Pharmacological Research 64 (2011) 289–297

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

In vitro inhibition of human and rat platelets by NO donors, nitrosoglutathione, sodium nitroprusside and SIN-1, through activation of cGMP-independent pathways

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ARTICLE INFO

Article history:
Received 14 January 2011
Received in revised form 28 March 2011
Accepted 29 March 2011

Keywords:
cGMP-independent response
GSNO
Sodium nitroprusside
Protein sulfhydryl groups (PSH)
SIN-1
Platelets

ABSTRACT

Three different NO donors, *S*-nitrosoglutathione (GSNO), sodium nitroprusside (SNP) and 3-morpholino-sydnonimine hydrochloride (SIN-1) were used in order to investigate mechanisms of platelet inhibition through cGMP-dependent and -independent pathways both in human and rat. To this purpose, we also evaluated to what extent cGMP-independent pathways were related with the entity of NO release from each drug.

SNP, GSNO and SIN-1 (100 μM) effects on platelet aggregation, in the presence or absence of a soluble guanylate cyclase inhibitor (ODQ), on fibrinogen receptor ($\alpha_{IIb}\beta_3$) binding to specific antibody (PAC-1), and on the entity of NO release from NO donors in human and rat platelet rich plasma (PRP) were measured.

Inhibition of platelet aggregation (induced by ADP) resulted to be greater in human than in rat. GSNO was the most powerful inhibitor (IC_{50} values, μM): (a) in human, GSNO = 0.52 ± 0.09, SNP = 2.83 ± 0.53, SIN-1 = 2.98 ± 1.06; (b) in rat, GSNO = 28.4 ± 6.9, SNP = 265 ± 73, SIN-1 = 108 ± 85. GSNO action in both species was mediated by cGMP-independent mechanisms and characterized by the highest NO release in PRP. SIN-1 and SNP displayed mixed mechanisms of inhibition of platelet aggregation (cGMP-dependent and independent), except for SIN-1 in rat (cGMP-dependent), and respectively lower or nearly absent NO delivery. Conversely, all NO-donors prevalently inhibited PAC-1 binding to $\alpha_{IIb}\beta_3$ through cGMP-dependent pathways.

A modest relationship between NO release from NO donors and cGMP-independent responses was found. Interestingly, the species difference in NO release from GSNO and inhibition by cGMP-independent mechanism was respectively attributed to *S*-nitrosylation of non-essential and essential protein SH groups.

1. Introduction

Nitric oxide (NO) is an important cellular messenger implicated in the regulation of various physiological functions in the cardiovascular, nervous and immune systems [1]. NO, synthesized by endothelial cells and platelets, is vital for maintaining a healthy cardiovascular apparatus [2] as it is involved in the regulation of vascular tone and platelet functionality through inhibition of platelet adhesion to the endothelium, platelet granule secretion and platelet aggregation. These functions, exerted by activation of soluble guanylate cyclase (sGC), formation of the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) and activation of cGMP-dependent protein kinases (cGMP-PK) with consequent phosphorylation of specific protein substrates, are called cGMP-dependent or NO-direct responses, to distinguish them from others successively hypothesized and defined as cGMP-independent or NO-indirect [2–4]. In fact, during the last decade there has been mounting evidence that NO inhibits platelets and other cells independently of its direct action on sGC, with effects generated by

Abbreviations: $\alpha_{IIb}\beta_3$, fibrinogen receptor; cGMP, guanosine 3′,5′-cyclic monophosphate; GSNO, *S*-nitrosoglutathione; IC_{50}, half maximal inhibitory concentration, the dose of drug that induces 50% of maximum inhibition of aggregation; NO, nitric oxide; PAC-1, fluorescein-isothiocyanate (FITC)-conjugated monoclonal antibody; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PDI, protein disulphide isomerase; PPP, platelet-poor plasma; PRP, platelet-rich plasma; sGC, soluble guanylate cyclase; SIN-1, 3-morpholinosydnonimine hydrochloride; SNP, sodium nitroprusside.

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doi:10.1016/j.phrs.2011.03.014

interaction of NO with reactive oxygen species under conditions of relatively high NO concentrations [4].

The molecular targets and the corresponding inhibition mechanisms of the NO-indirect pathway remain however unclear [5–8]. Various molecular modifications are caused by various agents (NO donors) that act by cGMP-independent pathways. They include protein phosphorylation (essential for the calcium entry in platelets), *S*-nitrosylation of cysteinyl residues, nitration of tyrosine residues (e.g. of α-actinin cytoskeletal protein), activation of ADP-ribosyltransferase, and modifications of various cell-surface receptors (such as those of fibrinogen, ADP, collagen, tromboxane A_2) with consequent platelet inactivation [4,6,9–17].

NO donors are a large heterogeneous group of compounds that release NO with different ways and kinetic properties [18], whose mechanisms of action, NO-direct and/or NO-indirect, are not yet well defined. For example, *S*-nitrosoglutathione (GSNO), sodium nitroprusside (SNP) and 3-morpholino-sydnonimine hydrochloride (SIN-1), may exhibit different pathways of platelet inhibition which for GSNO, the most investigated agent, may be cGMP-dependent, -independent or mixed (cGMP-dependent and -independent) [5–7,9,14,19,20].

The evaluation of the mechanism of action of the different NO donors is important for a better therapeutic characterization, as well as for a better knowledge of their possible adverse effects generated by relatively high NO concentrations and formation of reactive nitrogen species (RNS) [4,18]. For example, as indicated by other authors [21], the administration of NO donors acting through cGMP-independent pathways might resolve cases of reduced activity of sGC. To reduce possible toxic effects caused by high NO concentrations, it seems relevant to establish the relationship between the entity of NO release from each NO donor and the type of cGMP response.

In this work, we have compared the contribution of the cGMP-independent component on the inhibitory effects of SNP, GSNO and SIN-1 on human and rat platelet aggregation. Besides, we have investigated the release of NO from each drug to relate it with the corresponding inhibitory mechanism. For this purpose we evaluated: (i) the anti-aggregating effect of SNP, GSNO and SIN-1 in *in vitro* tests of platelet aggregation using platelet-rich plasma (PRP) according to the Born's method [22] in the presence or absence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a selective inhibitor of sGC [23]; (ii) the entity of NO release from NO donors in PRP and platelet-poor plasma (PPP) measured by NO electrode; (iii) the involvement of the fibrinogen receptor ($\alpha_{IIb}\beta_3$) in cGMP-independent responses by measuring its activation state through cytofluorimetric assay using PAC-1, a specific monoclonal antibody that recognizes an epitope present only on the activated receptor.

Our results have indicated that in both species GSNO acts by NO-indirect mechanisms, and delivers the highest NO concentration in comparison with SIN-1 and SNP which by contrast, except for SIN-1 in rat (NO-indirect mechanism), operate with mixed responses (NO-direct and -indirect). In particular, in PRP and PPP of both species, nearly equal NO amounts were released from GSNO. The NO delivery from GSNO in human was significantly higher than in rat, whereas no species difference was observed for SIN-1 and SNP.

A careful interpretation of the GSNO results induces to suggest that the human and rat responses are conditioned by thiol characteristics of essential and non-essential protein SH groups (PSH) of each species. We assume that GSNO causes platelet inhibition by *S*-nitrosylation reactions with essential PSH, as follows:

$$\mathrm{GSNO} + \mathrm{PSH} = \mathrm{PSNO} + \mathrm{GSH}, \quad (1)$$

and that the minor GSNO response in rat with respect to human is attributable to a more enhanced *S*-nitrosylation with non-essential PSH of the rat albumin, thus reducing the GSNO bioavailability for essential PSH. A putative molecular mechanism of GSNO action on PSH is also illustrated and discussed.

2. Materials and methods

2.1. Chemicals

Adenosine diphosphate (ADP), dimethyl sulphoxide (DMSO), 3-morpholino-sydnonimine hydrochloride (SIN-1), 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), *S*-nitrosoglutathione (GSNO), and sodium nitroprusside (SNP) were purchased from Sigma–Aldrich, Italy.

Fluorescein-isothiocyanate (FITC)-conjugated monoclonal antibody PAC-1 and corresponding isotypic control were from Becton Dickinson, Italy.

NO-donors were dissolved and diluted in saline (0.9%) at the moment of the analysis and conserved in the dark and at 0 °C during analyses.

Stock solutions of ODQ were prepared dissolving ODQ in dimethyl sulphoxide (DMSO) and stored at −20 °C. Dilution in saline was made before analysis and maintained in the dark and ice.

The final concentration of DMSO in PRP (0.37%, maximum final concentration) was unable to modify the platelet response (data not shown).

2.2. Human blood samples

The study was carried out in accordance with the principles of the Declaration of Helsinki. Human peripheral venous blood was drawn from healthy volunteers, who did not receive any drugs for 2 weeks; each of them gave an informed consent. The subjects were investigated after overnight fasting. Blood samples were withdrawn without stasis, anticoagulated with 3.8% trisodium citrate (v/v: 1/9). For cytofluorimetric analysis the first 2 mL of blood were discarded.

2.3. Rat blood samples

Animal experiments were carried out in accordance with the guidelines of the Council of the European Community 86/609/EEC and the Bioethical Committee of the University of Siena approved the protocols. Adult male Sprague-Dawley rats (Charles River), weighing ~400 g, were housed under controlled light (12 h on and 12 h off), temperature (20 ± 2 °C), and humidity (55 ± 5%) conditions. Rats consumed food and water ad libitum.

Rats were investigated after overnight fasting. Blood samples were drawn from abdominal aorta without stasis after anesthetization (50 mg/kg sodium pentobarbital and 0.4 mg/kg scopolamine, intraperitoneally) and anticoagulated with 3.8% trisodium citrate (v/v: 1/9).

2.4. Platelet preparation

Human platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 170 × *g* for 15 min and platelet-poor plasma (PPP) was prepared by further centrifuging at 2700 × *g* for 10 min. Human PRP was collected from healthy donors (5 males of age between 30 and 65). In the case of rat, platelets were prepared with slight modifications, as previously described [24]. Briefly, PRP was obtained after centrifugation of whole blood at 170 × *g* for 15 min and PPP after further centrifuging at 2000 × *g* for 10 min.

Platelet counts in PRP were obtained with a Burker chamber. The human platelet number ranged between 250,000 and 300,000 platelets/μL in PRP samples. Rat PRP platelet