# N-stearoylethanolamine inhibits integrin-mediated activation, aggregation and adhesion of human platelets

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**Running title:** N-stearoylethanolamine inhibits platelet reactivity

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**Abbreviations:** AA – arachidonic acid; ADP – adenosine diphosphate; AO – acridine orange; COX – cyclooxygenase; DAG – diacylglycerol; DMSO – dimethyl sulfoxide; FA – fatty acids; FS – forward scattering; GP – glycoprotein; GTP – guanosine triphosphate; IR – insulin resistance; HFD – high-fat diet; $IP_3$ – inositol-1,4,5-trisphosphate; IR – insulin-resistant; mAb – monoclonal antibody; NAEs – N-acyletanolamines; NOE – N-oleoylethanolamine; NSE – N-stearoylethanolamine; OPD – o-phenylenediamine; PAF – platelet activating factor; PAR – protease-activated receptor; PL – phospholipase; PRP – platelet-rich plasma; SEM – scanning electron microscopy; SS – side light scattering; vWF – von Willebrand factor; USFA – unsaturated fatty acids.

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### Abstract

N-stearoylethanolamine (NSE), a lipid mediator that belongs to the N-acylethanolamine (NAE) family, has anti-inflammatory, antioxidant and membranoprotective actions. In contrast to other NAEs, NSE does not interact with cannabinoid receptors. The exact mechanism of its action remains unclear. The aim of this study was to evaluate the action of NSE on activation, aggregation and adhesion of platelets that were chosen as a model of cellular response. Aggregation of platelets was measured to analyze the action of NSE ($10^{-6}$–$10^{-10}$ M) on platelet reactivity. Changes in granularity and shape of resting platelets and platelets stimulated with ADP in the presence of NSE were monitored by flow-cytometry, platelet deganulation was monitored by spectrofluorimetry. In vivo studies were performed using obese insulin-resistant rats. Binding of fibrinogen to the GPIIb/IIIa receptor was estimated using indirect ELISA and scanning electron microscopy (SEM). It was found that NSE inhibits the activation and aggregation of human platelets. Our results suggest that NSE may decrease the activation and subsequent aggregation of platelets induced by ristocetin, epinephrine and low doses of ADP. NSE also reduced the binding of fibrinogen to GPIIb/IIIa on activated platelets. These effects could be explained by the inhibition of platelet activation mediated by integrin receptors: the GPIb-IX-V complex for ristocetin-induced activation and GPIIb/IIIa when epinephrine and low doses of ADP were applied. The anti-platelet effect of NSE complements its anti-inflammatory effect and allows us to prioritize studies of NSE as a potent anti-thrombotic agent.

### 1. Significance Statement

N-stearoylethanolamine (NSE) was shown to possess inhibitory action on platelet activation, adhesion and aggregation. The mechanism of inhibition possibly involves integrin receptors. This finding complements the known anti-inflammatory effects of NSE.

### 2. Introduction

N-acyletanolamines (NAEs) are low-molecular-weight derivatives of saturated and unsaturated fatty acids, involved in the homeostatic response to cell damage. Their action on cells may be realized via both receptor-independent and receptor-dependent mechanisms (Mato et al., 2009).

The action of NAEs is directed at the neutralization of pathological damage in many physiological processes, and explains why the level of NAEs, normally in the picomolar range in (human) plasma, increases substantially during pathological or inflammatory processes (Kilaru et al., 2011). The concentration of NAEs in human plasma under certain pathological conditions can reach 2.70±3.37 ng/ml (vs 0.83±0.47 ng/ml in healthy controls) (Hauer et al., 21013).

N-stearoylethanolamine (NSE) is an NAE, with potent anti-inflammatory properties (Vitychuk et al., 2012) (Goridko et al., 2001). NSE is able to modulate voltage-gated sodium and calcium channels and, as a consequence, impacts the membrane potential of cardiac myocytes (Vitychuk et al., 2012). Previous studies showed that NSE had membranotropic action, and could influence the cholesterol level and balance between saturated and unsaturated fatty acids (Goridko et al., 2001). The modification of membrane lipid composition by NAEs led to the inhibition of veratridine-activated ion channels (Di Marzo et al., 1996) and decreased the production of 11-oxy steroids by the adrenal cortex in rats (Mikosha et al., 1998). NSE was also shown to prevent $Fe^{2+}$-dependent oxidation of lipids induced by free radicals in cardiac and liver mitochondria (Parinardi et al., 1988) (Gulaya et al., 1998). In contrast to other NAEs, NSE does not interact with cannabinoid receptors (Movahed et al., 2005) and its action may be realized via rearranging membrane lipids and modulation of proinflammatory signals (Onopchenko et al., 2018). However, the exact mechanisms of the action of NSE remain unclear.

The aim of our study was to evaluate NSE action on platelet activation, aggregation and adhesion. These processes, in particular, involve integrin signalling and platelet degranulation and are highly dependent on membrane lipid rearrangements and cholesterol-enriched domains. Platelets were chosen to study the mechanisms of NSE action on living cells because they are small anuclear cells that can be activated through diverse pathways and their responses to an inducer can be easily measured. By studying the activation, adhesion and aggregation of platelets using different approaches and comparing the results, we aimed to detect the peculiarities of NSE action on different branches of platelet signalling pathways separately. Taking into account that selective targeting of integrin outside-in signalling mechanisms provides potent inhibition of thrombosis, while maintaining blood coagulation in animal models (Estevez et al., 2015), we also studied the action of NSE on platelet reactivity *in vivo*.

## 3. Materials and methods
## 3.1 Materials

### 3.1.1 Chemicals

Thrombin (50 NIH/ml) and platelet activating factor (PAF) were purchased from Sigma (St. Louis, MO, USA). ADP, collagen, epinephrine and ristocetin were purchased from Tekhnologia-standard (Barnaul, Russia). Monoclonal antibody (mAb) 2d-2a to human fibrin(ogen) was kindly provided by I.M. Kolesnikova, Palladin Institute of biochemistry NAS of Ukraine. Rabbit anti-mouse IgG conjugated with horseradish peroxidase and chromogenic

substrate o-phenylenediamine (OPD) were purchased from Sigma (USA). Acridine orange (AO) was purchased from Invitrogen (Molecular Probes, Eugene, Oregon, USA).

### 3.1.2 NSE and NOE preparation and characterization

NSE was synthesized at the Department of Lipids Biochemistry of Palladin Institute of Biochemistry (Kyiv, Ukraine) by the condensation of ethanolamine and stearic acid (Gula et al., 2008).

Purity of the NSE preparation was confirmed by gas chromatography (HRGC 5300, Carlo Erba Instruments, Val De Reuil, France) using packed column Chromosorb W 100-125 (Supelco, New Jersey, USA) with phase 10% Silar 5 CP. The temperature of the injector was – 250°C and the temperature of the detector was –270°C.

The sample of NSE for gas chromatography was prepared as follows: 0.1 mg of NSE, and 1 ml of benzene and 1 ml of 3 M HCl in methanol were mixed in a glass ampoule. The ampoule was sealed and heated on a water bath at 100°C for 1.5 hours. Then the ampoule was cooled and opened. The solution was removed and put into a sample tube and mixed with 5 ml of hexane. The upper fraction of the solution was collected and transferred into a glass flask. The glass flask with the NSE sample was dried up using a rotary evaporator at 35°C. The dried sample was dissolved into 0.2 ml benzene. The solution was separated by a thin layer chromatography plate using benzene. The methyl ester of stearic acid was used as the marker probe. The zone which consisted of methyl ester NSE was extracted into benzene.

N-oleoylethanolamine (NOE) was synthesized and characterized in a manner similar to that described above for NSE, except oleic acid was used instead of the stearic acid.

Dried NSE and NOE powders were suspended in distilled water under sonication at a concentration of $10^{-3}$ M. Serial dilutions of NSE ($10^{-5} – 10^{-9}$ M) were prepared in dimethyl sulfoxide (DMSO) *ex tempore*. The final concentration of DMSO in the studied samples was 1 % for all experiments. The equal volume of DMSO was added to the control probes.

### 3.2 Methods

### 3.2.1 Platelet-rich plasma (PRP) and blood plasma preparation

Venous blood of healthy male volunteers (n = 4) who had not taken any medication for 7 days prior to blood sampling was collected into a 3.8 % sodium citrate solution (9 parts blood to 1 part sodium citrate). PRP was prepared by centrifuging blood at 160 g for 20 min at 25 °C. Washed platelets were obtained by spinning down the platelets at 300 g followed by

resuspension of the platelet pellet in 0.14 M NaCl, 0.01 M $KH_2PO_4$, pH 7.4 (phosphate buffered saline, PBS) (Korolova et al., 2014).

### 3.2.2 Flow cytometry

Platelet shape and cytoplasmic granularity were monitored on a Beckman Coulter Epics XL Flow Cytometer (Backman coulter, Ramsey, Minnesota, USA) (Konokhova et al., 2012). NSE ($10^{-8}$ M) was added to 1 ml of PRP and the samples were incubated for 60 min at 25 °C. Platelets were analyzed in comparison to thrombin-stimulated samples. Forward scattering (FS) and side light scattering (SS) were monitored to detect any changes of platelet granularity and shape.

### 3.2.3 Platelet aggregation

Platelet aggregation measurements were based on changes in the turbidity of human PRP (Cattaneo et al., 2013). Aggregation was registered for 10 min using the Aggregometer Solar AP2110 (Belorussia). The initial rate and final level of aggregation were estimated at 37 °C. In a typical experiment, 250 μl of PRP was activated by the following platelet agonists: ADP (2.5 μM, 12.5 μM), collagen (2 mg/ml), epinephrine (0.6 μg/ml), ristocetin (0.2 mg/ml, 0.4 mg/ml), thrombin (0.125 NIH/ml) or PAF (50 ng/ml)

### 3.2.4 Spectrofluorimetric analysis of platelet degranulation

Platelet degranulation was registered with pH-sensitive fluorescent AO dye that accumulates in platelet acidic compartments and is released after activation. PRP were preincubated with or without NSE ($10^{-8}$ M) at 37°C for 10 min and fluorescence measurements were started after the AO application. Changes in fluorescence intensity were measured as described previously (Kasatkina, 2016) at excitation and emission wavelengths of 490 and 530 nm, respectively (slit bands 5 nm each). The ADP (0.3 μM, 2 μM), epinephrine (0.2 μg/ml) collagen (0.1 mg/ml, 0.5 mg/ml) or ristocetin (0.4 mg/ml) were applied at the steady-state level of AO fluorescence. Fluorescence intensities were normalized to similar values for the absence of platelets ($F_t/F_0$). Degranulation was estimated at plateau level as % of released AO (from total accumulated dye).

### 3.2.5 Evaluation of fibrinogen binding to platelets

The modified ELISA technique was used to evaluate the binding of fibrinogen to activated platelets in the presence or absence of NSE ($10^{-7}$ M). Washed platelets were re-suspended in PBS and added to wells of a 96-well plate and kept for 1 hour at 37 °C. The 96-

well plate was washed three times with PBS containing 0.05% Tween-20 (TPBS). Then NSE ($10^{-7}$ M) and fibrinogen (3 mg/ml) in PBS were added to the wells and incubated for 1 hour at 37°C. The plate was washed three times with TPBS. A solution of mAb 2d-2a in PBS was added to the wells, the plate was incubated for 1 hour at 37°C and washed with TPBS. The rabbit anti-mouse IgG conjugated with horseradish peroxidase (1:1000) was added as a secondary antibody and incubated for 1 hour at 37°C. The plate was then washed three times with TPBS and 0.4 mg/ml OPD and 0.06% $H_2O_2$ were added. The reaction was terminated by the addition of 4 N $H_2SO_4$. The 2,3-diaminophenazine formed after OPD cleavage by horseradish peroxidase was determined at 492 nm using the Thermo Multiskan EX multiplate reader (Thermo Electron, USA).

For scanning electron microscopy (SEM) measurements of platelet binding to fibrinogen, plastic discs for cell culture dish were incubated with fibrinogen solution (2 mg/ml) for 1 hour at room temperature. Discs with adsorbed fibrinogen were washed gently and placed on the bottom of wells of a 24-well plate. Then 0.25 ml of PRP was added to the wells and incubated at 37 °C for 1 hour incubation, a 3% glutaraldehyde solution buffered with 0.05 M Tris-HCl buffer, pH 7.4, was poured onto the wells and the plate was incubated at room temperature for 1 hour. After 1 hour, the buffered glutaraldehyde solution was changed to a series of ethanol/water dilutions (with increasing ethanol concentrations) and the plate was incubated at each dilution for 30-60 min. As the final solution, 100% ethanol was added to the wells and the plate was left to dry. The dried samples were taken from the wells and placed onto stubs for coating with palladium by ion sputtering analysis by SEM.

### 3.2.6. Animal model

Male Wistar rats (170 ± 4 g, n = 40) were purchased from the Vivarium of the Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine. Only male rats were used in the study. According to the same logic as only male volunteers were involved as blood plasma donors for direct platelet studies. However, no sex-specific differences were expected.

Insulin resistance in rats (n = 30) was induced by feeding a prolonged high-fat diet far 6 month. The amount of lipids in the diet was increased by pork lard addition to the standard pellet chow, so the fat content was 58 %, proteins – 23 % and carbohydrates -10%. The lard contained a high level of palmitic and stearic acids, 24 % and 28 % of total fatty acids (FA), respectively. The FA composition was at a ratio of 55 % saturated (SFA) to 45 % unsaturated FA (USFA). Control rats (n = 10) during the experiment were on normal pellet diet (4 % fat:23 %

proteins: 65 % carbohydrates) with SFA/USFA ratio 38 % / 62 %, respectively. The cholesterol content of lard was 0.57 mg/g of lard.

Lard was provided to rats every day of the experiment in the same amount to each rat. All animals were keen to obtain their daily portions, eagerly took the lard from hands of laboratory assistant and ate it immediately. According to rats' preferences we provided lard to them thinly sliced and slightly chilled.

The animals were kept in standard cages (5 animals in each cage) under controlled conditions (22 °C ± 2, 12-h/12-h light/dark cycle) with unlimited access to drinking water and rodent chow (Animal Feed Manufacturer "Agrovita", Zhovtneve, Ukraine). Throughout the experiments, the rats were gaining weight gradually. On the 24th week, the average weight of HFD rats was 410-430 g (due to visceral fat) in comparison with control rats of 330-350 g.

Six months after HFD period, we conducted the oral glucose tolerance test according to (Collier et al., 1985). The rats with impaired glucose tolerance (the level of blood glucose within 150 min after the oral glucose administration was 6 ± 1.05  mmol/l) were selected and divided randomly into two groups: IR (n = 9) and IR + NSE (n = 10) with equal distribution of tolerance data in each test group. Control rats were further subdivided into control (n = 10) and NSE (n = 7) groups. The size of NSE group (control rats + NSE) was reasonably reduced according to previous data reported no physiological action of NSE in the case of healthy control animals (Onopchenko et al., 2018).

Animals in NSE and IR + NSE groups were orally received the water suspension of NSE for 2 weeks at the dose of 50 mg body weight kg$^{-1}$.

This particular dose of NSE has been chosen on the base of our previously experiments data, as an optimal reacting dose for the biological effect investigations. The exact concentration of NSE during the experiment of dog coronary artery occlusion was reported earlier (Epps et al., 1980).

Samples of rat blood were collected by heart puncture. Pentobarbital (Nembutal) anesthesia (dosage was 50 mg/kg of body weight) was used intraperitoneally according to ethical standards and principles (as specified below). A 3.8 % sodium citrate solution was added to the blood samples immediately after collection. PRP was prepared by centrifugation at 1000 rpm for 30 min.

Animals were decapitated immediately after blood collection still being under anesthesia. This study was carried out with the approval of animal care and use committee of the Palladin Institute of biochemistry of NAS of Ukraine (Protocol #08/09-2015).

*3.2.7 Statistical analysis*

Statistical analysis of the data was performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). All assays were performed in series of three replicates and the data were fitted with standard errors using Statistica 7 (Dell Technologies, Round Rock, Texas, USA). Results are presented as means ± standard deviation. The difference between the two significantly different groups (control and studied group) was analyzed by one-way ANOVA. Statistical data analysis of the groups of insulin resistant animals with or without administration of NSE was also performed using the Wilcoxon-Mann-Whitney test. Differences were considered significant for $p < 0.05$.

## 4. Results

*4.1 NSE specifically inhibits platelet aggregation*

To evaluate the concentrations of NSE that could affect platelets we used the standard test with ADP, the most common inducer of platelet aggregation in clinical tests (Born et al., 1963). The concentration range was selected based on previous observations of the biological action of NSE (Cattaneo et al., 2013). We studied the ADP-induced aggregation of platelets in the presence of NSE at concentrations of $10^{-5}$–$10^{-10}$ M and found it inhibited platelet aggregation in a dose-dependent manner. However, the correlation was not linear and the effective inhibition of platelet aggregation was observed when $10^{-7}$ and $10^{-8}$ M concentrations of NSE were applied (Figure 1). Lower or higher doses did not exhibit statistically significant effects on platelet aggregation induced by ADP. These results correspond to previous findings that showed maximal antiviral effects of NSE in the $10^{-7}$–$10^{-8}$ M range of concentrations (Gula et al., 2014). Thus, these concentrations were selected for all further experiments.

To confirm that the effect was specific, we performed similar experiments using NOE, which is analogous to NSE. However, no anti-aggregatory action of NOE was observed (Figure 1).

*4.2 NSE does not stimulate resting platelets*

To verify any possible effect of NSE on human platelets, resting platelets in PRP were analysed by flow cytometry in the presence or absence of NSE ($10^{-8}$ M). Non-activated PRP was incubated with NSE for 60 min. No changes of platelet granularity or shape were observed (Figure 2). The count of platelets with normal shape and granularity in control probes (Figure 2A) and in samples with NSE (Figure 2B) did not differ significantly ($61.5 \pm 3$ % vs $60.0 \pm 3$ %, respectively). Thus, we assumed that NSE did not activate platelets.

*4.3 NSE attenuates ristocetin-induced platelet activation*

To verify if NSE affects the platelet activation step we analyzed the action of NSE on platelet responses to agonists acting via G-protein-coupled receptors, i.e. ionotropic and metabotropic purinergic receptors P2X and P2Y, and estimated the secretion during ristocetin- and collagen-induced interaction of the platelet membrane glycoprotein Ib (GPIb) and von Willebrand factor (vWF). We used a range of inducers including "weak" inducers: ADP (agonist for P2Y1 and P2Y12 receptors (Lan Zhou et al., 2005), epinephrine (binds to α2-adrenergic receptor (Gachet, 2000), ristocetin (binds vWF and then adheres to GPIb-IX-V complex (Spalding et al., 1998) and "strong" inducers: thrombin (activates protease-activated receptors (PARs) (Koutts et al, 1976), collagen and PAF (binds to the PAF-receptor (Kahn et al., 1999).

The application of NSE ($10^{-8}$ and $10^{-7}$ M) or an equivalent volume of DMSO did not affect the proton gradient of platelet secretory granules. In the next set of experiments after 6 min of preincubation with NSE ($10^{-7}$ M) platelets were stimulated with ADP (0.3 μM), ristocetin (0.4 mg/ml), epinephrine (0.6 μg/ml) or collagen (0.5 mg/ml) to detect the release of secretory granule constituents. The degranulation was estimated in % from total accumulated dye.

ADP at a concentration of 2 μM promoted a fast secretion of granule constituents. The comparative analysis showed that ADP-stimulated platelet degranulation was not affected in the presence of $10^{-7}$ M NSE. However, at lower doses of ADP (0.3 μM) NSE inhibited platelet degranulation by 20.8±4% (Figure 3A). Epinephrine-induced degranulation of platelets was also partially inhibited by $10^{-7}$ M NSE ($9.5 \pm 2.1$ % vs $11.3 \pm 2.4$ % in control, Figure 3B).

We found that NSE slightly inhibited the degranulation induced by low doses of collagen (0.5 mg/ml), which did not induce platelet aggregation (Figure 3C).

Another approach was to test the platelet secretion produced by the interaction of vWF and GPIb in the presence of ristocetin. Such stimulation in a mode of outside-in signalling was typically characterized by a lower level of secretion and was substantially attenuated in the presence of NSE ($10^{-7}$ M) ($33.3 \pm 3.5$ % vs $44.8 \pm 4.2$ % in control, Figure 3D). As demonstrated in Figure 3D, platelets preincubated with NSE displayed attenuation of both the rate and the final level of ristocetin-induced secretion.

*4.4 NSE preferentially inhibits ristocetin-induced platelet aggregation*

Studies of platelet aggregation induced by different agonists allow specifying the action of inhibitors and provide a detailed pharmacological approach, which significantly increases the likelihood of detecting platelet function (Pacher et al., 2008).

The effects of low doses of ADP and epinephrine on platelet aggregation are in accordance with the data of activation studies. The effect of $10^{-8}$ and $10^{-7}$ M NSE on ADP-induced aggregation was moderate. When a low concentration of ADP (2 μM) was applied, the rate of the first wave of platelet aggregation decreased from 30±5% (control samples) to $25 \pm 6\%$ and $15 \pm 6$ % in the presence of $10^{-8}$ and $10^{-7}$ M NSE, respectively (Figure 4A). Such a concentration of ADP is normally assumed to be a weak stimulus for platelet aggregation. NSE also decreased the rate of platelet aggregation induced by epinephrine, from $24 \pm 3$ % (control samples) to $20 \pm 6$ % and $17 \pm 6$ % in the presence of $10^{-8}$ and $10^{-7}$ M NSE, respectively (Figure 4B).

It was observed that $10^{-8}$ and $10^{-7}$ M NSE did not affect the rate of platelet aggregation induced by collagen ($41 \pm 8$ % and $40 \pm 6$ %, respectively vs $50 \pm 6$ % in control), thrombin ($33 \pm 5$ % and $38 \pm 6$ %, respectively vs $40 \pm 6$ % in control) and PAF ($28 \pm 5$ % and $21 \pm 6$ %, respectively vs $27 \pm 4$ % in control). Typical curves are shown on Figures 3C, E and F.

The most prominent effect of NSE was shown for ristocetin-induced aggregation. NSE at concentrations of $10^{-8}$ and $10^{-7}$ M decreased the rate of platelet aggregation induced by ristocetin (0.4 mg/ml) from $45 \pm 8$ % in control samples to $31 \pm 6$ % and $10 \pm 3$ %, respectively (Figure 4D). This effect is in accordance with the prominent inhibition of ristocetin-induced platelet activation and suggests that NSE modulates platelet signalling at the early stage of blood clotting.

Thus, we showed the inhibitory effect of NSE on platelet activation and aggregation. This effect was more evident for "weak" inducers and the most prominent impact was observed for the ristocetin-induced process. As the binding of fibrinogen to the glycoprotein GPIIb/IIIa of activated platelets is the crucial step of platelet aggregation, we next studied the fibrinogen binding properties of platelets in the presence of NSE.

*4.5 NSE inhibits fibrinogen binding to platelets*

Fibrinogen has high affinity for GPIIb/IIIa receptors of activated platelets and much lower affinity for those of resting cells (Tselepis et al., 1999). Moreover, fibrinogen-GPIIb/IIIa interactions not only connect platelets to each other, but also induce outside-in signalling and promote the facilitation of platelet activation (Hantgan et al., 2010). The amount of fibrinogen that was bound to platelets was evaluated using specific anti-fibrinogen mAb. It was found that fibrinogen binding to platelets adhered to the surface was 15 % lower in the presence of NSE (Figure 5).

Thus, this approach allowed us to obtain measurable data. To reconfirm these results we also applied SEM as the traditional method for studying platelet adhesion to the fibrinogen-

coated surface. As seen in Figure 5B, platelets in the presence of $10^{-7}$ and $10^{-8}$ M of NSE were more spherical and did not expand pseudopodia as did those in the control sample or in samples in the presence of NSE in insufficient concentrations.

*4.6 NSE administration leads to normalization of platelet aggregation rate in animal model*

Previously we developed and characterized a model of obesity-induced insulin resistance in rats. Alongside with changes in blood coagulation parameters, this condition was shown to cause the over-reactivity of platelets (Dziuba et al., 2018). In the current study, we measured the aggregation of platelets from obese IR rats and compared it to that from rats that had been given NSE *per os* for two weeks until the end of the experiment.

As shown in Figure 6, we observed a statistically significant decrease in the rate of platelet aggregation in blood plasma of rats treated with NSE. This finding may be due to the known anti-inflammatory action of NSE, as well as by the direct action of NSE on platelets, similar to that observed *in vitro*.

## 5. Discussion

While the main mechanisms of platelet activation and aggregation induced by different agonists are similar, the intracellular signalling pathways differ significantly. All agonists have their unique receptors on the platelet surface, but the main result of their action is the activation of phospholipases leading to thromboxane A2 generation, granule secretion and integrin activation (Brantl et al., 2014) (Zhenyu, et al., 2010). A summarized scheme of platelet activation by the agonists we studied is shown in Figure 7.

ADP, PAF and thrombin act via G protein-coupled receptors and lead to the activation of phospholipase Cβ (PLCβ) (Roka-Moya et al., 2014) (Yang et al., 2002) (Offermanns, 2006). In our studies NSE did not affect aggregation stimulated by thrombin, PAF or high doses of ADP. However, NSE displayed inhibitory action on epinephrine-induced platelet aggregation and degranulation that is also dependent on G-protein signalling. In contrast to PAR, purinergic (i.e., P2Y1, P2Y12) and PAF receptors, the αA2 receptor of epinephrine induces the signalling pathway that preferentially activates phospholipase A2 (PLA2). In this case, phospholipase Cγ (PLCγ) is activated through a PLA2-dependent mechanism as a result of stimulation by mediators released from platelets after PLA2 action (Coughlin, 2000) (Banga et al., 1986).

We observed the prominent inhibitory effect of NSE on ristocetin-induced platelet degranulation. The rate and the final level of ristocetin-induced secretion of secretory granule constituents were attenuated in the presence of $10^{-7}$ M NSE. The receptor of ristocetin (in a complex with vWF) is GPIb-IX-V that is a complex of integrins. This complex generates the

signal in a G-protein-independent manner and mediates the activation of PLCγ (Wu et al., 1992). Signal transduction mediated by the GPIb-IX-V receptor is associated with the rearrangement of actin filaments (Rivera et al., 2009).

We did not observe prominent effects of NSE on collagen-induced activation or aggregation of platelets despite the fact that the collagen receptor GPVI is also an integrin and transduces the signal in a G-protein-independent manner (Bryckaert et al., 2015).

Surprisingly, it was reported that collagen and ristocetin can induce PLA2 activation and the subsequent enzymatic generation of free arachidonic acid (AA) and the formation of cyclooxygenase (COX) products also activate PLC (Nakano et al., 1989) (Börsch-Haubold et al., 1995). Under inflammatory conditions, COX-mediated formation of thromboxanes and prostaglandin provides the amplification signals in platelet activation; inhibition of their synthesis by anti-inflammatory agents is suggested as an effective mechanism for preventing platelet-dependent vascular occlusion (FitzGerald, 1991).

NSE also inhibited platelet degranulation induced by low doses of ADP (0.3 μM). The activation of the metabotropic purinergic receptors P2Y1 and P2Y12 leads to phosphorylation of PLCβ, but not PLA2. Low doses of ADP are known to induce two-wave aggregation with the second wave strongly dependent on GPIIb/IIIa activation and outside-in signalling (Kroll et al., 1991). Outside-in signalling after fibrinogen binding to GPIIb/IIIa and their clustering further increases platelet activation (Hantgan et al., 2010) (Buensuceso et al., 2003).

The last hypothesis is in accordance with the data on fibrinogen binding to platelets that was distinctly affected by NSE. The decreased binding of fibrinogen to activated and absorbed platelets could be evidence for the impaired GPIIb/IIIa activity in the presence of NSE.

As was previously shown, the localization of the GPIb-IX-V complex within the cholesterol-enriched domains is crucial for both platelet adhesion and post-adhesion signalling (Ginsberg et al., 2005). Thus, the NSE-mediated impact on cholesterol-enriched membrane microdomains may influence the stage of vWF and GPIb interaction, as well as the activation and clustering of GPIIb/IIIa receptors. This suggestion is supported by the prevailing effects of NSE on integrin-dependent steps of platelet responses.

## 6. Conclusions

NSE was shown for the first time to be an inhibitor of aggregation of human platelets. Our results suggest that NSE may decrease the activation and subsequent aggregation of platelets induced by ristocetin, epinephrine and low doses of ADP. NSE also reduced the binding of fibrinogen to GPIIb/IIIa on activated platelets. We concluded that these effects could be explained by the inhibition of platelet activation mediated by integrin receptors: the GPIb-IX-V

complex for ristocetin-induced activation and GPIIb/IIIa when epinephrine and low doses of ADP were applied. The anti-platelet effect of NSE complements its anti-inflammatory action and allows us to prioritize studies of NSE as a potent anti-thrombotic agent.

## 7. Ethics approval and consent to participate

The blood donors volunteers signed informed consent prior to blood sampling according to the Helsinki declaration. This study was approved by the Ethics Committee of the Palladin Institute of Biochemistry (23.08.2015, N5).

Animals were kept in accordance with the General Ethical Principles of Experiments on Animals (Ukraine, 2001), which are consistent with the provisions of "The European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986). This study was carried out with the approval of the Animal Care and Use Committee of the Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine (Protocol N1 from 08/09-2015).

## 8. Acknowledgements

## 9. Authorship contribution

Participated in research design: IH, VC, LK; Conducted experiments: IH, VC, VK, LK, OT, LU; Performed data analysis: NH, HK, TP; Contributed new reagents or analytic tools: VK, LU; Wrote or contributed to the writing of the manuscript: IH, VC, LK.

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## 11. Footnotes.

Conflicts of Interest: No author has an actual or perceived conflict of interest with the contents of this article.

## 12. Legends for figures

**Figure 1.** The action of NSE (grey line) and NOE (black line) on the rate of ADP-induced platelet aggregation in PRP. Final concentrations of NSE or NOE ranged from $10^{-5}$ to $10^{-9}$ M. Platelet aggregation was induced by ADP (12 µM). Results are presented as means ± standard deviation, n = 5. ADP – adenosine diphosphate; NOE – N-oleoylethanolamine; NSE – N-stearoylethanolamine; PRP – platelet-rich plasma.

**Figure 2.** Flow cytometry of resting human platelets in PRP incubated with (A) $10^{-8}$ M NSE or (B) an equal volume of solvent (DMSO) in a control probe. Forward scattering (FS) and side light scattering (SS) of platelets were monitored to detect any changes of platelet granularity and shape. The analysis was based on 20 000 particles after preincubation with NSE for 60 min. Then, 2.5 µM ADP was applied for platelet stimulation. n = 5. ADP – adenosine diphosphate; DMSO – dimethyl sulfoxide; NSE – N-stearoylethanolamine; PRP – platelet-rich plasma.

**Figure 3.** The action of NSE on the acidification of platelet secretory granules and the release of granule constituents during agonist-induced activation. Platelets were loaded with the pH-sensitive fluorescent dye acridine orange in the presence of an equivalent volume of DMSO (control) or $10^{-7}$ M NSE and stimulated with (A) 0.3 µM ADP, (B) 0.6 µg/ml epinephrine, (C) 0.5 mg/ml collagen or (D) 0.4 mg/ml ristocetin. The concentration of DMSO in the control samples was the same as that used in the preparation of $10^{-7}$ M NSE. Fluorescence intensities were normalized to similar values for the absence of platelets ($F_t/F_0$). Represented traces were selected as the most common for independent experiments with the use of samples of PRP of 2 donors (each in triplicate). ADP – adenosine diphosphate; DMSO – dimethyl sulfoxide; NSE – N-stearoylethanolamine.

**Figure 4.** The action of NSE on platelet aggregation in PRP induced by (A) 2 µM ADP, (B) 0.6 µg/ml epinephrine, (C) 3 mg/ml of collagen, (D) 0.4 mg/ml of ristocetin, (E) 0.125 NIH/ml of thrombin or (F) 50 nM PAF. C – control samples; $10^{-7}$, $10^{-8}$ – samples with $10^{-7}$ and $10^{-8}$ M NAEs added. Represented traces were selected as the most common for independent experiments with the use of samples of PRP of 2 donors (each in triplicate). ADP – adenosine diphosphate; NAEs – N-acyletanolamines; NSE – N-stearoylethanolamine; PAF – platelet activating factor; PRP – platelet-rich plasma.

**Figure 5.** (A) Immunoassay of fibrinogen binding to activated platelets in the presence of NSE (from $10^{-6}$ to $10^{-9}$ M, K – control sample). Platelets were adsorbed to the microtiter wells in the presence of ADP, the amount of fibrinogen bound to platelets was estimated using mAb 2d-2a. Results are presented as means ± standard deviation, n = 8. * $p < 0.05$. (B) Scanning electron microscopy (SEM) of platelets attached to the plastic coated with fibrinogen in the presence of different concentrations of NSE (from $10^{-6}$ to $10^{-9}$ M, Control – control sample). ADP – adenosine diphosphate; mAb – monoclonal antibody; NSE – N-stearoylethanolamine.

**Figure 6.** Aggregation rate of platelets from obese insulin-resistant (IR) rats after 2 weeks of NSE administration (IR+NSE). Platelets were activated by 12.5 μM of ADP (IR vs IR+NSE, $p < 0.05$ according to Mann-Whitney U test), n = 9 (IR), n = 10 (IR+NSE). * $p < 0.05$. ADP – adenosine diphosphate; NSE – N-stearoylethanolamine.

**Figure 7.** Platelet signalling pathways during activation by the studied agonists. ADP, PAF and thrombin act via G protein-coupled receptors and promote the activation of $PLA_2$ and PLCβ. Ristocetin-vWF and collagen bind to GPIb-IX-V and GPVI, respectively, via kinases and adaptor proteins that stimulate PLCγ and increase the production of $IP_3$. $IP_3$ interacts with the $IP_3$-receptor on the dense tubular system and stimulates the release of intracellular $Ca^{2+}$, which is the main second messenger of platelet activation. Small GTP-ase Rap1b is controlled by $Ca^{2+}$ and DAG-regulated guanine nucleotide exchange factor I (CalDAG-GEFI). Downstream effectors of Rap1, Rap1-GTP-interacting adaptor molecule (RIAM), and Akt are important molecules involved in talin-1 recruitment to the cytoplasmic integrin tail [47]. Binding of talin-1 and kindlin-3 to cytoplasmic domains of β3-integrin triggers a conformational change in the extracellular domains that increases its affinity for ligands, such as fibrinogen and vWF. AA – arachidonic acid; ADP – adenosine diphosphate; COX – cyclooxygenase; DAG – diacylglycerol; GP – glycoprotein; GTP – guanosine triphosphate; IP3 – inositol-1,4,5-trisphosphate; P2Y1, P2Y12 – purinergic receptors; PAF – platelet activating factor; PAR – protease-activated receptor; PL – phospholipase; vWF – von Willebrand factor. Artwork by Ludmila Kasatkina.
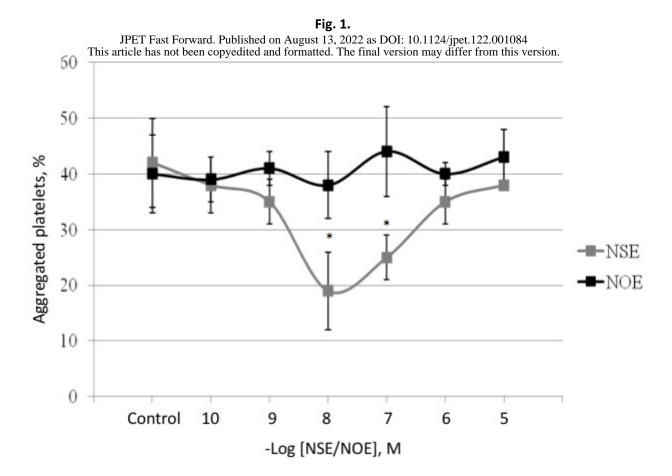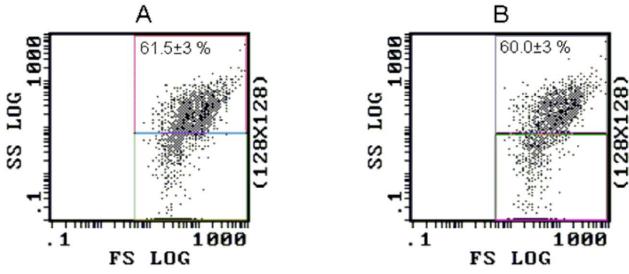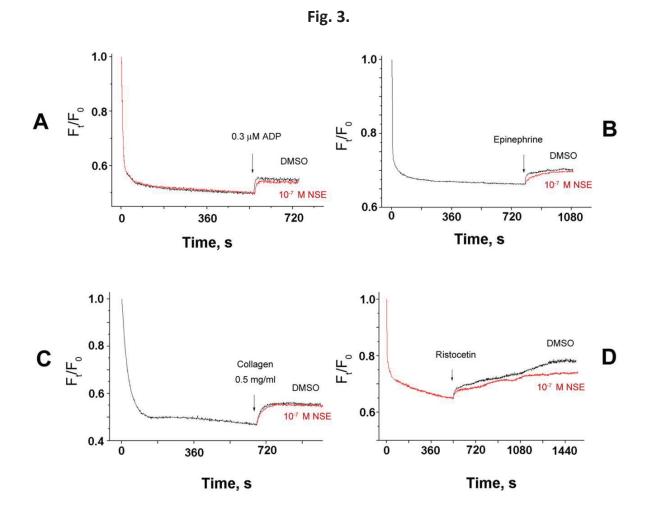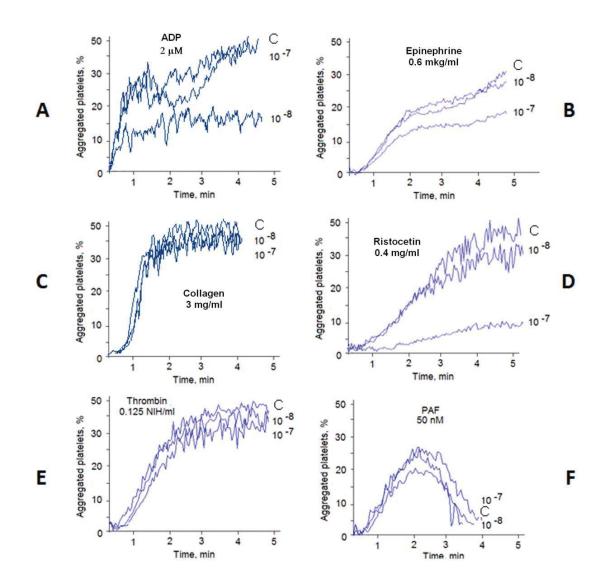
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