeicosatetraenoic acid (5-HETE) (O'Flaherty et al., 1983).

During the past decades, inflammation has been increasingly recognized as a key event in the formation of atherosclerotic plaques (Ross, 1999). *C. pneumoniae*-induced release of growth factors, cytokines and ROS from activated platelets may trigger and support the inflammatory responses in the vessel wall. This emphasizes the requirement of specific therapies to prevent a harmful platelet activation, thrombosis and atherosclerosis in

C. pneumoniae-infected patients. When used in the treatment of cardiovascular diseases, the efficacy of aspirin in inhibiting platelet functions varies markedly between different patients. Furthermore, hazardous cardiovascular events preferentially occur in patients with low responses to aspirin therapy (Horiuchi, 2006). The findings in this study may explain why some patients show no benefiting effect of aspirin treatment. The presence of *C. pneumoniae* bacteria in the circulation and in atherosclerotic plaques may permit platelet activation despite ongoing aspirin treatment.

In conclusion, this study shows that inhibition of purinergic receptors, PAF and 12-LOX, but not COX, counteracts harmful *C. pneumoniae*-induced platelet activation. These findings may be of importance in the development of new pharmacological strategies to avoid uncontrolled platelet activation and thrombosis.

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