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Reduced nicotinamide adenine dinucleotide phosphate inhibits rat platelet aggregation and p38 phosphorylation

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ABSTRACT

Previous studies found that reduced nicotinamide adenine dinucleotide phosphate (NADPH) protected neurons against ischemia/reperfusion-induced injury. In addition to ROS reduction and ATP increment, preliminary data suggested that NADPH inhibited ADP and thrombin-induced platelet aggregation. As the effect of NADPH on platelet function was not reported by other investigators, the actions of NADPH on platelet function and mechanisms of actions were investigated in the present study. In vitro studies, the effects of different concentrations of NADPH on platelet aggregation induced by ADP (10 μM), thrombin (0.05 U/mL) or AA (50 μM) were determined. The results showed that NADPH could inhibit platelet aggregation induced by ADP, thrombin or AA in a concentration dependent manner. When the inhibitory effects of NAD^+, NADH, $NADP^+$ and NADPH on platelet aggregation were compared, NADPH demonstrated the relatively best effect on platelet aggregation. In vivo studies, the effects of NADPH on platelet aggregation, tail bleeding time, coagulation response and ferric chloride-induced thrombosis were determined in mice or rats. The maximum aggregation rate of platelets of rats injected with NADPH (5 mg/kg) was lower than platelets from control rats. NADPH transiently prolonged tail bleeding time in mice at 30 min after the injection of NADPH (7.5 mg/kg), while aspirin (15 mg/kg) significantly prolonged the tail bleeding time in mice at all time points examined. NADPH (5 mg/kg), as well as aspirin (10 mg/kg), had no effect on coagulation response in rats. Using a $FeCl_3$-induced abdominal aorta injury thrombosis model, administration of NADPH (5 mg/kg) significantly delayed the onset of vessel occlusion, while aspirin (10 mg/kg) almost completely prevented the vessel occlusion. With microscopic examination the thrombi in injured vessel sections of rats received NADPH were much smaller and less dense than that of rats received vehicle treatment. ADP induced an increase in phosphorylation of p38 and the effect was markedly inhibited by the p38 inhibitor SB203580. Similarly, NADPH also inhibited ADP-induced phosphorylation of p38. Similar to NADPH, SB203580 robustly inhibited ADP- and thrombin-induced platelet aggregation. In addition, NADPH also reduced ADP-induced increases in ROS in platelets. The current results demonstrated that NADPH inhibited platelet aggregation, oxidative stress and p38 phosphorylation, suggesting that NADPH might be a novel compound for management of high risk of cardiovascular disease.

1. Introduction

The platelet is the second largest population of blood cells in mammals and its main function is to stop bleeding by forming a clot together with fibrin at the site of disrupted vessels. However, hyperactivity of platelet aggregation is recognized as an important risk factor for formation of the thrombus [1], which is commonly associated with multiple cardio-cerebral vascular diseases, such as acute myocardial infarction (clots take place in the coronary circulation) and ischemic stroke (clots take place in the cerebral circulation) [2]. These diseases are leading causes of death and disability worldwide. Thus, the inhibition of excessive platelet aggregation is an important measure to prevent and treat cardio-cerebral vascular diseases and of vital importance to human health. Aspirin is a widely used drug for this application and it is often combined with clopidogrel, eptifibatide or other antiplatelet drugs to seek a better efficacy, reduce adverse reactions or for other reasons. For instance, when aspirin fails to prevent stroke, clopidogrel is usually applied. Aspirin is an inhibitor of cyclooxygenase (COX) and inhibits platelet aggregation by reducing the synthesis of thromboxane A_2 (TXA_2), while clopidogrel inhibits platelet aggregation by inhibiting the binding of ADP to its receptor. These antiplatelet drugs have many adverse effects, including internal bleeding,

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prolonged bleeding time, gastrointestinal irritation and risk of inhibiting PGI_2 [3]. In particular, the increased risk of bleeding, carries a similar risk of death as recurrent ischemia [4]. Furthermore, bleeding is a distinct problem in older people with cardiovascular diseases. Therefore, finding an agent with fewer adverse effects but greater efficacy for chronic prevention of thrombosis is vitally important.

Reduced nicotinamide adenine dinucleotide phosphate (NADPH), a metabolic product of pentose phosphate pathway (PPP) [5], has many important biological functions, including its role as the key component in cellular antioxidation systems, an electron source for reductive synthesis and the substrate for NADPH oxidase [6]. In previous studies, NADPH showed protective effects on neurons against ischemia/reperfusion-induced injury [7] and its inhibitory action on platelet aggregation induced by ADP or thrombin was found [8]. Therefore, we hypothesized that NADPH may prevent thrombosis by inhibiting platelet aggregation.

Here, report that NADPH could inhibit platelet aggregation in vitro and in vivo and partially prevent thrombosis with less influence on tail bleeding time than aspirin. The inhibitory effects of NADPH on platelet function were associated with reduction in p38 phosphorylation and oxidative stress.

2. Materials and methods

2.1. Reagents and materials

Adenosine 5′-diphosphate (ADP), arachidonic acid (AA), NAD^+, NADH, $NADP^+$, acetylsalicylicacid (aspirin) and dihydroethidium (DHE) were from Sigma Aldrich (St. Louis, MO, USA), whereas thrombin was from Solarbio (Beijing, China) and NADPH was from Renben Pharmaceutical Company (Suzhou, China). The anti-p38 and anti-phospho-p38 (Thr180/Tyr182) were from Cell Signal Inc. (Beverly, MA, USA). SB203580 was from Selleck (Houston, TX, USA). $NADP^+$/NADPH assay kit was from Bioassay Systems (Hayward, CA, USA). Other reagents were all of analytical grade.

2.2. Animals

Male ICR mice weighing 25–30 g and male Sprague-Dawley rats weighing 300–350 g were purchased from SLACCAL Lab Animal Ltd. (Shanghai, China). All animals used for experiments were housed in groups in a humidity- and temperature-controlled environment under 12/12 light/dark cycles, with access to a standard diet and water ad libitum. All experiments involving animals were performed in accordance with the institutional guidelines for animal use and care, and the research protocol was approved by the Ethical Committee of Soochow University.

2.3. Preparation of rat platelets

Rat platelets were prepared as previously described [7]. Briefly, normal rats were anesthetized with 1% pentobarbital sodium and blood was drawn from the abdominal aorta and anti-coagulated with acid-citrate dextrose buffer (9:1 vol/vol). Then, the whole blood was centrifuged at 250*g* for 5 min to gain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by centrifuging the remaining blood sample at 2500*g* for 15 min. Platelet-rich plasma (PRP) was pelleted at 1000*g* for 10 min and then washed twice with a modified Tyrode's buffer without Ca^{2+} and resuspended again. Platelet number was adjusted to a final concentration at 2×10^8 cells/mL.

2.4. Platelet aggregation assay

Aggregation of platelets was determined using a platelet aggregator (Tailikangxin Technology, Beijing, China). In vitro studies, platelets were first incubated with NAD^+, NADH, $NADP^+$, NADPH or SB203580 for 5 min at 37 °C and aggregation was induced by ADP (10 μM), thrombin (0.05 U/mL) or AA (50 μM) [9]. In vivo studies, rats were IV injected with NADPH (5 mg/kg) 30 min before collected blood samples. The platelet aggregation was induced by ADP (10 μM) directly. The aggregation curve and the maximum aggregation rate were recorded.

2.5. Measurement of NADPH levels

In vitro studies, platelets were incubated with NADPH (60 μM) for 5 min and washed twice for the measurement of NADPH level with the enzychrom $NADP^+$/NADPH assay kit following the manufacturer's instructions. In vivo studies, rats were IV injected with NADPH (5 mg/kg) 30 min before collecting blood samples. Then, blood was obtained and NADPH concentrations in plasma and platelets were measured with the enzychrom $NADP^+$/NADPH assay kit following the manufacturer's instructions.

2.6. Tail bleeding time assay

Mice were IV injected with different concentrations of NADPH or normal saline or IG given with aspirin (15 mg/kg) or distilled water and then anesthetized with 1% pentobarbital sodium at different time points. The tail was transected at a distance of 3 mm from the tip and then immersed in 3 mL of normal saline warmed to 37 °C. The time to bleeding cessation was measured and blood loss was determined by measuring the absorbance of the mixture of normal saline and blood at 405 nm using a microplate reader. The maximum observation time is 1800 s [10].

2.7. Coagulation response

Rats were IV injected with NADPH (5 mg/kg) or normal saline or given IG aspirin (10 mg/kg) or distilled water and then anesthetized with 1% pentobarbital sodium after 30 min or 60 min. Blood was drawn from abdominal aorta of the rats and anti-coagulated with acid-citrate dextrose buffer (9:1 vol/vol). After that, the coagulation function was tested in 2 h.

2.8. Ferric chloride–induced thrombosis model

Rats were IV injected with NADPH (5 mg/kg) or normal saline or given IG aspirin (10 mg/kg) or distilled water and the $FeCl_3$-induced thrombus in abdominal aorta was performed after 30 min or 60 min. The rats were anesthetized with 1% pentobarbital sodium and a filter paper (10 × 5 mm) with 15% $FeCl_3$ was attached to the exposed abdominal aorta after the normal blood flow was monitored for 5 min, and then removed after 20 min of $FeCl_3$ exposure [11]. The blood flow was continuously monitored with a laser Doppler perfusion and temperature monitor and time to vessel occlusion was calculated as the difference in time between the placement of the filter paper and the stable occlusion [12]. Finally, the impaired vessel segment was dissected, made into paraffin sections, stained with hematoxylin-eosin (HE) and examined with microscopy.

2.9. Western blot analysis

Western blot was performed according to the method described previously [3]. Briefly, after platelet aggregation assay, the platelets were lysed and proteins were separated on 10% SDS-polyacrylamide gel, and then transferred onto a nitrocellulose filter membrane. After that, the nitrocellulose filter membrane was blocked with 5% fatty-free milk for 60 min and incubated with primary antibodies. The working dilutions of the antibodies were used as the suggestions of the manufacturers. The images were captured with the Odyssey infrared imaging system and analyzed with the Image J software [13].

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