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Lipopolysaccharide as trigger of platelet aggregation via eicosanoid over-production

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Summary

The effect of lipopolysaccharide (LPS) on platelet aggregation is still controversial. We performed *in vitro* and *ex vivo* studies in controls and in patients with community-acquired pneumonia (CAP) to assess the effect of LPS on platelet activation (PA). LPS (15–100 pg/ml) significantly increased PA only if combined with sub-threshold concentrations (STC) of collagen or ADP; this effect was associated with increased platelet H_2O_2 production, Nox2 activation, PLA2 phosphorylation, thromboxane (Tx)A_2 and 8-iso-PGF2α-III, and was inhibited by aspirin, TxA2 receptor antagonist or by Toll-like receptor 4 blocking peptide (TLR4bp). Analysis of up-stream signalling potentially responsible for Nox2 and PLA2 activation demonstrated that LPS-mediated PA was associated with phosphorylation of AKT, p38 and p47phox translocation. In 10 consecutive CAP patients serum endotoxins were significantly higher compared to 10 controls (145 [115–187] vs 18 [6–21] pg/ml; $p<0.01$). *Ex vivo* study showed that agonist-stimulated platelets were associated with enhanced PA ($p<0.01$), Toll-like receptor 4 (TLR4) expression ($p<0.05$), TxA_2 ($p<0.01$) and 8-iso-PGF2α-III ($p<0.01$) production in CAP patients compared to controls. The study provides evidence that LPS amplifies the platelet response to common agonists via TLR4-mediated eicosanoid production and suggests LPS as a potential trigger for PA in CAP.

Keywords

LPS, platelet activation, oxidative stress, community-acquired pneumonia

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Received: November 15, 2016
Accepted after major revision: April 9, 2017
Epub ahead of print: May 11, 2017
https://doi.org/10.1160/TH16-11-0857
Thromb Haemost 2017; 117: 1558–1570

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Introduction

Systemic inflammation secondary to an infection may be associated to artery and venous thrombosis; thus, recent studies have shown that patients with sepsis secondary to community-acquired pneumonia (CAP) are characterised by an enhanced risk of myocardial infarction (MI) and stroke in the early phase of hospitalisation (1). Such cardiovascular complications have a negative impact in short- and long-term follow-up as they enhance the risk of mortality and recurrence of cardiovascular events (2). Among the mechanisms potentially accounting for the association between sepsis and thrombosis, platelets may play a central role. Thus, a recent study from our group demonstrated an enhanced risk of MI in CAP patients with systemic signs of platelet activation; more in particular we found that platelet thromboxane(Tx)A_2 over-production and *in vivo* platelet activation were significantly associated with MI. Platelet activation was suggested to be dependent upon infection severity but the intrinsic mechanism was not identified (3). Recent studies suggested that, during infection, patients disclose a gut barrier dysfunction, which could cause lipopolysaccharide (LPS) translocation into systemic circulation (4). Bacterial LPS could account for platelet activation but, so far, there is uncertainty as to whether LPS is actually capable of activating platelets. Thus experimental studies demonstrated that LPS may have null, inhibitory and pro-aggregating effects but the reason for these discrepancies is still unclear (5, 6). A recent study by Zhang et al. (7) shed light on this issue by demonstrating that LPS per se is unable to aggregate platelets but amplifies the platelet response to common agonists upon interaction with its Toll-like receptor 4 (TLR4) (7). The study, however, did not fully solve this issue because LPS concentration used for *in vitro* experiments was in a range between 1 and 10 µg/ml, which is much higher than that potentially achievable in human circulation during infections or sepsis (8). Based on this, we undertook an *in vitro* and *ex vivo* study with the main objective to investigate if endotoxins are implicated in platelet aggregation and the mechanism potentially accounting for it in CAP patients. The study encompassed two phases: 1) *in vitro* experiments to assess if LPS, in a range of concentrations detected in blood of CAP, was capable of activating platelets, and 2) *ex vivo* platelets aggregation in CAP patients and controls.

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Materials and methods

In vitro study

Platelet aggregation

Citrated blood samples were taken between 8 and 9 am from healthy subjects (HS) who had fasted for at least 12 hours (h)**.** To obtain platelet-rich plasma (PRP), citrated blood samples were centrifuged for 15 minutes (min) at 180g. To avoid leukocyte contamination, only the top 75 % of the PRP was collected according to Pignatelli et al. (9).

To obtain washed platelets, PRP was treated with acid citrate dextrose (ACD) (10/1 v/v), centrifuged at 300g for 10 min (twice) and suspended in fatty acid free Tyrode's buffer (2×10^8 platelets/ml). Before activation, samples were pre-incubated (20 min at 37 °C) with TLR4 inhibitor peptide, that inhibits TLR4 signalling by blocking interactions between TLR4 and its adaptors proteins (4 µmol/l, Novus Biologicals, Littleton, CO, USA), or control peptide (CP) (4 µmol/l, Novus Biologicals), aspirin (ASA), which inhibits the COX-1 pathway preventing TxA_2 biosynthesis (100 µmol/l) (9) or SQ29548, a TxA_2/isoprostane receptor inhibitor (0.1 µmol/l, Calbiochem, San Diego, CA, USA) (9) or Nox2dstat, the most specific and efficacious Nox inhibitor that specifically inhibits interactions between Nox2 and p47phox (50 µmol/l, Anaspec, Fremont, CA, USA) (10). To study the intra-signalling pathway, we used the p38 inhibitor SB202190 (5 µmol/l, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as p38 is involved in ROS (11) and eicosanoids production (12). After incubation, samples were treated with LPS from *Escherichia coli* 0111:B4 (Sigma Cat# L3015, protein contamination <1 %, Sigma Aldrich, St. Louis, MO, USA) (15–100 pg/ml) 5 min before activation with sub-threshold (STC) concentration of agonists such as collagen (0.25 µg/ml, Mascia Brunelli, Milan, Italy) or ADP (2 µmol/l) for 10 min at 37 °C. STC concentration of agonists was defined as the highest concentration that elicited <20 % platelet aggregation. After stimulation with agonists, samples were centrifuged for 3 min at 3000g. Supernatants were stored at –80 °C for analysis of sNox2-dp, TxB2, 8-Iso-PGF2α -III and pellets were stored at –80 °C for analysis of AKT, cPLA2, p38 phosphorylation and p47phox translocation.

The intra-assay and inter-assay coefficients of variation were 5.7 % and 6.8 %, respectively.

H_2O_2 production

Hydrogen peroxide (H_2O_2) was evaluated by a Colorimetric Detection Kit (Arbor Assays, Ann Arbor, MI, USA) and expressed as µmol/l. Intra-assay and inter-assay coefficients of variation were 2.1 % and 3.7 %, respectively.

Platelet soluble Nox2-derived peptide

Extracellular levels of soluble Nox2-derived peptide (sNox2-dp), a marker of NADPH oxidase activation, were detected by ELISA as previously described (13). The peptide was recognised by the specific monoclonal antibody against the amino acidic sequence (224–268) of the extra membrane portion of Nox2 (catalytic core of NADPH oxidase), which was released in the medium upon platelet activation. Values were expressed as pg/ml; intra-assay and inter-assay coefficients of variation were 5.2 % and 6 %, respectively.

Platelet and urinary TxB_2 and 8-Iso-PGF2α assays

Platelet TxA_2 was analysed as previously described (14) by evaluating its stable metabolite TxB_2 by ELISA commercial kit (Cusabio, College Park, MD, USA) and expressed as pg/ml $\times10^8$ cells. Intra- and inter-assay coefficients of variation for TxB_2 were 4.0 % and 3.6 %, respectively. Urinary 11-dehydro TXB_2 was analysed by ELISA commercial kit (DRG International, Springfield, NJ, USA) and expressed as pg/mg creatinine.

Platelet and urinary isoprostane (8-iso-PGF2α -III) were measured by the enzyme immunoassay method (DRG International) and expressed as pmol/l and pg/mg creatinine, respectively. Intra-assay and inter-assay coefficients of variation were 5.8 % and 5.0 %, respectively.

Endotoxin levels

Endotoxin (LPS) serum and plasma levels were measured by ELISA Kit (Cusabio). Antibody specific for LPS has been precoated onto a microplate and 100 µl of standards or sample was plated for 2 h at room temperature. After incubation, samples were read at 450 nm. Values were expressed as pg/ml; intra-assay and inter-assay coefficients of variation were 8 % and 10 %, respectively.

Membrane and cytoplasmic proteins extraction

Briefly, the extraction of membrane and cytoplasmic proteins was performed by using the ProteoJET Membrane Protein Extraction Kit (Fermentas International Inc, Burlington, ON, Canada) as reported by Fortuño et al. (15).

Western blot analysis of TLR4, AKT, cPLA2, p38MAP-Kinase and $p47^{phox}$

Platelet pellets were suspended in a 2X Lysis buffer (5 mM EDTA, 0.15 mol NaCl, 0.1 mol Tris pH 8.0, 1 % triton and 10 µg/ml of protease and phosphatase inhibitors cocktail). Equal amounts of protein (30 µg/lane) estimated by Bradford protein assay were solubilised in a 2X Laemmli sample buffer containing 20 % of 2-mercaptoethanol. Proteins were separated by SDS-PAGE on 10 % polyacrylamide gel and then electro-transferred to nitrocellulose membranes. After blocking, membranes were incubated with different antibodies (all Santa Cruz Biotechnology): mouse monoclonal anti-p-AKT antibody raised against an amino acid sequence recognising the phosphorylation in the Ser473 site of AKT; mouse monoclonal anti-AKT antibody; polyclonal anti-p-$cPLA_2$ antibody raised against an amino acid sequence recognising the phosphorylation in the Ser505 site of $cPLA_2$; polyclonal anti-$cPLA_2$

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antibody; polyclonal anti-p-p38 antibody raised against an amino acid sequence recognising the phosphorylation in the Thr180 site of p38; polyclonal p38α antibody; mouse monoclonal anti-p47^{phox} antibody; polyclonal anti-TLR4 antibody or mouse monoclonal anti-β actin antibody and incubated overnight at 4°C. After, the membranes were incubated with secondary antibody (Santa Cruz Biotechnology, 1:5000) and then the immune complexes were detected by enhanced chemiluminescence substrate. Densitometric analysis of the bands was performed using Image J software.

Measurement of $[Ca^{2+}]i$ mobilisation

Platelet [Ca2+]i mobilisation was measured in PRP incubated for 20 min at 37 °C with 8 µmol/l FLUO 4-acetoxymethyl ester (FLUO 4-AM; Invitrogen, Carlsbad, CA, USA) in dimethyl sulfoxide. One ml of Tyrode buffer was added and the mixture was analysed by the Epics XL-MCL Cytometer (Coulter Electronics, Cooper City, FL, USA) equipped with an argon laser at 488 nm. The basal fluorescence intensity corresponding to FLUO 4-AM was measured for 1 min in the platelet population identified in the gate as CD61-positive events. LPS (15–100 pg/ml) or STC of agonists alone or LPS 15 pg/ml in presence of STC of agonists with or without TLR4bp were added and data acquired for 1 min.

Assessment of apoptosis

Apoptosis was determined by staining cells with Annexin V according to Hai et al. (16). Briefly, cells were harvested after activation with LPS (15–100 pg/ml) in presence of STC of collagen or ADP. As positive control of apoptosis, cells were treated with 50 mmol/l of arachidonic acid (AA). After incubation, cells were washed twice with cold phosphate-buffered saline and then suspended in 1 ml binding buffer (10 mmol/l HEPES/NaOH (pH 7.4), 140 mmol/l NaCl, 2.5 mmol/l $CaCl_2$) at a concentration of 1×10^6 cells/ml. Platelets were incubated with 5 µl Annexin V (8.3 mmol/l) (Invitrogen) for 10 min in the dark. Cell fluorescence was measured on Epics XL-MCL Cytometer (Coulter Electronics) equipped with an argon laser at 488 nm.

Ex vivo study

PA was evaluated in 15 consecutive aspirin-free patients (9 males, 6 females; age 65.0 ± 9.8 years), who were enrolled in a prospective study aimed at assessing the incidence of cardiovascular disease in CAP patients (3, 17); 10 age- and sex-matched controls (6 males, 4 females; age 64.8 ± 11.5 years), not taking antiplatelet drugs were used as controls. Patients with CAP and controls were consecutively recruited within a prospective, observational study, which was registered at ClinicalTrials.gov (Identifier: NCT01773863) at the Internal Medicine ward of "Sapienza" University of Rome. CAP patients were part of the SIXTUS study (3), which included consecutive patients according to the following criteria after giving written informed consent. Patients' characteristics have been previously reported (3). Severity of illness at presentation was quantified by the Pneumonia Severity Index (PSI), a validated prediction score for 30-day mortality in patients with CAP. In the same period of CAP enrolment, 10 hospitalised patients without acute infections matched for sex, age, and comorbidities were used as controls. *Ex vivo* PA was performed using citrated blood samples, which were taken and treated as above reported.

This study was conducted according to the principles stated in the Declaration of Helsinki. The institutional review board approved this observational study.

Statistical analysis

Categorical variables were reported as counts (percentage). Continuous variables with normal distribution were expressed as means ± standard deviation (SD). Non-parametric variables were expressed as median and interquartile range (IQR) or median and minimum-maximum values. We tested the independence of categorical variables by Chi^2-test and the normal distribution of parameters by Kolmogorov-Smirnov test. We used a Student t-test to evaluate normally distributed continuous variables. Appropriate nonparametric tests were employed for all the other variables. As an overall non-parametric ANOVA, the Friedman test for the analysis of intragroup variations was used. In cases of significance, we compared pair related samples using the Wilcoxon test. The intergroup analysis was performed with the non parametric Mann–Whitney U-test. Only p values lower than 0.05 were regarded as statistically significant. All tests were two-tailed and analyses were performed using computer software packages (SPSS-22.0, IBM, NY, USA or R version 2.15.2, R Development Core Team, Vienna, Austria).

Results

In vitro study

LPS and platelet aggregation

Platelets from healthy volunteers (n=5, males 3, females 2, age 33.8 ± 4.1) were incubated with scalar concentrations of LPS up to 100 pg/ml; no significant changes of PA were detected compared to baseline for each concentration used (►Figure 1A-B). The choice of this concentration was based on serum and plasma LPS analysis performed in 10 CAP and 10 controls; serum and plasma endotoxins levels were significantly higher in CAP patients compared to controls: 145 [115–187] vs 18 [6–21] pg/ml for serum and 107 [88–131] vs 13 [9–15] pg/ml for plasma (Suppl. Table 1, available online at www.thrombosis-online.com).

LPS-treated platelets stimulated with STC of collagen (0.25 µg/ml), showed a significant increase of PA compared to LPS-treated platelets alone (►Figure 1A, C). This effect was already evident at concentrations of 15 pg/ml. Similar findings were observed with LPS-treated platelets stimulated with STC of ADP (2 µmol/l) (►Figure 1B-C).

To investigate the underlying mechanism, we pre-treated platelets with different inhibitors, such as TLR4 inhibitor peptide (4 µmol/l), aspirin (100 µmol/l) or SQ29548 (0.1 µmol/l) which in-

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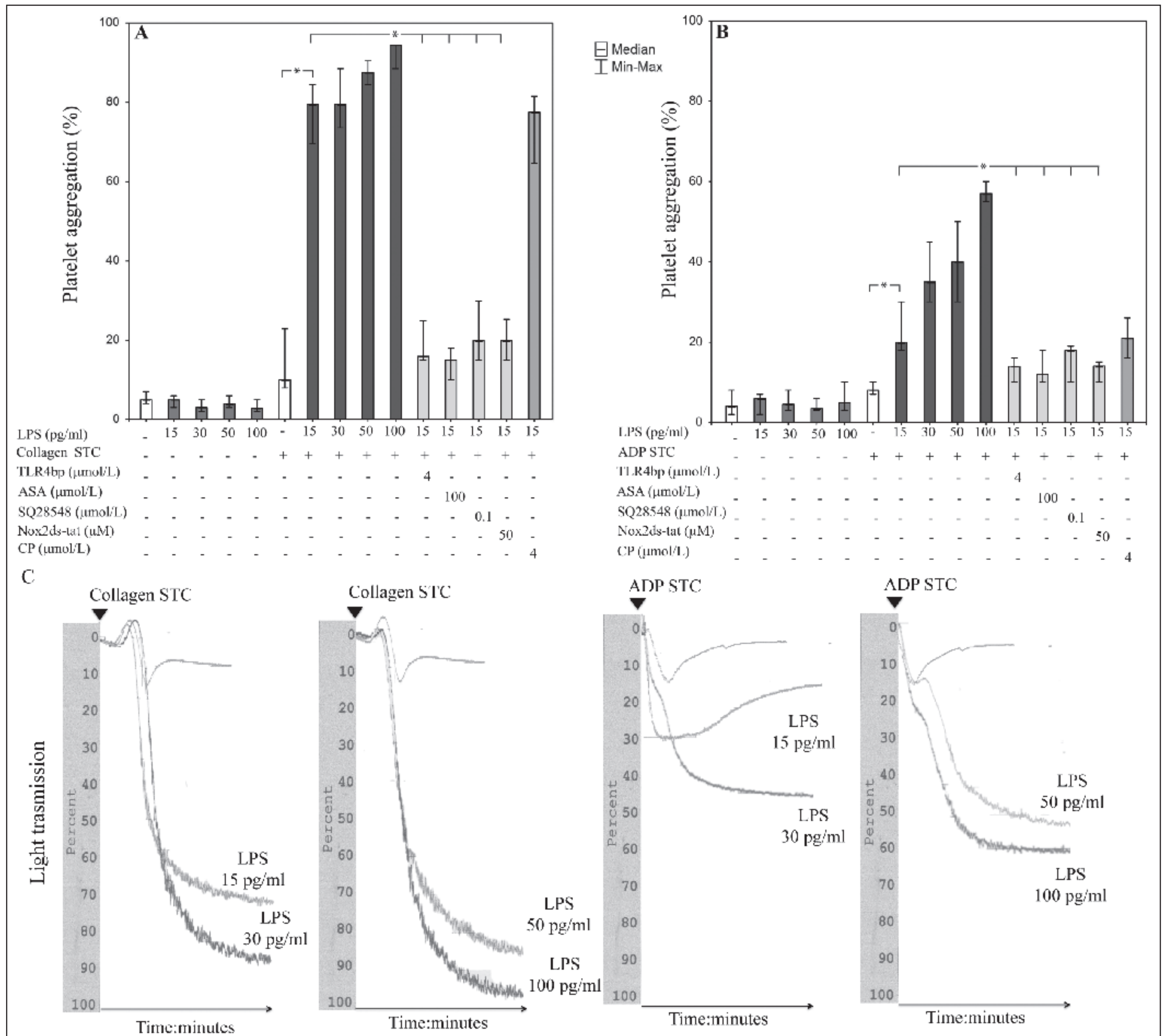

Figure 1: LPS and platelet aggregation. Platelets were incubated with scalar concentrations of LPS (15–100 pg/ml) and stimulated with or without subthreshold concentration (STC) of collagen (0.25 µg/ml) (A) or ADP (2 µmol/l) (B) in presence or less of TLR4 inhibitor (4 µmol/l), ASA (100 µmol/l), inhibitor of TxA_2 receptors SQ28548 (0.1 µmol/l), control peptide (CP) (4 µmol/l) or Nox2ds-tat (50 µmol/l) (A and B) (n=5) (*$p<0.05$ for paired analyses; $p<0.001$ as an overall analysis). Representative tracing of platelet aggregation in presence of LPS (15–100 pg/ml) and STC of collagen or ADP (C).

hibits TxA_2 receptors (9) and Nox2ds-tat (50 µmol/l). TLR4 inhibitor, aspirin, SQ28548 and Nox2ds-tat significantly inhibited PA elicited by LPS-treated platelets added with STC of collagen (0.25 µg/ml) or ADP (2 µM) (▶Figure 1A-B).

LPS and eicosanoids production

LPS (15–100 pg/ml)-treated platelets did not modify TxB_2 (▶Figure 2A-B) or 8-iso-PGF2α-III production (▶Figure 2C-D) compared to un-stimulated platelets. LPS-treated platelets stimulated with STC of collagen or ADP elicited TxB_2 and 8-iso-PGF2α-III formation (▶Figure 2A-D). This effect was already evident at concentrations of 15 pg/ml and was significantly reduced in platelets treated with TLR4 inhibitor (▶Figure 2A-D) or aspirin (▶Figure 2A-B).

LPS and platelet oxidative stress

Platelets incubated with scalar concentrations of LPS (15–100 pg/ml) did not modify platelet H_2O_2 production (▶Figure 3A-B) and

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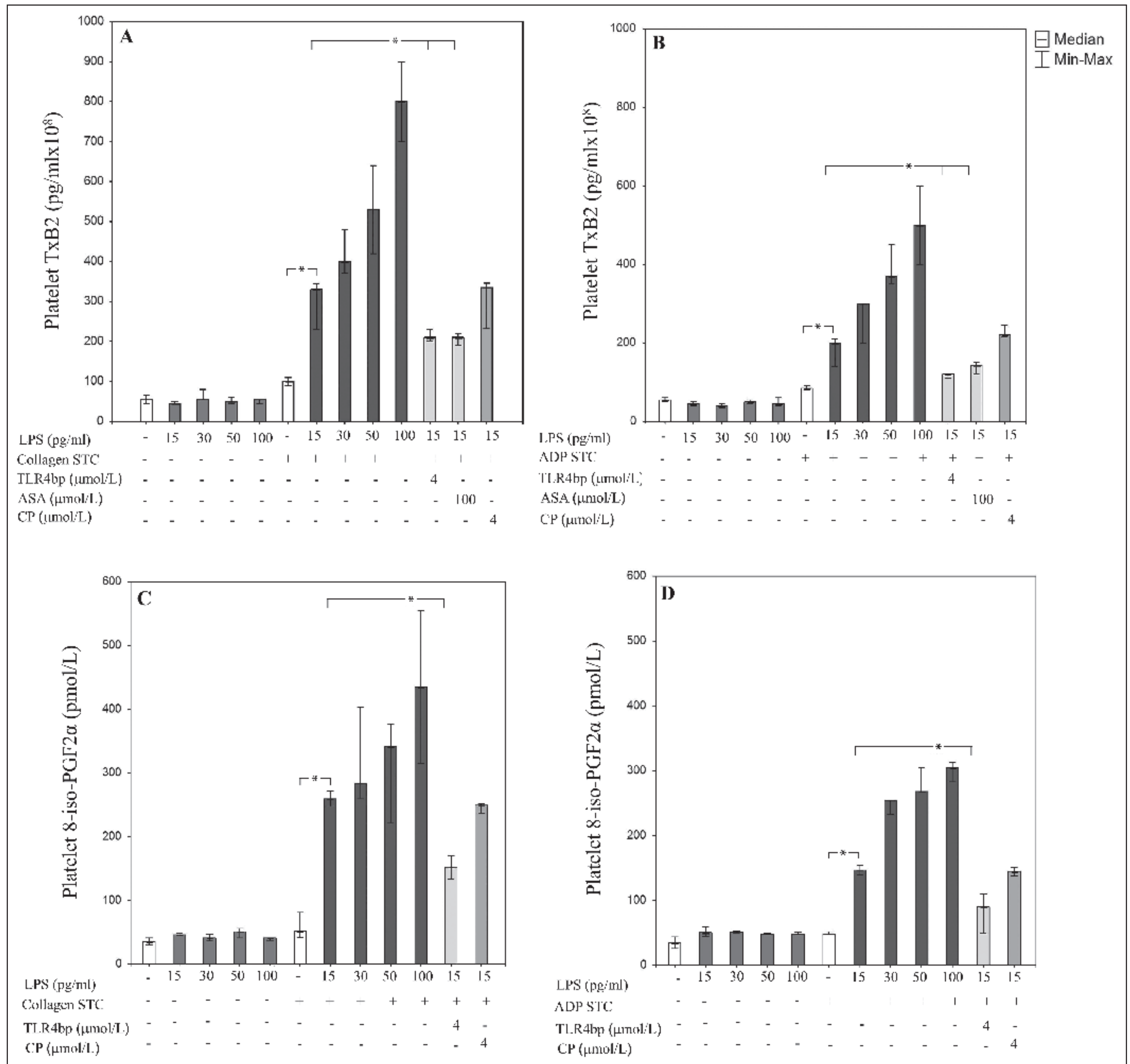

Figure 2: LPS and eicosanoids production. TxB_2 production was evaluated in platelets incubated with scalar concentrations of LPS (15–100 pg/ml) and stimulated with or without subthreshold concentration (STC) of collagen (0.25 µg/ml) (A) or ADP (2 µmol/l) (B) in presence or less of TLR4 inhibitor (4 µmol/l), ASA (100 µmol/l), or control peptide (CP) (4 µmol/l) (A and B) (n=5) (*$p<0.05$ for paired analyses; $p<0.001$ as an overall analysis). 8-iso-PGF2α-III formation was evaluated in platelets incubated with scalar concentrations of LPS (15–100 pg/ml) and stimulated with or without subthreshold concentration (STC) of collagen (0.25 µg/ml) (C) or ADP (2 µmol/l) (D) in presence or less of TLR4 inhibitor (4 µmol/l) or control peptide (CP) (4 µmol/l) (C and D) (n=5) (*$p<0.05$ for paired analyses; $p<0.001$ as an overall analysis).

sNox2-dp release (►Figure 3C-D) compared to baseline. LPS-treated platelet stimulated with STC of collagen or ADP, increased platelet H_2O_2 and sNox2-dp release (►Figure 3A-D). This effect was already evident at concentrations of 15 pg/ml. In LPS-treated platelet stimulated with STC of agonists TLR4 inhibitor significantly inhibited H_2O_2 production (►Figure 3A-B) and sNox2-dp release (►Figure 3C-D).

Intra-signalling pathway of platelet activation by LPS

AKT and p38MAPkinase

To investigate upstream pathways implicated in Nox2 activation, we analysed the role of AKT and p38MAP kinase, which are both implicated in $p47^{phox}$ activation (11, 18). LPS (15–100 pg/ml)-treated platelets did not show significant changes in AKT and

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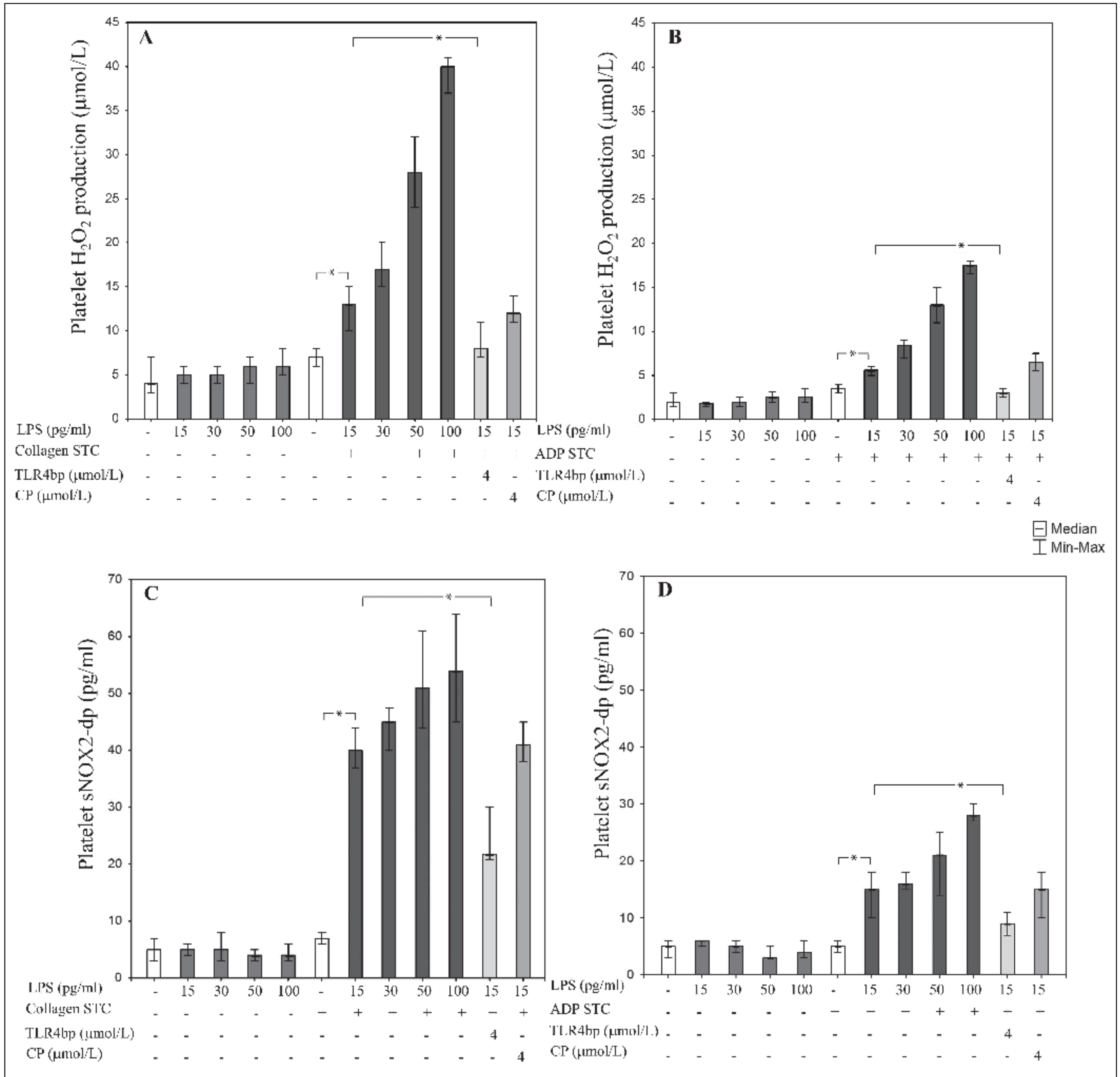

Figure 3: LPS and platelet oxidative stress. H_2O_2 production was evaluated in platelets incubated with scalar concentrations of LPS (15–100 pg/ml) and stimulated with or without subthreshold concentration (STC) of collagen (0.25 µg/ml) (A) or ADP (2 µmol/l) (B) in presence or less of TLR4 inhibitor (4 µmol/l) or control peptide (CP) (4 µmol/l) (A and B) (n=5) (*p<0.05 for paired analyses; p<0.001 as an overall analysis). Nox2 activation was evaluated in platelets incubated with scalar concentrations of LPS (15–100 pg/ml) and stimulated with or without subthreshold concentration (STC) of collagen (0.25 µg/ml) (C) or ADP (2 µmol/l) (D) in presence or less of TLR4 inhibitor (4 µmol/l) or control peptide (CP) (4 µmol/l) (C and D) (n=5) (*p<0.05 for paired analyses; p<0.001 as an overall analysis).

p38MAPkinase phosphorylation compared to baseline (▶Figure 4A-F). LPS-treated platelets stimulated with STC of collagen or ADP showed AKT and p38 phosphorylation (▶Figure 4A-F); this effect was inhibited in platelets pre-incubated with TLR4 inhibitor (▶Figure 4A-F).

Finally we investigated if p38MAPKinase inhibition resulted in impaired production of platelet eicosanoids; the experiment showed that platelet TxB_2 and 8-iso-PGF2α -III were significantly inhibited in LPS-treated platelets pre-incubated with SB202190, a selective inhibitor of p38MAP kinase (▶Figure 4G-H).

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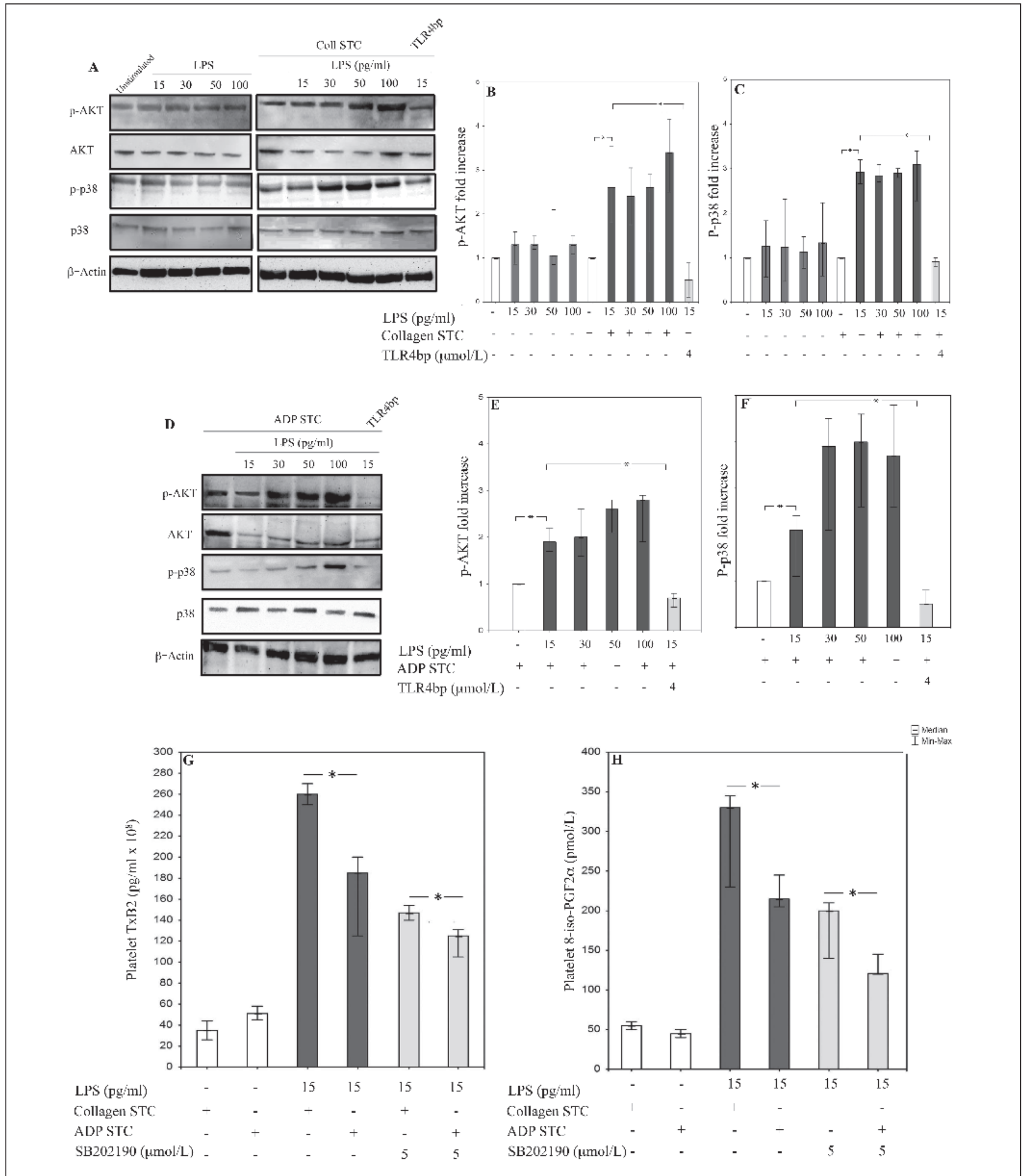

Figure 4: Intra-signalling pathway of platelet activation by LPS: AKT and p38MAPKinase. AKT and p38 phosphorylation was analysed in platelets incubated with scalar concentrations of LPS (15–100 pg/ml) and stimulated with or without subthreshold concentration (STC) of collagen (0.25 µg/ml) (A-C) or ADP (2 µmol/l) (D-F) in presence or less of TLR4 inhibitor (4 µmol/l) (A-F) (n=5) (*p<0.05 in quantitative analysis). A representative western blot of AKT and p38 phosphorylation (A and D). TxB_2 production and 8-iso-PGF2α-III formation was evaluated in platelets pre-treated with LPS 15 pg/ml and stimulated with subthreshold concentration (STC) of collagen (0.25 µg/ml) or ADP (2 µmol/l) in presence or less of SB202190 (5 µmol/l) (G and H) (n=5) (*p<0.05 for paired analyses; p<0.001 as an overall analysis).

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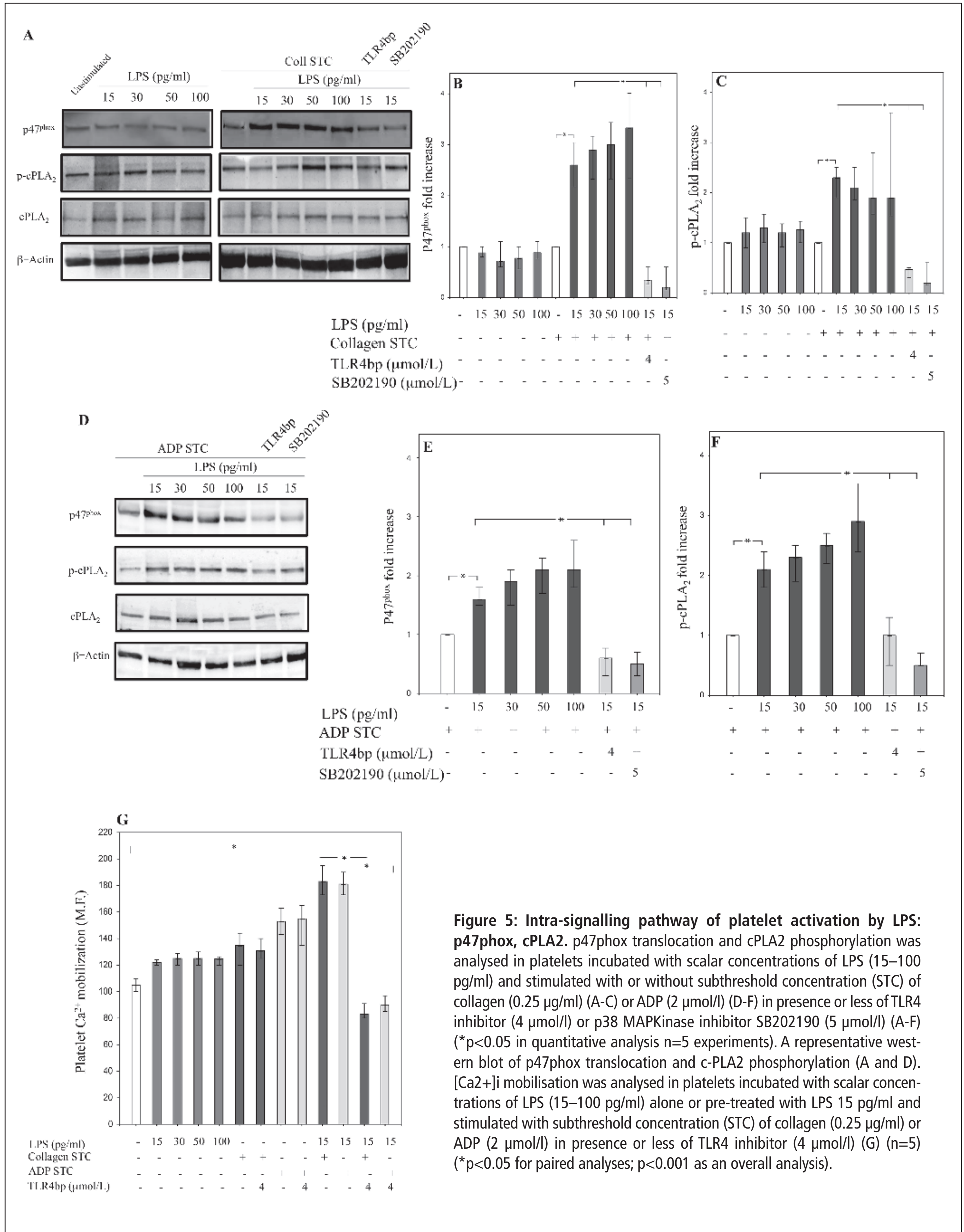

Figure 5: Intra-signalling pathway of platelet activation by LPS: p47phox, cPLA2. p47phox translocation and cPLA2 phosphorylation was analysed in platelets incubated with scalar concentrations of LPS (15–100 pg/ml) and stimulated with or without subthreshold concentration (STC) of collagen (0.25 μg/ml) (A-C) or ADP (2 μmol/l) (D-F) in presence or less of TLR4 inhibitor (4 μmol/l) or p38 MAPKinase inhibitor SB202190 (5 μmol/l) (A-F) (*$p<0.05$ in quantitative analysis n=5 experiments). A representative western blot of p47phox translocation and c-PLA2 phosphorylation (A and D). [Ca2+]i mobilisation was analysed in platelets incubated with scalar concentrations of LPS (15–100 pg/ml) alone or pre-treated with LPS 15 pg/ml and stimulated with subthreshold concentration (STC) of collagen (0.25 μg/ml) or ADP (2 μmol/l) in presence or less of TLR4 inhibitor (4 μmol/l) (G) (n=5) (*$p<0.05$ for paired analyses; $p<0.001$ as an overall analysis).

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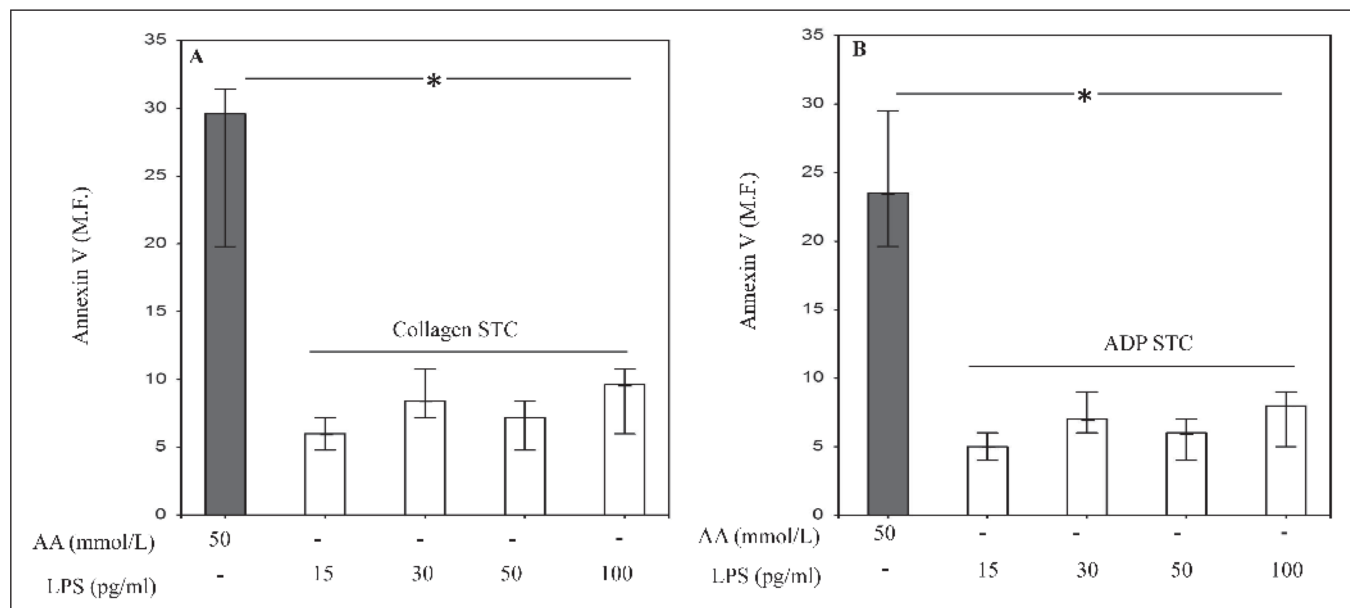

Figure 6: Apoptosis analysis. Apoptosis analysis was performed in platelets treated with AA (50 mmol/l) or scalar concentrations of LPS (15–100 pg/ml) and stimulated with subthreshold concentration (STC) of collagen (0.25 μg/ml) (A) or ADP (2 μmol/l) (B) (*p<0.05).

p47, cPLA$_2$ and [Ca^{2+}]i mobilisation

To analyse the pathway involved in LPS-dependent platelet activation and oxidative stress, we studied p47^{phox} translocation on platelet membrane, as final and key event upstream of Nox2 activation, and cPLA$_2$ phosphorylation, a key enzyme for generation of eicosanoids and related bioactive lipid mediators.

LPS (15–100 pg/ml)-treated platelets did not affect p47 translocation or cPLA2 phosphorylation compared to control (▶Figure 5A-F). Conversely, in LPS-treated platelets stimulated with STC of collagen or ADP, an increase of p47phox cytosol/membrane translocation and cPLA2 phosphorylation were detected (▶Figure 5A-F); this effect was significantly inhibited in samples pre-incubated with TLR4 inhibitor and SB202190 (▶Figure 5A-F). Finally, TLR4 inhibitor did not affect p47 translocation or cPLA2 phosphorylation in platelet stimulated with STC of collagen or ADP alone (Suppl. Figure 1, available online at www.thrombosis-online.com).

[Ca2+]i mobilisation is a fundamental step for platelet activation (19). Compared to platelet alone, LPS up 100 pg/ml or STC of agonists slightly but not significantly increased [Ca2+]i mobilisation compared to control. LPS (15 pg/ml)-treated platelets stimulated with STC of agonists showed a significant increase of [Ca2+]i mobilisation, which was blunted in the presence of TLR4 inhibitor (▶Figure 5G).

TLR4 inhibitor did not affect [Ca2+]i mobilisation in platelet stimulated with STC of collagen or ADP alone (▶Figure 5G).

Apoptosis analysis

To exclude laboratory artefact related to a non-specific activation of platelets, we analyses if LPS could determine platelet apoptosis. LPS treated-platelets stimulated with STC of either agonist did not result in an increase of apoptosis, as assessed by Annexin V membrane expression (▶Figure 6A-B).

Ex vivo study

Clinical characteristics of patients with CAP and controls are summarised in Suppl. Table 1 (available online at www.thrombosis-online.com).

About two third of CAP patients had moderate-severe CAP as assessed by the PSI scoring system, belonging 45 % to PSI class IV and 21 % to class V. Compared to controls, CAP patients displayed higher urinary levels of 11-dehydro-TxB$_2$ and 8-iso-PGF2α (Suppl. Table 1, available online at www.thrombosis-online.com).

Endotoxin serum levels were 145 [115–187] pg/ml in CAP patients and 18 [6–21] pg/ml in controls (Suppl. Table 1, available online at www.thrombosis-online.com); moreover, TLR4 was expressed significantly more in platelets from CAP compared to controls (▶Figure 7A). While control PRP and WP stimulated by STC of collagen did not aggregate, PRP and WP from CAP patients fully aggregated (▶Figure 7B-C and Suppl. Figure 2A, available online at www.thrombosis-online.com) coincidentally with a significant increase of 8-iso-PGF2α (▶Figure 7D) and TxB$_2$ (▶Figure 7E). Platelet aggregation and eicosanoids production

Figure 7: LPS in controls and CAP patients. In 10 community-acquired pneumonia (CAP) patients and 10 controls, platelet TLR4 expression (A), PA (B), 8-iso-PGF2α-III (D) and TxB$_2$ (E) production were evaluated in PRP pre-incubated or not with TLR4bp and stimulated or not with sub-threshold concentration (STC) of collagen (n=10) (**p<0.01 and *p<0.05 in quantitative analysis for TLR4 expression). Representative tracing of platelet aggregation in presence of STC of collagen and TLR4bp (C).

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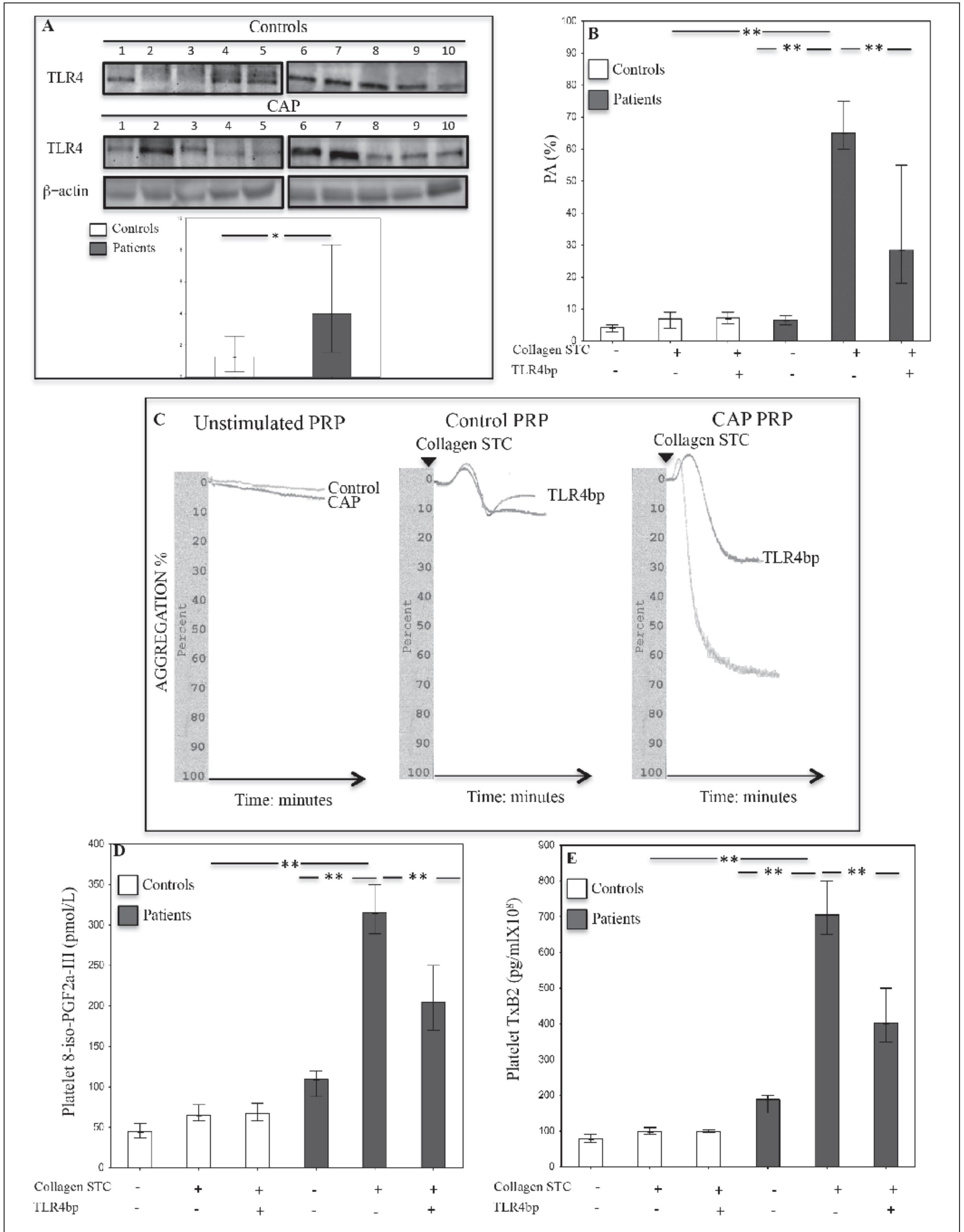

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were blunted in the presence of TLR4 inhibitor in CAP patients (►Figure 7B-E).

In order to support the finding that PA is mediated by increasing TxA_2 production, we pre-incubated platelets from CAP patients with or without 100 µmol/l ASA. The results showed that ASA inhibits LPS-mediated platelet aggregation and TxB_2 production (Suppl. Figure 2B-C, available online at www.thrombosis-online.com).

Similar findings were obtained using STC of ADP as agonist (data not shown).

Discussion

The results of the present study indicate bacteria-derived LPS as a trigger of platelet activation so suggesting a role for LPS in eliciting platelet activation in CAP patients.

In the present study, we explored if bacteria-derived LPS is responsible for platelet activation and the underlying mechanism. Platelets from healthy volunteers were incubated with LPS in a range of concentration similar to that found in the blood of CAP; the experiment showed that LPS per se is not an aggregating molecule but is able to amplify platelet response to common agonists such as collagen or ADP. This finding parallels the *ex vivo* study showing that, while platelets from healthy subjects did not respond to STC of agonists, platelets from CAP patients fully aggregated suggesting that an endotoxin-rich milieu may be a trigger for platelet aggregation; in accordance with this, CAP patients disclosed high values of blood LPS coincidentally with platelet TLR4 over-expression.

Eicosanoids are a family of molecules derived from enzymatic and non-enzymatic oxidation of AA (20). Among them, TxA_2, which is an unstable product of AA oxidation by COX1, and 8-iso-PGF2α-III, that is a chemically stable product of arachidonic interaction with oxygen free radicals (ROS) (21), elicit PA via a common receptor (9). Our study shows that both eicosanoids are increased in patients and are implicated in LPS-mediated platelet aggregation as TxB_2, the stable metabolite of TxA_2, and 8-iso-PGF2α-III significantly increased in platelets exposed to LPS and stimulated with STC of agonists. This finding provides a plausible explanation for the *ex vivo* study where platelets from CAP stimulated with STC of agonists produced more eicosanoids compared to controls. The key role played by the two eicosanoids in amplifying the platelet response to the STC of agonists was further supported by the fact that aspirin or a TXA_2 receptor antagonist inhibited LPS-mediated PA.

In accordance with previous studies, we confirm that also in our experimental model TLR4 is crucial for LPS-mediated PA, as the amplification of platelet response to common agonist was no more detected in platelets incubated with a TLR4 receptor antagonist; similarly, the increase of either TxA_2 or 8-iso-PGF2α-III by LPS was significantly inhibited by a TLR4 antagonist.

PA by common agonists is associated with a burst of ROS that contribute to platelet aggregation via several mechanisms including nitric oxide inactivation, ADP release and 8-iso-PGF2α-III formation (9, 22, 23). Up-regulation of Nox2, the catalytic sub-unit of NADPH oxidase, has a key role in platelet ROS formation as shown by almost complete absence of ROS in platelets from patients with hereditary deficiency of Nox2 (24). In our experimental

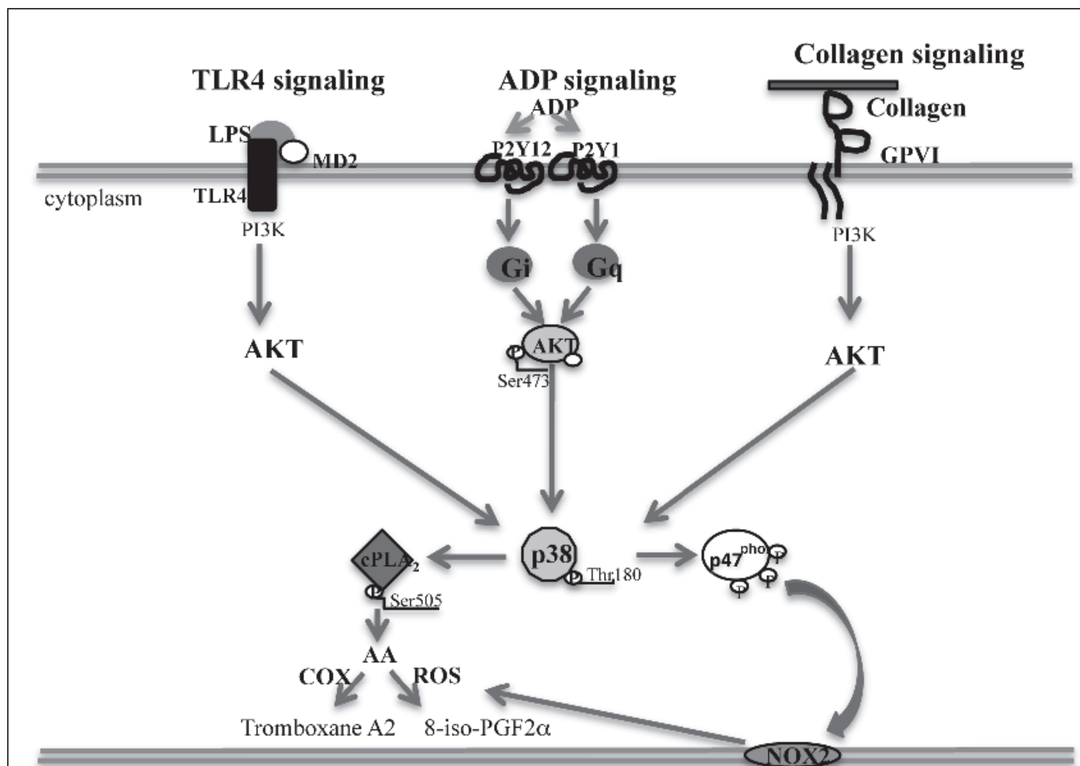

Figure 8: Schematic representation of LPS-amplified platelet activation. LPS (15–100 pg/ml) combined with sub-threshold concentrations of collagen or ADP is able to amplify platelet activation via AKT, p38 and cPLA2 phosphorylation and $p47^{phox}$ translocation. cPLA2 is key enzyme for generation of thromboxane $(Tx)A_2$ and 8-iso-PGF2α-III whereas $p47^{phox}$ is a key cytosolic subunit upstream from Nox2 activation and H_2O_2 production.

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What is known about this topic?

- LPS might activate platelets but at concentrations several time higher than those found in human circulation.
- Platelet activation has been suggested to play a role in increased risk of cardiovascular events in patients with community-acquired pneumonia (CAP) but the underlying mechanism is still unclear.

What does this paper add?

- At concentrations detectable in the human blood of CAP patients, LPS amplifies the response to common agonists upon interaction with Toll-like receptor 4.
- LPS behaves as a pro-thrombotic molecule potentially capable of favouring vascular disease.

model, Nox2-derived ROS seems to be essential for LPS-mediated PA as platelet incubation with LPS was associated with ROS overproduction coincidentally with Nox2 activation; both changes were inhibited in platelets treated with a TLR4 antagonist suggesting that LPS yields platelet ROS formation via interaction with TLR4. As cellular up-stream activation by TLR4 are AKT and p38, which are also involved in platelet activation by ADP or collagen (20) (▶Figure 8), we investigated if AKT and p38 were phosphorylated by LPS-mediated PA and the role of TLR4 receptor; the experiment showed that both were activated by LPS and significantly inhibited in platelets incubated with a TLR4 antagonist.

Activation of the AKT-p38MAP kinase axis is of particular interest to explain LPS-mediated PA because this pathway is implicated in Nox2 activation via p47phox translocation to the membrane Nox2; furthermore, this intracellular pathway is involved in PLA2 activation, that is essential for AA release from platelet membrane and eventually eicosanoid formation (25). In our experimental model, LPS-mediated PA was associated with either p47phox or cPLA2 activation, which likely accounted for the enhanced formation of 8-iso-PGF2α-III and TxA_2, respectively. Thus, p47phox translocation to platelet membrane provides further support to the role of Nox2 in platelet ROS formation and ultimately 8-iso-PGF2α-III formation while activation of PLA2 is crucial for AA release from platelet membrane and ultimately TxA2 production (▶Figure 8).

Abbreviations

8-iso-PGF2α: 8-Iso Prostaglandin F2alpha; AA: arachidonic acid; ACD: acid citrate dextrose; ASA: acetylsalicylic acid; CAP: community-acquired pneumonia; COPD: chronic obstructive pulmonary disease; CP: control peptide; cPLA2: cytosolic phospholipase A2; PAD: peripheral arterial disease; PRP: platelet-rich plasma; ROS: reactive oxygen species; sNOX2dp: soluble NOX2-derived peptide; STC: sub-threshold concentration; TLR4bp: TLR4 inhibitor; TxB_2: Thromboxane B2; WP: washed platelet.

To explain the synergism between LPS and low doses of platelet agonists we focused on intracellular [Ca2+]i mobilisation, which a key step for initiation of PA (24). Of interest is the fact that either LPS or low doses of agonists slightly increased [Ca2+]i mobilisation while a combination of both significantly mobilised intraplatelet [Ca2+]i, suggesting that this effect is likely to play a pivotal role in PA elicited by LPS and STC of agonists.

The results of the present study have implications and limitations. The fact that LPS may activate platelets at concentration commonly found in human circulation during infectious disease provides further support to the role of LPS as a pro-thrombotic molecule in different clinical settings including sepsis or vascular diseases complicating the early phase of infections such as CAP (26–28).

We did not identify the pathogens responsible for CAP. We cannot exclude that viruses, which are implicated in CAP more than Gram-negative bacteria are also implicated in platelet activation detected in CAP patients (29). The enhanced circulating levels of circulating of LPS may be dependent upon gut barrier dysfunction with ensuing translocation of LPS in the systemic circulation (5); this issue should be investigated in the future. Finally, we cannot exclude that LPS from other bacteria might have similar effects.

In conclusion, we demonstrated that LPS is a potential stimulus for PA via overproduction of TxA_2 and 8-iso-PGF2α-III so providing novel insight into the mechanism accounting for platelet activation in patients with infectious disease such as those with CAP.

Conflicts of interest
None declared.

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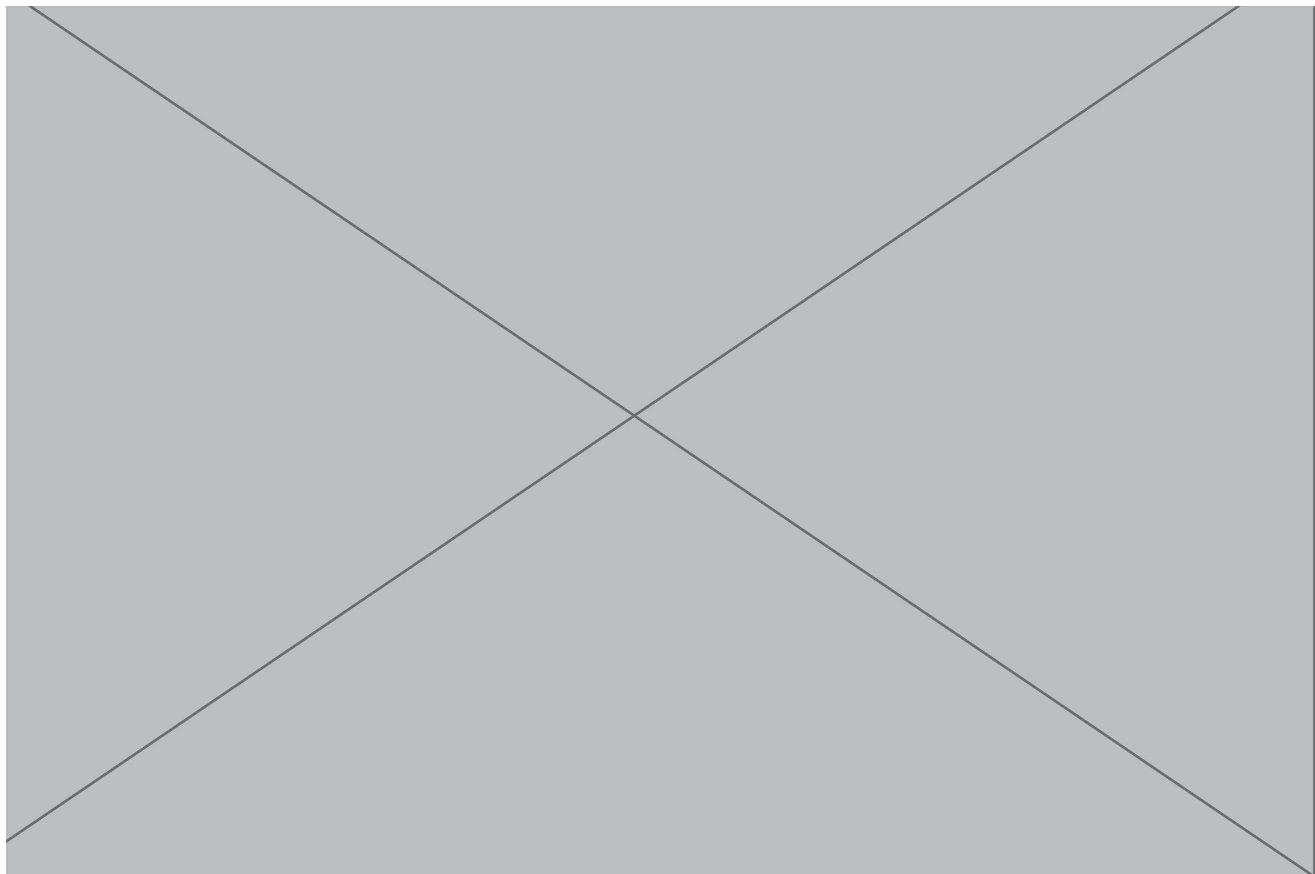

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