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Original article

An ex vivo standardized assay to measure human platelet cGMP

Roberta d'Emmanuele di Villa Bianca [a,b], Emma Mitidieri [a], Vincenzo Mirone [b], Ferdinando Fusco [b], Ciro Imbimbo [b], Giuseppe Cirino [a,b,*], Raffaella Sorrentino [a,b]

[a] Department of Experimental Pharmacology, University of Naples Federico II, Italy
[b] Interdipartimental Research Center for Sexual Medicine, University of Naples Federico II, Italy

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ABSTRACT

Introduction: Nitric oxide (NO) acts a pleiotropic biomodulator in several systems, including the cardiovascular, nervous and immune systems. The intracellular levels of cyclic guanylate monophosphate (cGMP) can be increased by NO or by inhibiting the breakdown to 5′cGMP operated by the cyclic nucleotide phosphodiesterases (PDEs). Platelets are anuclear circulating cells that are rich in both soluble guanylyl cyclase and PDEs. The purpose of this study was to standardize cGMP determination in human platelets. **Methods:** Fresh blood samples were obtained from a group of healthy volunteers in order to obtain washed platelets. Platelet (3×10^4/μl or 5×10^5/μl) cGMP levels were measured in basal and stimulated conditions. To stimulate platelets two different NO-donors were used: sodium nitroprusside (SNP; 10, 100, 1000 μM) or diethylamine NONOate (DEA-NONOate; 1, 10, 100 μM). Different times of incubation were also studied (5, 15 and 30 min). As positive control has been used ODQ a well known inhibitor of guanylyl cyclase. Platelet cGMP accumulation was measured by using a standard ELISA kit using different sample dilutions. **Results:** The optimal *stimulus* was DEA-NONOate, the optimal washed platelet concentration was 5×10^5/μl, incubation time was 30 min and dilution to be used was 1:2. **Discussion:** Platelets represent a valuable marker to investigate the effect of drugs interfering with the cGMP cascade. This standardized assay allows to measure ex vivo the inhibitory (PDE inhibitors) or stimulatory effect (NO donors) of drugs given in vivo to humans.

1. Introduction

Nitric oxide (NO) is a pivotal signaling messenger in the cardiovascular system in physiological and pathophysiological conditions. NO contributes to regulatory functions including control of hemostasis, fibrinolysis, platelet and leukocyte interactions with the arterial wall and regulation of vascular homeostasis. It is constitutively generated by nitric oxide synthase (NOS) in endothelial cells and platelets as well as it is released from nitrergic nerves. NO activates the NO-sensitive guanylyl cyclase through its binding to the prosthetic heme group of the enzyme leading to the conversion of guanosine 5′-triphosphate (GTP) to the second messenger cyclic guanylate monophosphate (cGMP) (Koesling & Friebe, 1999). Levels of cGMP in a cell may reflect the interplay between the rate of synthesis and the rate of degradation by phosphodiesterases (PDEs). The involvement of cGMP in platelet has been demonstrated in 1978 (Hamet & Coquil, 1978). Since, the platelet, is rich in guanylyl cyclase (GC) and PDEs, (specially PDE5), it represents a target for NO (Halcox et al., 2002; Wallis et al., 1999). In particular, the PDE5, the cGMP-binding, cGMP-hydrolyzing PDE first characterized in lung in 1980 (Francis et al., 1980) was identified as the major PDE isoform in cardiovascular system as well as in platelets (Wallis et al., 1999).

Thus, the platelet represents a good candidate to investigate on molecular mechanism(s) involving cGMP modulation. The platelet is also the simplest cell available, it is very small and homogeneous. On this basis, we have set up a method in order to evaluate platelet cGMP level in basal and stimulated conditions.

2. Materials and methods

2.1. Experimental design

A group of 10 healthy volunteers were enrolled at the University of Naples Federico II at the department of Urology. The subjects gave a blood sample (20 ml) in order to obtain platelets. The protocol was approved by the ethical committee of Federico II University of Naples.

2.2. Washed platelets

Fresh blood samples (20 ml) anticoagulated with citrate (3.8% w/v 1:10 ratio) were centrifuged at 150×*g* for 10 min to obtain platelet-rich plasma as a supernatant. At 1/10 volume ACD solution (85 mM Na_3-citrate, 11 mM D-glucose, 71 mM citric acid, pH 4.4) was added to 9/10 volume of blood. After centrifugation (800×*g* for 12 min) the cell

* Corresponding author at: Department of Experimental Pharmacology, University of Naples Federico II, Italy Via D. Montesano 49, 80131 Naples, Italy. Tel.: +39 081678105; fax: +39 081678403.
E-mail address: cirino@unina.it (G. Cirino).

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pellet was resuspended in Ca^{+2}/Mg^{+2}-free HEPES-Tyrode buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM Na_2HPO_4, 5 mM HEPES, 5 mM glucose, 0.5% (w/v) bovine serum albumin, pH 7.4) and the platelet number was determined by using a cell counter (AcT diff 2, Instrument Laboratory, Milan, Italy).

2.3. Study to evaluate cGMP accumulation in washed platelets

In order to determine the optimal conditions to measure the platelet cGMP, we have evaluated and determined the following parameters i) the optimal concentration of washed platelets to be used ii) the optimal incubation time to have the best cGMP signal and iii) the NO-donor to be used and its optimal concentration.

In order to define the best concentration of platelets, we used a concentration of 3×10^4/μl or 5×10^5/μl of platelets. The samples were equilibrated at 37 °C and continuously and gently horizontally mixed by using a thermostatic Dubnoff bath. After the equilibration period the samples were incubated with sodium nitroprusside (SNP; 10, 100, 1000 μM, Sigma, Milan, Italy) or diethylamine NONOate (DEA-NONOate; 1, 10, 100 μM, Alexis, Vinci Biochem, Vinci, Italy) for 5, 15 and 30 min. In order to assess the effectiveness of the method, washed human platelets were incubated for 15 min with 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 10 μM, Tocris, UK), a well known and characterized guanylyl cyclase inhibitor, prior to the addition of DEA-NONOate (1, 10, 100 μM). The samples obtained were frozen in liquid nitrogen to stop the reaction.

2.4. cGMP measurement in washed platelets

In order to measure cGMP content, cell pellet was hydrolyzed with 15 μl of 3.3 M HCl. The lysates were centrifuged ($600\times g$ for 10 min) and cGMP measured in supernatants as described in the manufactures protocol of cGMP EIA Kit (Cayman, Vinci Biochem, Vinci, Italy) tested by using neat or dilution 1:2 and 1:3 with EIA buffer in order to assess the best dilution to detect the cGMP accumulation.

2.5. Statistical analysis

The cGMP concentrations were obtained by using a standard curve and the software GraphPad (GraphPad Software Inc, USA). Platelets were harvested from 10 healthy subjects. Data were expressed as mean ± s.e.m. of sample run in duplicate. Data were analyzed by One Way Anova following by Bonferroni as post test. P values less than 0.05 were considered significant.

3. Results

3.1. Platelet study: SNP

In order to assess the optimal concentration of platelets as well as the best time of accumulation of cGMP, we incubated 300 μl of human washed platelet at final concentration of 3×10^4/μl or 5×10^5/μl with SNP (10, 100, 1000 μM) or vehicle. Samples were incubated for 5, 15 or 30 min. cGMP content was measured in undiluted samples (neat) or in samples diluted at 1:2 and 1:3.

The neat did not give reliable results (data not shown). By using 3×10^4/μl platelets, the levels of cGMP stimulated with SNP (10, 100, 1000 μM) at 5, 10 and 30 min tested in the dilution 1:2 or 1:3 did not give a clear dose-response (Fig. 1A, *$p<0.05$ vs vehicle 5 min, and Fig. 1B). The cGMP levels measured using 3×10^4/μl platelets were less of 1 pmol/ml in both dilutions used. Increasing the amount of platelets to 5×10^5/μl the cGMP accumulation improved and was in the order of about 5–6 pmol/ml (Fig. 2A and B). Also in this case there was not a clear dose-effect.

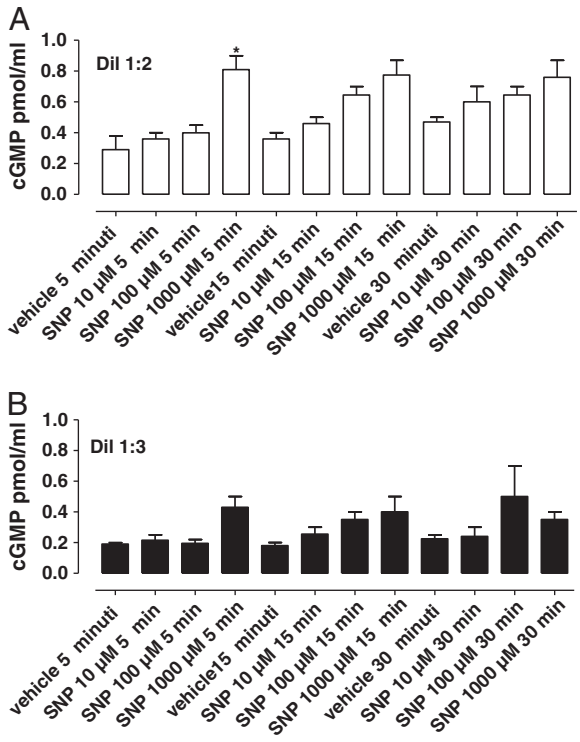

Fig. 1. Effect of SNP on platelet cGMP-induced accumulation. The levels of cGMP, expressed as pmol/ml, were measured in 3×10^4/μl platelet, obtained from three donors, following SNP (10, 100 and 1000 μM) incubation for 5, 10 and 30 min, Panel A: Samples were diluted 1:2 and then assayed as described; *$p<0.05$ *vs* vehicle 5 min Panel B: samples were diluted 1:3 and then assayed as described. Samples we obtained by four volunteers.

3.2. Platelet study: DEA-NONOate

In order to address the problem raised by the studies performed with SNP described in the above paragraph, we used the higher concentration of platelets i.e. 5×10^5/μl and as *stimulus* we used DEA-NONOate (1, 10, 100 μM). The incubation times were 5, 15 and 30 min and samples were diluted, as above, 1:2 and 1:3. The neat was not used.

Using a platelet concentration of 5×10^5/μl we found that DEA-NONOate elicited a dose and time response in cGMP accumulation (Fig. 3A and B). Between the two dilutions used, the most reliable was 1:2 (Fig. 3A). Indeed, by using the dilution 1:2 we observed a dose-related increase in cGMP at each time of incubation point. The data analysis demonstrated that 30 min was the optimal incubation time to achieve a significant and reproducible cGMP accumulation (Fig. 3A, *$p<0.05$ *vs* vehicle and *vs* DEA-NONOate 100 μM 30 min; ***$p<0.001$ vs vehicle and DEA-NONOate 10 μM 30 min, **$p<0.01$ *vs* DEA-NONOate 10 μM 30 min). The dilution 1:3 was not reliable (Fig. 3B).

3.3. Platelet study: standard assay

On this basis in order to confirm the results described above we performed the same protocol in another set of experiments. We used DEA-NONOate as NO-donor and 30 min as the best time of incubation. Samples were diluted at 1:2 and 1:3.

The incubation of 5×10^5/μl platelets for 30 min with DEA-NONOate leads to a significant increase in cGMP production by a dilution 1:2 either as pmol/ml (Fig. 4A, **$p<0.001$ *vs* DEA 10 μM, ***$p<0.0001$ *vs* DEA 10 and 100; ###$p<0.001$ vs DEA 100 μM, °°°$p<0.001$ *vs* DEA 100 μM) or as fold increase (Fig. 4B,***$p<0.001$ vs DEA 1 μM and *$p<0.05$ vs DEA 10 μM). Similarly, by using dilution 1:3 we observed a significant dose response in cGMP production as