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Article

Synergistic inhibition of platelet function by NADPH oxidase inhibitors and clopidogrel

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Abstract— **Previous studies have shown that platelets play an essential role in prothrombotic complications due to several factors such as hyperglycemia, oxidative stress, and hypercholesterolemia, which affect platelet reactivity. Platelet activation involves ADP stimulation via the P2Y12 receptor. In contrast, reactive oxygen species (ROS), including superoxide anion and hydrogen peroxide (H_2O_2), produced by NADPH oxidase (Nox), act as the second messenger. It plays a role in platelet activation and may contribute to thrombus formation. The present study aimed to investigate the influence of Nox on the purinergic receptor (P2Y12 receptor) in the activation of human platelet function stimulated by platelet agonists. Our study explored the effects of Nox inhibitors (diphenylene iodonium and apocynin) and gp91ds-tat, a selective NADPH oxidase peptide inhibitor with P2Y12 receptor inhibitor, clopidogrel either alone or in combination, on ADP-stimulated human platelets aggregation and adhesion measured by modified light transmission aggregometry (LTA). In our observation, we found that pre-incubation of PRP with clopidogrel (0.1µM) with DPI (50 µM) or apocynin (50µM), either alone or in combination, resulted in concentration-related inhibition in platelets aggregation and adhesion in response to ADP. In addition, our data showed a synergistic reduction of platelet aggregation in combined clopidogrel and gp91ds-tat when induced with 30µM and 100µM ADP. Our findings suggest that a combination of clopidogrel and Nox inhibitors synergistically reduced platelet function. These results showed that the activation of NADPH oxidase influenced P2Y12 receptor reactivity. Thus, these data demonstrated a potential combination therapy to reduce the risk of thrombosis formation.**

Keywords— **NADPH oxidase; clopidogrel; platelets.**

I. INTRODUCTION

Cardiovascular diseases (CVDs), such as ischemic heart disease and cerebrovascular disease/stroke, are the top leading diseases in Malaysia and are predicted to remain so. The common cardiovascular risk factors such as insufficient physical activity, current tobacco smoking, overweight, hyperglycemia, and hypertension are strongly associated with atherothrombosis, subsequently resulting in heart failure and mortality (Lam, 2015; Falk & Fuster, 2011). Many studies have reported that these pathological conditions are influenced by platelet activation (Nording et al., 2015; Engelmann & Massberg, 2013).

Platelets are small, anucleate cell fragments that consist of numerous receptors, enzymatic systems, and other protein molecules that contribute to platelet activation. Agonists such as collagen, ADP, arachidonic acid, thrombin, and epinephrine are agents that physiologically stimulate platelet activation *in vivo* through specific platelet receptors. These agonists synergize to activate platelets (Alexandru et al., 2012;

Zhou & Schmaier, 2005). In addition to platelets agonist, ADP targeted two G-protein-coupled purinergic receptors: P2Y1 and P2Y12, which resulted in shape change and platelet aggregation. The P2Y12 receptor is considered a major receptor responsible for completing and amplifying platelet activation and aggregation (Gachet, 2005). Platelet activation and inhibition in preventing thrombotic events have received considerable critical condition, and more research is needed for better understanding.

Platelets activation is contributed by reactive oxygen species (ROS) mainly produced by NADPH oxidase (Nox). Platelets-derived ROS such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) acts as the second messenger via calcium (Ca^{2+}) mobilization, nitric oxide (NO) inactivation, and isoprostane formation. At physiologic concentration, ROS is responsible for normal cellular function. However, ROS can contribute to pathological disease if overproduced (Pastori et al., 2015; Violi & Pignatelli, 2014; Brandes et al., 2010).

Since the P2Y12 receptor mechanism has still not yet been extensively studied, there is a need to focus on the scientific exploration of the role of purinergic receptor signaling and NAPDH oxidase activation and their interaction towards platelets reactivity.

This study aimed to investigate the influence of NADPH oxidase on purinergic receptor activation of human platelet function stimulated by platelet agonists.

II. MATERIALS AND METHOD

A. Subjects

Venous blood samples were drawn from healthy volunteers aged ≥ 18 years. Inclusion criteria include no long-term medical therapy, no history of any cardiovascular-related disease such as diabetes mellitus, hypercholesterolemia, acute inflammation or infection, non-smoker, no known allergy to medicines, and abstained from drugs known to affect platelets function and numbers in the previous two weeks. Menstruating women, pregnant women, and participants who had phlebotomy procedures (e.g., blood donation or blood taking) were excluded from the study due to the possibility of unreliable results or platelet non-responsiveness. Ethical clearance was obtained from the institution, where all studies performed followed the values included in the Declaration of Helsinki. Informed consent was obtained from all subjects before conducting the study.

B. Blood Collections

Blood was collected by venipuncture through a 21-gauge butterfly needle, carefully drawn into a plastic polypropylene tube containing anticoagulant 3.2% (0.1032M) sodium citrate. Platelets-rich plasma (PRP) was prepared by centrifugation of blood from healthy volunteers at 1100 rpm for 15 minutes. Platelets-poor plasma (PPP) was then obtained by centrifugation of remaining blood at 3000 rpm for 2 minutes at room temperature. PRP and PPP were kept in a water bath at 37ºC before treated and used within 3 hours after centrifugation.

PRP was centrifuged at 7900 rpm for 10 minutes at room temperature to pellet the platelets, and the supernatant was then removed. Pellet of platelets was resuspended in Tyrode's buffer using Pasteur pipette. The buffer was released along the wall of the microcentrifuge tube, resuspending the whole pellet slowly. These are known as 'washed' platelets (WP).

Platelets were activated adenosine diphosphate (ADP) from Chrono-Par (USA) and inhibited with clopidogrel (hydrochloride) from Cayman Chemical (USA), while Nox inhibitors include diphenylene iodonium (DPI). Moreover, apocynin was purchased from Sigma-Aldrich (St. Louis, MO), while peptide gp91ds-tat from Anaspec (USA).

C. Platelet Aggregation and Adhesion Assay

Platelets aggregation was assessed as described previously (Armstrong et al., 2008). PRP obtained was pre-incubated with clopidogrel (0.1µM), apocynin (50µM), DPI (50µM) and gp91ds-tat (100µM) alone or in combination of apocynin, DPI or gp91ds-tat with clopidogrel for 30 minutes in 37ºC. These inhibitors were added into a plate in the presence of ADP (1,3,10,30,100,300µM) with an automated multichannel pipette (Eppendorf, Germany). Then, the plate was quickly placed on a microplate reader (Sunrise™, Tecan, Switzerland) and read with absorbance 595 nm for 64 cycles. Each cycle consists of vigorous shaking for 7 seconds and reading for 8 seconds with temperature setting at 37ºC. This resulted in a total assay time of approximately 16 minutes for each 96-well plate.

Platelets adhesion was measured using colorimetric assay by modifying the procedure described previously (Bellavite et al., 1994). Briefly, buffer (0.1M citric acid, 43.6 mM sodium citrate, 0.1% Triton X-100) containing p-nitrophenyl phosphate (0.2mg/ml) was added to each well and incubated for 15 minutes at room temperature before addition of NaOH (2mM). The solution would turn yellow if *p*-nitrophenol were present. The plate was read at 405 nm using a microplate reader.

D. Statistical Analysis

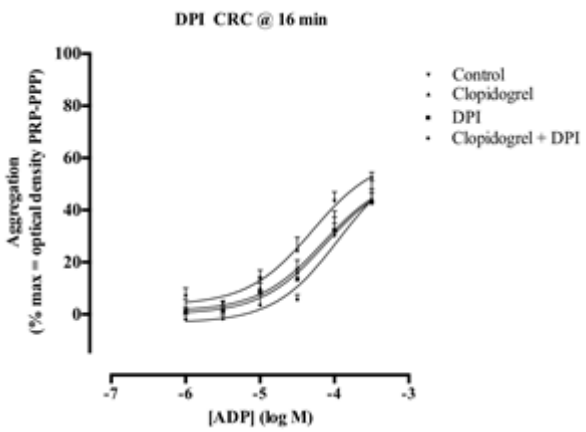
Pharmacological parameters were analyzed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA) software. The results were tested with one-way analysis of variance (ANOVA), with Bonferroni post-test (for multiple comparisons). All data in the text, tables, and figures were expressed as mean ± standard error of mean (SEM) of all values in each experiment. All experiments were performed in duplicates. p values <0.05 were considered as significant.

III. RESULT AND DISCUSSION

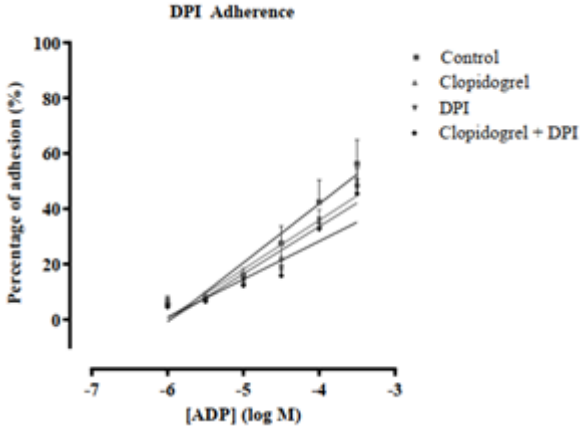
A. Effect on platelet aggregation and adhesion

Pre-incubation of PRP with clopidogrel (0.1 µM), DPI (50 µM), apocynin (50 µM), and gp91ds-tat (100 µM) either alone or in combination resulted in concentration-related inhibition in platelets aggregation and adhesion in response to ADP. Clopidogrel alone reduced platelet aggregation to a lesser extent than that found for DPI alone, apocynin alone, and gp91ds-tat alone (Figure 1). Combination of DPI with clopidogrel and combined apocynin with clopidogrel, when induced by 30µM and 100µM ADP, showed a synergistic reduction of platelets aggregation to 5.7%±1.9; 30.3%±4 and 10.6%±3.0; 25.5%±2.6 respectively as compared to control and single inhibitor (Figure 2a). Similarly, with the same concentration of ADP, the combined gp91ds-tat with clopidogrel showed a synergistic reduction of platelets aggregation to 6.1%±3.8 and 21.1%±4.6 (Figure 3a).

Similar inhibitory patterns of adhesion were also seen in clopidogrel, DPI, apocynin, and gp91ds-tat either alone or in combination upon stimulation by ADP in 30 and 100 µM. Platelets adhesion was being reduced synergistically to 15.9%±2.0 and 32.9%±3.5 in combined DPI with clopidogrel (Figure 1b); 19.1%±4.7 and 29.2%±3.6 in apocynin with clopidogrel (Figure 2b), 13.0%±1.5 and 38.7%±5.8 in combined gp91ds-tat with clopidogrel (Figure 3b) as compared to control and inhibitor alone.

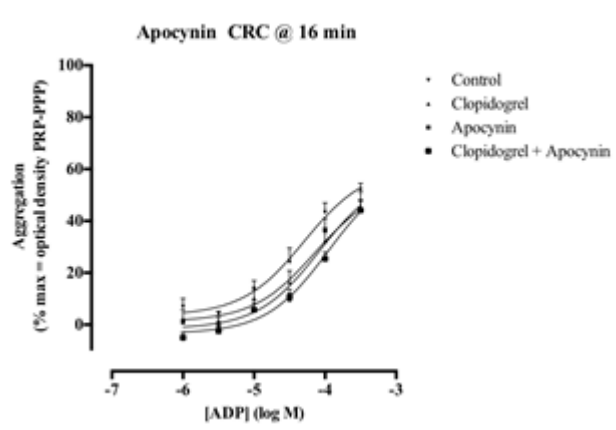


(1a)

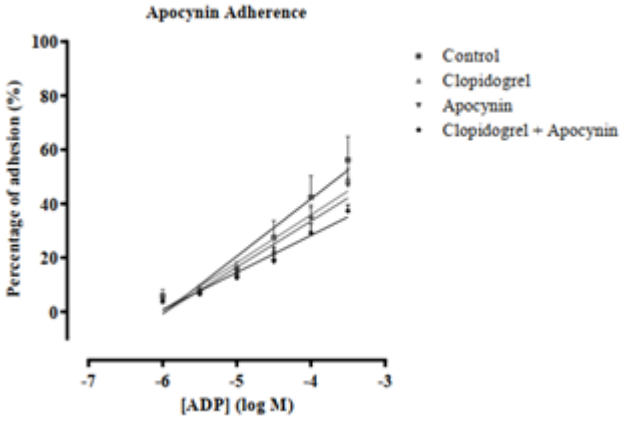


(1b)

Fig. 1 Concentration-response curves of platelet aggregation (1a) and adhesion (1b) obtained from pre-incubation with clopidogrel (0.1 µM) and DPI (50 µM) alone or in combination before platelet activation by ADP. The aggregation and adhesion were expressed as percentages (%). Results are shown as mean ± SEM (N=4).

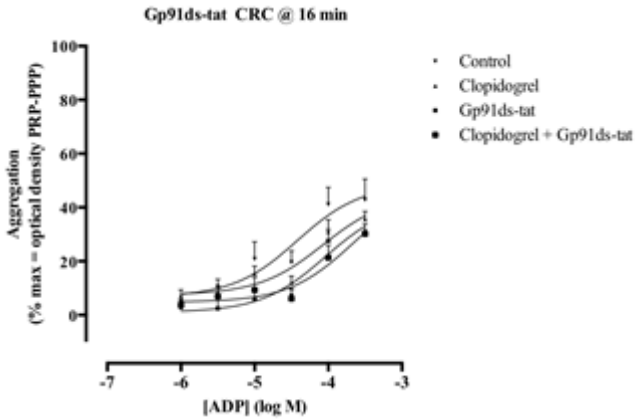


(2a)

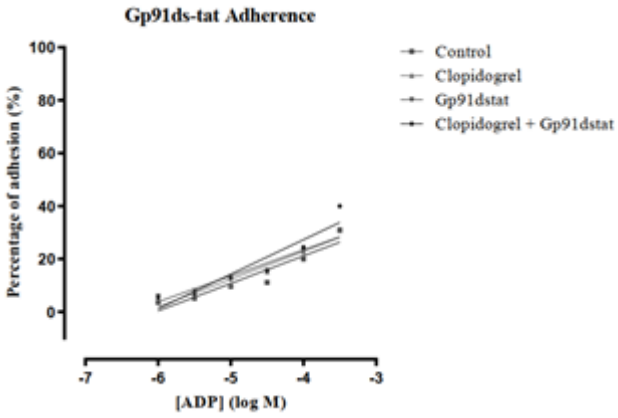


(2b)

Fig. 2 Concentration-response curves of platelet aggregation (2a) and adhesion (2b) obtained from pre-incubation with clopidogrel (0.1 µM) and apocynin (50 µM) alone or in combination before platelet activation by ADP. The aggregation and adhesion were expressed as percentages (%). Results are shown as mean ± SEM (N=4).



(3a)



(3b)

Fig. 3 Concentration-response curves of platelet aggregation and adhesion obtained from pre-incubation with clopidogrel (0.1µM) and gp91ds-tat (100µM) alone or in combination before platelet activation by ADP. Pre-incubation of platelet with clopidogrel (0.1µM) and gp91ds-tat (100µM) alone or in combination before platelet activation by a various concentration of ADP showed a reduction in platelet aggregation (3a) and adhesion (3b) as compared to control. The aggregation and adhesion were expressed as percentages (%). Results are shown as mean ± SEM (N=4).

B. Discussion

Data presented herein show that the combination of Nox inhibitors with P2Y12 receptor inhibitor; clopidogrel synergistically inhibited platelet aggregation and adhesion. Therefore, we suggest that in ADP-induced platelet function, Nox plays a role in P2Y12 downstream platelet activation and partially responsible for platelet aggregation and adhesion. ADP activates platelets via P2Y1 and P2Y12 receptors, in which activation of both P2Y receptors is mandatory for a full platelet response to ADP (Storey, 2006). The present finding suggests that inhibition of the P2Y12 receptor reduces platelet function and eventually mediates weak response compared to control. The synergism between Nox inhibitors and clopidogrel might be explained by the fact that they inhibit two physiologic pathways of amplification of platelet activation: the Nox pathway and P2Y12 receptor pathway, and these pathways are suggested to influence by each other.

In this study, DPI and apocynin have shown inhibitory property on platelet aggregation and adhesion in response to agonists explained through different mechanisms. Nox inhibitors, particularly DPI and apocynin, share the common ability to reduce Nox activity through different mechanisms (Barbieri et al., 2004). Broad-spectrum flavoprotein inhibitor DPI is the best known Nox antagonist and until today has become the *in vitro* gold standard for Nox inhibition (Bedard et al., 2015). DPI directly inhibits the activity of the gp91phox subunit and also targets`the FAD binding sequence found in other flavoproteins; thus, DPI is not specific for Nox2 (El-Benna et al., 2012).

Apocynin oligomer blocked the Nox2 system by covalently modifying Cys196 of the p47phox subunit and also preventing its translocation to the membrane. Hence, this inhibitor prevents the assembling of p47phox with p22phox.
Apocynin also has shown antioxidant properties; therefore, it is possible that the effects of apocynin are not related to Nox inhibition and ROS signaling at all (Diebold et al., 2014; Heumüller et al., 2008). Superoxide is known to amplify the platelet aggregation responses, and the previous study has demonstrated a significant reduction of platelet aggregation in the presence of DPI and apocynin (Begonja et al., 2005). The present study demonstrated that ADP-induced platelet aggregation and adhesion were reduced prior to the treatment with Nox inhibitors alone; thus, these present results aligned with previous studies.

Specific peptide inhibitor, gp91ds-tat, was designed to prevent the interaction of the p47phox subunit with the large spanning gp91phox subunit by binding to the docking sequence (ds) of phox47 at gp91phox (Rey et al., 2001). We have demonstrated that gp91ds-tat, when used in combination with clopidogrel, inhibited platelet aggregation and adhesion at high concentrations of ADP, suggesting initial aggregation is not affected by O_2^-. Platelet Nox1 and Nox2 isoforms play different roles in regulating platelet activation. Both platelet Nox1 and Nox2 are responsible for GPCR-mediated activation. However, Nox2 is also crucial for activating GPIV-dependent platelet activation (Delaney et al., 2016). It is noteworthy that increasing evidence of abundant ROS production by activating platelet Nox suggests that the Nox pathway, particularly Nox2, may represent a key target for controlling thrombosis associated with oxidative stress (Fuentes et al., 2018).

IV. CONCLUSION

Our study shows that the combination of Nox inhibitors or specific gp91phox inhibitors with clopidogrel inhibited platelet function. Mechanism of platelet function and signaling by Nox inhibitors may be beneficial for cardiovascular treatment. It can be inferred that combined Nox inhibitors with clopidogrel have a synergistic effect on platelet function. The results of this study indicate that P2Y12 receptor activation is influenced by stimulation of the Nox pathway.

Findings from this current study may enhance our understanding of the platelet activation mechanism. The present findings may also contribute to the anti-inflammatory and antithrombotic benefits of combined Nox inhibitors with clopidogrel treatment. Thus, this study makes several noteworthy contributions to the potential combination therapy for thrombosis and cardiovascular disease.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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