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High-dose acetylsalicylic acid is superior to low dose as well as to clopidogrel in preventing LPS-induced lung injury in mice

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Abstract

Background: Use of aspirin (ASA) was found to improve outcome in animal models of acute lung injury (ALI) or its more severe form ARDS. In ARDS patients, data indicating a protective effect of ASA are less convincing. We hypothesize that ASA in a high dose is superior to low dose ASA in preventing lung injury. Also, the effect on lung injury of inhibiting platelet activation by clopidogrel was investigated.

Methods: ALI was induced by intranasal instillation of 10 μg lipopolysaccharide (LPS). Before LPS, BALB/c mice were pre-treated with either high dose of ASA (100 μg/g intraperitoneally (i.p.), low dose ASA (12.5 μg/g i.p), clopidogrel (50 μg/g i.p) or clopidogrel in combination with low dose of ASA. Controls received vehicle or LPS without intervention. Five hours after LPS, bronchoalveolar lavage fluid (BALF) and plasma were obtained.

Measurements and Main Results: All treatment regimes reduced neutrophil influx in the BALF compared to LPS controls (high dose ASA 75±2 (mean±SD), low dose ASA 86±3, clopidogrel 82±1 and low dose ASA-clopidogrel 82±3 vs LPS control 88±2%, P ≤0.05). High dose ASA reduced BALF levels of protein compared to LPS controls (0.2[15] (median [IQR]) vs 75[20] pg/mL, P < 0.01), to a greater extent than after low dose ASA (48[32] pg/mL), clopidogrel (37[23]pg/mL) or low dose ASA-clopidogrel (57[8] pg/mL).

Conclusion: High dose ASA is superior to low dose ASA, clopidogrel and to a combination of clopidogrel and low dose ASA in attenuating LPS-induced lung injury in mice, suggesting high dose ASA to be the antiplatelet therapy of choice in further research on preventing ALI.
Introduction

Acute lung injury (ALI) and its more severe form, Acute Respiratory Distress Syndrome (ARDS), are a frequent complication in critically ill patients, with an additional mortality of up to 60% [1]. Treatment options for ALI/ARDS are limited. Of interest, a large part of patients develops lung injury after a median of two days following hospital presentation, as a consequence of an initial predisposing acute injury such as aspiration, pneumonia or sepsis. This time frame presents a window of opportunity for interventions to prevent the development of ALI [2].

Platelets play a prominent role in ALI. After activation, resulting in expression of adenosine diphosphate (ADP) receptor, platelets aggregate and interact with neutrophils [3,4]. Platelet-neutrophil interactions depend on P-selectin expression on platelets, associated with thromboxane-2 (TXA₂) formation, resulting in increased pulmonary vascular permeability [5-9].

In accordance, platelet depletion as well as inhibition of platelets by acetylsalicylic acid (ASA) was found to abrogate lung injury in preclinical ALI models inflicted by acid instillation [5] and in transfusion-related acute lung injury (TRALI) [10-12]. Observational studies have shown an association between ASA and other antiplatelet drugs and reduced organ failure and mortality in the critically ill [13]. However, data on the effect of ASA on ALI are conflicting. Whereas use of ASA was associated with prevention of onset of ALI in a retrospective analysis in medical ICU patients [14], this benefit could not be confirmed in a large multicenter observational study, when adjusted for the propensity score [15]. Also, the protective effect of ASA in an animal TRALI model was not confirmed in an observational study in critically ill TRALI patients [12,16]. Differences may be explained by dose-dependent effects of ASA.

ASA has inhibitory effects on cyclooxygenase-1 (COX-1) and COX-2 [17]. Whereas a low dose of ASA mainly blocks COX-1, ASA in a high dose also blocks COX-2. COX-1 is an inhibitor of platelet activation through inhibiting TXA₂, whereas COX-2 inhibits inflammation [18]. In the clinical studies, ASA is used in a dose that only inhibits COX-1. In contrast, in all the animal studies a high dose is used, which inhibits both COX-1 and COX-2.

Furthermore, there is evidence that clopidogrel, a specific antagonist of the platelet ADP-receptor, is also capable of reducing inflammation [17,19].

While the first clinical interventional trial testing the efficacy of ASA in preventing ARDS is underway [2], we think there is a need to elucidate which type and dose of antiplatelet therapy may be most protective. In this study, we investigate the role of the most commonly used platelet aggregation inhibitors in preventing lung injury. In addition, we hypothesized that a combination ASA and clopidogrel exerts a better protective effect in lipopolysaccharide (LPS)-induced ALI than ASA low dose.
Materials and Methods

This study was approved by the Animal Care and Use Committee of the Academic Medical Center (IRB#102283). Animal procedures were carried out in compliance with the institutional Standards for Human Care and Use of Laboratory Animals.

Experiments were performed with healthy male BALB/c mice (n=56, Charles River Someren, The Netherlands), aged 8-10 weeks, with weights ranging from 20-25 grams.

Interventions

The mice were randomly assigned to 6 groups (n=8 per group). All antiplatelet therapy was administered intraperitoneally (i.p.) 1 hour prior to LPS. Two treatment groups received ASA (Carbasalaatcalcium cardio, APOTEX Europe B.V, Leiden, The Netherlands). Of which one in a low dose of 12.5 μg/g, which is a calculated comparable dose as used in humans for platelet aggregation inhibition [20] and one in a high dose of 100 μg/g, which is comparable with the dose used in humans for anti-inflammatory effects. The third group was pre-treated with clopidogrel (50 μg/g, Plavix, Sanofi-Aventis Netherlands B.V., Gouda, The Netherlands), with a dose comparable with a loading dose in humans. The fourth group was pre-treated with clopidogrel (50 μg/g) in combination with ASA in a low dose (12.5 μg/g). Two groups served as controls as further explained in the experimental study protocol section.

Experimental study protocol

One hour before LPS administration, mice were pre-hydrated with 1 mL NaCl 0.9% i.p. to prevent dehydration induced by the illness. Mice were anesthetized by isoflurane 2-3% inhalation. ALI was induced by intranasal (i.n.) LPS (10 μg/50 μL from E. coli 0111:B, Sigma-Aldrich, St. Louis, USA) [21,22]. Control animals received vehicle (50 μL NaCl 0.9%) i.n. The mice were then placed back in their cages. Five hours after LPS inoculation, anaesthesia was achieved with i.p. injection of a mix of Ketamine (EurovetAnimal Health B.V., Bladel, The Netherlands), Dexmedetomidine (Pfizer Animal Health B.V., Capelle a/d Ijssel, The Netherlands) and Atropine (Pharmachemie, Haarlem, The Netherland) (KMA) of 7.5 μL per gram body weight of 1.26 mL 100 mg/ml ketamine, 0.2 mL 0.5 mg/mL dexmedetomidine, and 1 mL 0.5 mg/mL atropine in 5 mL normal saline. Tail bleeding time was assessed by briskly cutting exactly 0.5 cm of the distal tip of the tail of the mice using a sharp new razor blade. The tail was then immediately submersed in a pre-warmed tube of saline. A stopclock was started at this time. The stopclock was stopped, when visible bleeding was no longer detected. Bleeding times between 50-70 seconds are considered normal [23]. Then mice were exsanguinated by drawing blood from the carotic artery. The lung was lavaged three times with 0.5 mL of normal saline. Approximately 1.0 mL of lavage fluid was retrieved per mouse. Blood and lavage fluid were centrifugized and supernatant was stored at -20⁰C for total protein level and cytokine measurement. Differential counts were done (up to 100 cells per slide) on cytospin preparations stained with a modified Giemsa stain (Diff-Quick, Dade
Behring AG, Düdingen, Switzerland). Neutrophil influx is then defined as the number of neutrophils according to total amount of cells on cytospin preparations.

Assays
Total protein levels (Bradford Protein Assay Kit, OZ Bioscience, Marseille, France) were measured in BALF. Interleukin (IL)-6, IL-1β, Keratinocyte-derived Chemokine (KC), Macrophage inflammatory protein-2 (MIP-2) were measured in BALF and plasma using Enzyme-Linked Immuno Sorbent Assay (ELISA) according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN). Prostaglandin E-2 (PGE-2) was measured in BALF according to the instructions of the manufacturer (R&D).

Statistical analysis
Data were expressed as mean ± standard deviation (SD) or median [interquartile range] when appropriate. Comparisons between experimental groups were performed using one way ANOVA or Kruskall-Wallis analysis to allow for multiple comparisons, followed by students T-test or Mann-Whitney test depending on data distribution. In figures boxplots are used. The lower hinge is defined as the 25th percentile, middle as 50th percentile and upper hinge as the 75th percentile. Whiskers define lowest and highest observation. A p-value <0.05 is considered statistically significant. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, La Jolla, Ca).

Results
Intranasal LPS induced ALI
All animals survived the experiment. Intranasal LPS resulted in an increase in BALF levels of protein compared to saline controls, reflecting lung edema due to increased vascular permeability. Also, pulmonary neutrophil influx BALF MIP-2 and BALF KC levels were increased (Figure 1A-D). BALF levels of IL-1β were also increased (140[15] vs 8[2] pg/mL, P <0.001). Furthermore, LPS resulted in an increase in median levels of KC and IL-6 in plasma compared to saline controls (4714[11643] vs 484[771] and 43[7] vs 8[3] respectively, P < 0.05 for both).

Tail bleeding time
To investigate whether the interventions with ASA and clopidogrel sufficiently inhibited platelet aggregation, tail bleeding time was measured. Both ASA in a low and high dose prolonged tail bleeding time (Fig. 2). Also clopidogrel prolonged tail bleeding time. Tail bleeding was not assessed in the ASA-clopidogrel group since a prolonged tail bleeding was already achieved in the other groups.
Figure 1. Markers of lung injury in bronchoalveolar lavage fluid in LPS-induced acute lung injury and after treatment with platelet inhibitors.

A: neutrophil influx; B: total protein; C: Macrophage inflammatory protein-2 (MIP-2); D: Keratinocyte-derived Chemokine (KC). NaCl: control group with vehicle i.n.; LPS: control group with LPS i.n.; ASA low: acetylsalicylic acid (ASA) in a low dose; ASA high: ASA in a high dose. For fig. 1A parametric and 1B, 1C and 1D nonparametric tests were used for analysis. * P < 0.05; ** P < 0.01.

Figure 2. Tail bleeding time in mice with LPS-induced lung injury treated with platelet inhibitors.

NaCl: control group with vehicle (i.n); LPS: control group with LPS (i.n.); ASA low: acetylsalicylic acid (ASA) in a low dose; ASA high: ASA in a high dose. Nonparametric tests were used for analysis. ** P < 0.01.
Effect of different treatment regimes on inflammation

Treatment with ASA in a low and high dose, as well as treatment with clopidogrel alone or in combination with a low dose of ASA, decreased neutrophil recruitment into the lung (Fig. 1A). In mice treated with ASA high dose, a significantly lower influx of neutrophils in the BALF was found compared to ASA low dose and compared to clopidogrel ($P \lt 0.01$ for both), but not compared to ASA/clopidogrel group ($P = 0.08$).

Protein levels in BALF were significantly reduced following ASA high dose and clopidogrel group compared to controls (Fig. 1.B). The inhibitory effect of high dose ASA on BALF protein level was significantly greater compared to the other treatment groups ($P \lt 0.05$ for all).

BALF levels of MIP-2 were significantly reduced in all groups ($P \lt 0.05$ for all), except for ASA/clopidogrel group which only showed a trend for lowering BALF MIP-2 levels (Fig. 1C). BALF KC-levels were significantly reduced by the combination of ASA and clopidogrel compared to LPS (Fig. 1D). Other treatment regimes did not influence KC levels. There was a trend for lower levels of IL-1$\beta$ in the treatment groups compared to LPS (LPS 140[15] vs ASA low 128[20], ASA high 114[22], clopidogrel 133[9] or ASA+ clopidogrel 87[19], one way ANOVA $P = 0.06$). No effect was found on IL-6 (data not shown).

To investigate whether the protective effect of ASA was associated with inhibition of prostaglandin production, which is generated by COX-2, PGE-2 levels in the BALF were measured. ASA high dose reduced BALF levels of PGE-2 compared to LPS-controls and ASA low dose, although this effect only reached statistical significance when compared to ASA low dose ($P = 0.01$) (Fig. 3).

Antiplatelet treatment groups did not reduce plasma levels of KC and IL-6 compared to LPS controls (data not shown). For MIP-2, plasma levels were below the detection limit of the assay in all groups.

Figure 3. Levels of prostaglandin E2 (PGE-2) in bronchoalveolar lavage fluid in LPS-induced acute lung injury and after treatment with aspirin in low and high dose.

NaCl: control group with vehicle (i.n); LPS: control group with LPS (i.n.); ASA low: acetylsalicylic acid (ASA) in a low dose; ASA high: ASA in a high dose. Nonparametric tests were used for analysis. * $P \lt 0.05$ compared to ASA low dose.
Discussion

We found that different antiplatelet agents protect against lung injury, but there are marked differences between the regimes that we tested. High dose ASA is superior to low dose ASA and to clopidogrel in preventing lung inflammation. Adding clopidogrel to low dose ASA had no additional beneficial effect in abrogating lung inflammation. Our results suggest that high dose ASA seems to be the most effective antiplatelet therapy in preventing onset of ALI.

A pivotal role for platelets in the pathogenesis of lung injury represents an opportunity for a preventive treatment strategy with antiplatelet therapies. However, the protective effect of ASA in preventing ALI found in animal models [5,11,12,24] has not been consequently reproduced in observational studies [14-16]. Conflicting results may have been due to dosing of ASA. In all animal studies, ASA dosing is 5-8 times higher than the dose used in patients for platelet inhibition [20]. In humans, a dose of 30 mg ASA per day results in virtually complete suppression of platelet TXA₂ production via inhibition of the isoform COX-1 [25,26], sufficient for the antithrombotic effect. In line with this, low dose ASA in this study was already sufficient to increase tail-bleeding time, indicating a sufficient dose for platelet inhibition, i.e. a COX-1 effect. The anti-inflammatory effects of ASA occur via inhibition of isoform COX-2, whose expression is upregulated by cytokines, inflammatory stimuli, and some growth factors [18]. COX-2 generates prostanoids such as prostaglandin-E₂, which are present in high concentrations in the lung of animals and humans with sepsis and ARDS [9,27,28]. As ASA is an approximately 150-200 fold more potent inhibitor of COX-1 than COX-2, there is a difference in dosage requirements of ASA as an antithrombotic (COX-1) and an anti-inflammatory drug (COX-2) [18]. To test the level of COX-2 inhibition, we measured levels of PGE-2, which is a product of prostaglandin synthesis and thereby of COX-2 activity [29]. ASA high dose suppressed PGE-2 levels compared to ASA low dose and there was a trend for lower PGE-2 levels compared to LPS. These results suggest COX-2 inhibition by ASA high dose and not by ASA low dose. Our study shows that ASA low dose attenuates lung injury, but the effect of ASA in a high dose is clearly stronger. Together, these results suggest that both COX-1 and COX-2 inhibition might be required for a full anti-inflammatory effect of ASA in attenuating ARDS. Our results may offer an explanation for contrasting results in humans and support the use of a higher ASA dose in further research.

Clopidogrel, a specific antagonist of the platelet ADP-receptor, also attenuates lung injury, suggesting that platelet inhibition has an anti-inflammatory effect independent of the mechanism of inhibition. We used a combination of ASA low dose and clopidogrel to determine whether inhibition of platelets via alternative mechanisms would enhance protection. This combination is commonly used in clinical practice in patients who have received coronary stents [30]. We found no significant additional effect of this combination. ASA high dose was superior to clopidogrel as well as to the combination of ASA low dose and clopidogrel. Both
ASA and clopidogrel reduced neutrophil migration in the alveolar compartment, possibly through inhibition of platelet-neutrophil interaction [4,5].

This study has several limitations. First, not all parameters were ubiquitously influenced by the antiplatelet agents. This may have been due to low numbers per group, thereby hampering statistical significant differences. Also, there are limitations to the model. First, LPS induced ALI in mice is a simplification of human ALI. Furthermore, our hit is modest. Despite this modest hit, differences between treatment groups are found. Our hit may be hampered by the anti-inflammatory effects of the anaesthetics used, but as these agents are used in all groups, they cannot account for the differences found. In addition, the number of circulating platelets is higher and the number of polymorphonuclear neutrophils is lower in mice compared to humans, rendering the platelet/neutrophil ratio in circulating blood substantially different in the two species [31,32]. Furthermore, the doses used are a calculation of comparable doses in humans as we were not able to measure plasma levels of ASA [20]. Lastly, this study does not show direct evidence of COX-1 and COX-2 inhibition. High dose ASA has potentially other mechanisms to prevent lung injury, such as attenuating P-selectin levels, lipoxin, neutrophil extracellular traps or enhanced formation of lipid anti-inflammatory mediators [2,33]. Since no clear difference in IL-1β levels was found, an inflammasome-mediated pathway may not contribute to the effects of platelet inhibition in this study [34]. The possible mediators involved have to be elucidated in further studies.

Conclusions

High dose ASA is superior to low dose ASA, clopidogrel and to clopidogrel in combination with low dose ASA in attenuating LPS-induced lung injury in mice. These results show that clinical trials exploring the effects of antiplatelet agents in ALI patients should take into account the dose and type of antiplatelet drug.
Reference List


