DL-3-n-butylphthalide inhibits platelet activation via inhibition of cPLA2-mediated TXA2 synthesis and phosphodiesterase

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Abstract

Aberrant platelet activation plays a critical role in the pathogenesis of heart attack and stroke. DL-3-n-butylphthalide (NBP) has been approved in China to treat stroke with multiple mechanisms. The anti-stroke effects of NBP may be related to its antiplatelet effects reported in rats in addition to its antioxidative, antiapoptotic, and angiogenic effects. However, the effects and the underlying mechanisms of NBP on human platelets are not yet clear. In this study, we found that NBP concentration-dependently inhibited human platelet aggregation and ATP release induced by ADP, thrombin, U46619, arachidonic acid, or collagen. NBP also inhibited PAC-1 binding induced by ADP or thrombin and platelet spreading on immobilized fibrinogen. NBP reduced TXA2 synthesis induced by thrombin or collagen via inhibiting cPLA2 phosphorylation, concomitantly with a marked decrease in intracellular calcium mobilization. Moreover, NBP also inhibited human platelet phosphodiesterase (PDE) and elevated 3,5-cyclic adenosine monophosphate level in platelets. In conclusion, NBP significantly inhibits human platelet activation via inhibition of cPLA2-mediated TXA2 synthesis and PDE, and may be effective as an antiplatelet drug to treat other arterial thrombotic diseases.

Keywords

Antiplatelet, cPLA2, DL-3-n-butylphthalide, phosphodiesterase, TXA2

Introduction

Arterial thrombotic diseases heart attack and stroke, are the leading cause of morbidity and mortality in industrialized countries. Platelet activation elicited by atherosclerotic plaque rupture or local vessel injury caused by percutaneous coronary intervention and the resulting intravascular arterial thrombogenesis are the common pathological basis of arterial thrombotic diseases [1]. Therefore, antiplatelet drugs are very effective for the prevention and treatment of arterial thrombotic diseases. Multiple pathways contribute to platelet activation, and the pathways for protective hemostasis may differ from the pathways of pathologic thrombosis [2]. Although current oral antiplatelet therapy with cyclooxygenase inhibitor aspirin and P2Y12 receptor antagonists have demonstrated proved clinical benefit, residual morbidity and mortality still remains high [3, 4], and novel antiplatelet agents with improved efficacy and safety profile are still needed.

1-3-n-Butylphthalide (1-NBP), a pure component extracted from celery seeds, has antiplatelet and antithrombotic activity in rats and improves cognitive deficits in rats with chronic cerebral ischemia [5, 6]. DL-3-n-Butylphthalide (NBP), the racemate of 1-NBP, has been synthesized and approved for clinical use in stroke patients in 2002 by China Food and Drug Administration [5, 7]. In a multiple-center, double-blind clinical trial, Cui et al. [8] found that NBP is safe and more effective than TXA2 synthase inhibitor ozagrel and aspirin to treat acute ischemic stroke. NBP has also been shown to decrease brain infarct size in mouse and rat ischemic stroke models [9–11]. However, the molecular mechanism of NBP is still obscure. NBP has been reported to have antiplatelet [5, 12], antiapoptotic [10], antiangiogenic [13–15], and antioxidant properties [10], which may all contribute to its beneficial effects in stroke patients. Among the multiple effects of NBP, the antiplatelet effect was only investigated in rats and NBP was found to increase cAMP in rabbit platelets [5, 12].

In this study, we investigated the effects of NBP on human platelets and further explored its mechanism. We found that NBP inhibits human platelet activation via inhibition of TXA2 synthesis and phosphodiesterase (PDE).

Materials and methods

Reagents and chemicals

ADP, thrombin, arachidonic acid (AA), collagen, and luciferin–luciferase were purchased from Chrono-Log Corp (Havertown, PA) and U46619 from Enzo Life Sciences (Lausen, Switzerland). AR-C69931MX was a gift from AstraZeneca (Loughborough, United Kingdom). Apyrase grade VII, human fibrinogen, forskolin, 3-isobutyl-1-methylxanthine (IBMX), cAMP, and acetylsalicylic acid (aspirin) were purchased from Sigma-Aldrich (St. Louis, MO). [3H] cAMP was obtained from

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PerkinElmer (Waltham, MA) and TXB₂ enzyme immunoassay (EIA) kits from Suzhou University (Suzhou, China). Fluo-3/AM and F127 were purchased from Biotium (Hayward, CA). Anti-phospho-cytosolic phospholipase A2 (cPLA2) (Ser505) and anti-GAPDH antibody were from Cell Signaling Technology (Beverly, MA). All other reagents were of reagent grade, and deionized water was used throughout.

**Preparation of human platelet-rich plasma and washed platelets**

All experiments using human subjects were carried out in accordance with the Declaration of Helsinki and approved by the Fudan University Institutional Review Board. Only healthy volunteers without taking aspirin or other nonsteroidal anti-inflammatory drugs for at least 14 days were recruited and informed consent was obtained before blood collection. Blood (36 ml) was drawn into tubes containing 6 ml ACD (85 mM sodium citrate, 71.38 mM citric acid, and 27.78 mM glucose) solution. PRP and washed platelets were prepared as described previously [16–18]. If indicated, PRP was incubated with 1 mM aspirin for 30 minutes at 37°C. Platelet number was adjusted to 2–3 × 10⁸ platelets/ml.

**Measurement of platelet aggregation and platelet secretion**

Aggregation of 0.5 ml human washed platelets in response to agonists or antagonists was analyzed using a lumi-aggregometer (Model 400VS; Chrono-Log) under stirring conditions (900 rpm) at 37°C as reported before [17–20]. In some experiments, platelet secretion was simultaneously monitored by measuring ATP release using luciferin–luciferase reagent [17].

**Measurement of PAC-1 binding in whole blood using flow cytometry**

First, 2 ml blood were discarded and the whole blood was diluted by isotonic HEPES buffer (1:10 V/V). Platelets were pre-treated by different antagonists for 3 minutes and subsequently activated by ADP or thrombin for 3 minutes. The samples were incubated with allophycocyanin (APC)-conjugated anti-CD42b monoclonal antibody (BioLegend, San Diego, CA) and fluorescein isothiocyanate (FITC)-conjugated PAC-1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in the dark at room temperature for 15 minutes without stirring, and then fixed with 400 μl 1% paraformaldehyde. Platelets were identified with anti-CD42b and the level of platelet activation was expressed as percentage of PAC-1-positive cells [21].

**Measurement of cAMP level in human platelets**

Intracellular cAMP of platelets was measured using chromatography and expressed as cAMP conversion from ATP calculated as described before [22] using the following formula: cAMP conversion from ATP = ([3H]cAMP/([3H]ATP + [3H]cAMP)) × 10⁻³.

In addition, we also detected the cAMP level using ELISA. Human washed platelets were pre-incubated with 300 μM NBP, 100 nM AR-C69931MX, or 100 μM IBMX for 3 minutes and reactions were terminated by addition of 0.1 M HCl. After being incubated for 20 minutes at room temperature, the platelets were centrifuged at 1000 g for 10 minutes. The supernatant was decanted into a clean test tube and subjected to cAMP assay using cAMP EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s protocols.

**Assay of the activity of PDE extracted from human platelets**

Human platelet PDE extracts were prepared and the assay for cAMP-PDE activity was performed using high-performance liquid chromatography (HPLC) as previously reported [18]. The inhibition of PDE activity was calculated using the following formula: % inhibition of PDE activity = (1 – converted cAMP in enzyme reaction system treated by different agent/converted cAMP in untreated enzyme reaction system) × 100%.

**Measurement of TXA₂ generation in human platelets**

Human washed platelets pre-treated with or without NBP were stimulated with or without agonists at 37°C in an aggregometer under stirring at 900 rpm. After 5 minutes, the reaction was stopped by adding 5 mM EDTA. The samples were centrifuged at 3000 g for 10 minutes at 4°C to remove lysed platelets [23, 24]. The supernatants were diluted with assay buffer and used to measure the content of TXB₂, a stable metabolite of TXA₂, using an ELISA commercial kit according to the manufacturer’s instruction.

**Western blot analysis**

Human washed platelets pre-treated with vehicle or NBP were stimulated with agonists for 5 minutes and lysed using five times sample loading buffer and then boiled for 5 minutes. The platelet lysates were further subjected to western blot analysis using anti-phospho-cPLA2 and anti-GAPDH antibody. The optical density of the bands was measured using Image J (National Institutes of Health, Bethesda, MD).

**Measurement of intracellular Ca²⁺ release in human platelets**

Human PRP was centrifuged and then resuspended in Tyrode’s buffer containing no calcium. Platelets were subsequently loaded with 2 μM fluo-3/AM for 30 minutes at 37°C in the dark. Platelets incubated with DMSO (0.5%) or NBP (300 μM) for 5 minutes before treatment with ADP (10 μM). Fluorescence measurements were performed under continuous stirring using a Hitachi F4500 Fluorescence spectrophotometer [25].

**Platelet spreading experiment**

Glass coverslips were coated with 20 μg/ml fibrinogen in 0.1 M NaHCO₃ (pH 8.3) at 4°C overnight. Human washed platelets (2 × 10⁷/ml) pre-incubated with or without inhibitors were allowed to adhere and spread on the fibrinogen-coated glass coverslips at 37°C for 90 minutes. After washing with PBS, attached platelets were fixed, permeabilized, and then stained with FITC-labeled phalloidin as previously described [26, 27]. After washing with PBS, adherent platelets were viewed by confocal microscopy using a Leica SPE confocal microscope. The images were processed with Leica LAS AF Lite software (Leica Microsystems Inc., Buffalo Grove, IL).

**Statistical analysis**

All data are expressed as mean ± SEM. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by a Newman–Keuls test using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA) unless otherwise stated. p Values less than 0.05 were considered statistically significant.
Results

NBP inhibits platelet activation induced by ADP, thrombin, U46619, AA, and collagen

As shown in Figure 1, in the range of 30–300 μM, NBP concentration-dependently inhibited platelet aggregation and ATP release of human washed platelets stimulated by 10 μM ADP, 0.05 U/ml thrombin, 1 μM U46619, 0.5 mM AA, or 1 μg/ml collagen. When aspirinated platelets were used, the inhibitory effects of NBP on platelet activation induced by ADP or thrombin were obviously dampened, while the inhibition on platelet activation induced by U46619 (Figure 2A and B) remains similar to that in non-aspirinated platelets (Figure 1). This result suggests that NBP may inhibit platelet activation by suppressing TXA2 production. Compared to the vehicle control, 300 μM NBP shifted the concentration–response curve of U46619-induced platelet aggregation to the right (Figure 2C), suggesting that the TXA2 receptor (TP)-antagonism may also contribute to the antiplatelet effect of NBP.

The inhibitory effects of NBP on ADP-induced platelet activation were further confirmed by PAC-1 binding using whole blood from healthy donors. NBP 300 μM reduced PAC-1 binding on human platelets stimulated by 10 μM ADP or 0.05 U/ml thrombin (Figure 3), further confirming the antiplatelet activity of NBP on human platelets.

NBP inhibits platelet spreading on immobilized fibrinogen

Fibrinogen binding to activated integrin αIIbβ3 results in platelet aggregation by a classical inside-out signaling. Then, it triggers outside-in signaling, leading to platelet spreading and clot retraction, which play a crucial role in thrombosis. We have shown that NBP inhibits platelet aggregation induced by multiple agonists and integrin αIIbβ3 activation induced by ADP, two typical inside-out signaling events. We next investigated whether NBP also influence platelet outside-in signaling. As shown in Figure 4, NBP 300 μM significantly inhibited platelet spreading on immobilized fibrinogen, similar to LY294002, a PI3K inhibitor, which inhibits platelet spreading [26]. NBP slightly inhibited platelet spreading at 100 μM, a concentration that significantly inhibited platelet aggregation (Figure 4).

NBP inhibits TXA2 synthesis via inhibiting cPLA2 phosphorylation

The more robust inhibition of NBP on non-aspirin-treated platelets induced by ADP or thrombin but not by U46619 (Figures 1 and 2) suggests that the antiplatelet effects of NBP may depend on TXA2 synthesis inhibition. As shown in Figure 5(A) and (B), in the range of 30–300 μM, NBP concentration-dependently inhibited platelet TXA2 production as measured by TXB2, the stable metabolite of TXA2. At 100 and 300 μM, NBP inhibited TXA2 synthesis similarly to that caused by aspirin (100 μM) or indomethacin (10 μM), two typical TXA2 synthesis inhibitors.

TXA2 is produced by activated platelets via the sequential conversion of AA by cPLA2, cyclooxygenase-1 (COX-1), and thromboxane synthase. Since cPLA2 phosphorylation is the first step to liberate AA from platelet membrane phospholipid, we investigated whether NBP influences cPLA2 phosphorylation and therefore inhibits TXA2 synthesis. As shown in Figure 5(C), the phosphorylation of cPLA2 in human washed platelets stimulated...
Figure 2. NBP inhibits platelet aggregation and ATP release in aspirin-treated human washed platelets induced by ADP, thrombin, and U46619. (A) NBP (30-300 µM) inhibited platelet aggregation in aspirin-treated human washed platelets induced by 10 µM ADP, 0.05 U/ml thrombin, or 1 µM U46619. Simultaneously recorded ATP release was inhibited by NBP. Tracings shown were representative of five experiments using platelets from different donors. DMSO was used as a vehicle control. (B) Quantification of NBP inhibition on platelet aggregation in aspirin-treated human washed platelets. Data are expressed as mean ± SEM, n = 5. (C) Concentration-response curve of U46619-induced platelet aggregation was shifted to the right by 300 µM NBP. Each data point is expressed as mean ± SEM of four experiments from different donors.
with ADP was inhibited by NBP 30–300 μM. Furthermore, we showed that NBP did not influence the phosphorylation of p38 and Src, two signaling molecules upstream of cPLA2 [28, 29] (data not shown). The inhibition of cPLA2 phosphorylation by 300 μM NBP was lower than that caused by 100 nM AR-C69931MX, a typical P2Y12 antagonist. We therefore investigated whether NBP influenced P2Y12 signaling. We found that 300 μM NBP did not influence ADP-induced platelet cAMP decrease (Supplemental Figure S1), ruling out the involvement of P2Y12 signaling pathway. Taken together, these findings suggested that TXA2 synthesis inhibition by NBP might be through inhibiting cPLA2 phosphorylation.

**NBP reduces ADP-induced platelet intracellular Ca2+ mobilization**

TXA2 activates platelets via the TXA2 receptor, which couples to Gq and G13. The activation of Gq increases intracellular Ca2+ mobilization. Therefore, we investigated whether NBP affects intracellular Ca2+ mobilization. As shown in Figure 5(D), in the
ADP, further confirming the antiplatelet effect of NBP via TXA₂ synthesis. NBP 100 μM markedly reduced Ca²⁺ rise, which then dropped back to baseline due to Ca²⁺ re-uptake or extrusion. NBP 100 μM markedly reduced Ca²⁺ rise elicited by ADP, further confirming the antiplatelet effect of NBP via TXA₂ synthesis inhibition.

**NBP inhibits the activity of PDE extracts from human platelets**

NBP has been reported to increase cAMP level in rabbit resting platelets [12], which could be a result of PDE inhibition. Therefore, we prepared PDE extracts from human platelets and investigated the effects of NBP on the activity of PDE indicated by residual cAMP, measured by HPLC. As shown in Figure 6(A) and (B), 300 μM NBP inhibited the activity of platelet PDE extracts as evidenced by cAMP increase in the reaction mixture. Compared with 100 μM IBMX, a non-specific PDE inhibitor, 300 μM NBP moderately inhibited platelet PDE activity, which may also contribute to the antiplatelet effects of NBP, especially in aspirin-treated platelets (Figure 2).

Moreover, we examined the effects of NBP on platelet cAMP. As shown in Figure 6(C), 300 μM NBP increased intracellular cAMP level in resting human platelets. This result further confirms the PDE inhibition activity of NBP.

**Discussion**

NBP is mainly used as an anti-stroke drug clinically with multiple mechanisms including the antiplatelet and antithrombotic activities reported in rats [12]. The antiplatelet mechanism of NBP is not clear. Xu and Feng [12] ever reported that NBP increased rat platelet intercellular cAMP levels and thought this may account for its antiplatelet effects. In this study, using human platelets, we found that NBP inhibits platelet activation induced by multiple agonists and revealed that NBP exerts its antiplatelet effects by inhibiting PDE and TXA₂ synthesis.

Previously, Xu et al. found that NBP 100 μM inhibits platelet aggregation induced by ADP, AA, and collagen in rat PRP. Consistently, our present study demonstrated that NBP (30–300 μM) inhibited platelet aggregation and ATP release induced by multiple agonists including the aforementioned agonists and thrombin in non-aspirinated human washed platelets. NBP also inhibited platelet aggregation and ATP release in aspirinated human washed platelets stimulated with thrombin and U46619.

When ADP was used as platelet agonist, we noticed that NBP 100 μM inhibited platelet aggregation more robustly in non-aspirinated platelets than in aspirinated platelets (80% vs. 40%). These results suggest that the antiplatelet effects of NBP may be a result of inhibition of TXA₂ biosynthesis, a mechanism similar to aspirin and GIRK blockers which inhibit platelet activation via suppressing TXA₂ generation [29]. TXB₂ assay confirmed that NBP significantly inhibited the TXA₂ synthesis induced by thrombin or collagen, similar to aspirin or indomethacin. We further investigated the mechanism of TXA₂ synthesis inhibition and found that NBP inhibited cPLA₂ phosphorylation in human washed platelets induced by ADP. More work is needed to define the detailed mechanism of TXA₂ synthesis inhibition by NBP.

The antiplatelet effects of NBP observed in aspirated platelets stimulated with U46619 (Figure 2) cannot be explained by TXA₂ synthesis inhibition. Therefore, NBP might bear other antiplatelet mechanism. Xu and Feng [12] ever reported that NBP increased platelet intracellular cAMP, we therefore explored the possible role of NBP on P2Y₁₂ receptor and PDE, both influencing platelet cAMP level and platelet activation [30]. Even at 300 μM, NBP does not affect ADP-induced cAMP decrease in platelets, ruling out the P2Y₁₂ receptor as the target of NBP. In contrast, NBP significantly suppressed the activity of platelet PDE, correlating with its cAMP rising and antiplatelet effects. Therefore, we think that NBP exerts its antiplatelet effects via PDE inhibition in addition to TXA₂ synthesis inhibition.

Inside-out signaling of integrin αIIbβ3 results in the binding of fibrinogen to integrin αIIbβ3 and subsequently triggers outside-in signaling, which leads to stable platelet adhesion, spreading, and clot retraction [31]. In this study, we found that at the antiplatelet concentration (300 μM), NBP dramatically inhibited platelet spreading on immobilized fibrinogen, which may also contribute to its antiplatelet effects.

In conclusion, for the first time, we show that NBP inhibits human platelet activation via inhibition of TXA₂ synthesis and PDE. Previous studies have demonstrated that NBP markedly inhibited thrombus formation in rats [5, 32]. Given that NBP has been safely used in stroke patients as a multitarget drug, NBP may be also useful to treat other arterial thrombotic diseases involving
Figure 5. NBP reduces TXA2 generation by inhibiting cPLA2 phosphorylation. Human washed platelets were preincubated with DMSO (0.5%), aspirin (100 μM), indomethacin (10 μM), or NBP (30–300 μM) at 37 °C for 3 minutes and subsequently stimulated by 0.05 U/ml thrombin (A) or 1 μg/ml collagen (B) for 5 minutes. The levels of TXB2, a stable metabolite of TXA2, in supernatants were determined and expressed as mean ± SEM representing at least three separate experiments. (C) Human washed platelets were preincubated with DMSO (0.5%), AR-C69931MX (100 nM), or NBP (30–300 μM) at 37 °C for 3 minutes and subsequently stimulated by 10 μM ADP for 5 minutes. Platelets were solubilized and immunoblotted with antibodies against p-cPLA2 (Ser505) and GAPDH. Data shown are representative of four experiments using platelets from different donors. Quantification of cPLA2 phosphorylation normalized to GAPDH was also shown (mean ± SEM, n = 4). (D) NBP inhibited ADP-induced platelet intracellular calcium mobilization. Fluo-3/AM loaded platelets were preincubated with DMSO (0.5%) or NBP 100 μM at 37 °C for 90 seconds, then ADP 10 μM was added. Tracings shown were representative of three separate experiments.
aberrant platelet activation such as coronary heart diseases and peripheral vascular diseases. Further investigation is still needed to answer this question.

Declaration of interest

The authors report no potential conflicts of interest. This work was partially supported by National Natural Science of Foundation of China (No.81173053, 81100344), Drug Innovative Program from Shanghai Municipal Science and Technology Commission (No. 11431920103).

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