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SHORT COMMUNICATION

Differential Interactions of Nitric Oxide Donors with Rat Oxyhemoglobin

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ABSTRACT. To estimate the reaction of two primary redox-related species of nitric oxide (i.e. NO^+ vs $NO^\bullet$) from a variety of NO donors, we employed the differential interactions of these NO forms with oxyhemoglobin (oxyHb) as a chemical assay. NO^+ formation was estimated by the *S*-nitrosation reaction with oxyHb, and $NO^\bullet$ formation via its reaction with the oxygen-heme complex of oxyHb. Under the conditions employed, all NO donors caused concentration-dependent formation of methemoglobin, indicative of $NO^\bullet$ liberation. However, the extent of *S*-nitrosation was substantially different among the NO donors studied. A representative *S*-nitrosothiol, *S*-nitroso-*N*-acetyl-penicillamine, caused significantly more *S*-nitrosation than nitroglycerin, isobutyl nitrite, sodium nitroprusside, and 3-morpholino-sydnonimine (ANOVA, $P < 0.05$). These results indicated that NO donors can differ in their interactions with oxyHb, and possibly with other target proteins, in part because they liberate or transfer different ratios of NO redox forms. This difference may contribute, in part, to the diversity of pharmacological effects elicited by NO donors. BIOCHEM PHARMACOL **58**;4:671–674, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. nitric oxide donors; redox-related species; oxyhemoglobin; *S*-nitrosation

NO† is an important mediator involved in the regulation of various physiological processes. Its diversity in biological action has been attributed to the existence of various redox forms [1, 2]. In the brain, for example, the neuroprotective effects of NO appear to be attributable to the transfer of nitrosonium (NO^+) cation to thiol groups (–SH) on the NMDA receptors, whereas the neurotoxic effects are due to peroxynitrite anion ($ONOO^-$) generated from the reaction of $NO^\bullet$ (free radical) with superoxide anion [3]. It has been demonstrated recently that RSNOs and NO are markedly different in their reactions with Hb. RSNOs contain a NO group that is believed to possess NO^+ character capable of undergoing transnitrosation reactions with thiol groups on Hb to form nitrosated proteins [4]. On the other hand, the reaction of NO with the oxygen-heme complex of oxyhemoglobin (oxyHb, Fe^{2+}) to form methemoglobin (metHb, Fe^{3+}), which can be readily detected by spectrophotometric changes [4, 5], has been considered to be a $NO^\bullet$ characteristic.

NO donors, as a class, are believed to elicit their pharmacological effects through a common mediator, i.e. NO. Interestingly, differences in pharmacology exist among this class of agents. For example, continuous exposure to organic nitrates results in the development of hemodynamic tolerance in rats with congestive heart failure, but constant infusion of RSNOs and organic nitrites in the same animal model does not produce tolerance [6, 7]. Since these different pharmacological effects may arise from dissimilar compositions of the NO-related species produced by these compounds and the reactions of these species with target proteins, we seek to determine the relative reactivities of several NO donors with the two principal reactive sites of oxyHb, viz. the oxygen-Fe^{2+} complex and the –SH groups. Furthermore, it has been demonstrated that the interaction of NO with different sites on Hb may be responsible for the regulation of blood flow and pulmonary vasodilatation [8, 9]. Thus, an understanding of the relative ability of NO donors to interact with the Hb sites may provide valuable insights into their potential therapeutic applications.

MATERIALS AND METHODS

Materials

Crystallized rat Hb, sodium dithionite, SNP, and ISBN were obtained from the Sigma Chemical Co. SIN-1 and SNAP were purchased from Alexis. NTG (in sucrose) was obtained from ICI America Inc., and concentrated solutions of NTG in ethanol were prepared according to a procedure described previously [10]. Sephadex G-25 was obtained from Pharmacia Biotech.

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† *Abbreviations*: NO, nitric oxide; NMDA, *N*-methyl-D-aspartate; RSNO, nitrosothiol; Hb, hemoglobin; oxyHb, oxyhemoglobin; metHb, methemoglobin; NTG, nitroglycerin; SNP, sodium nitroprusside; ISBN, isobutyl nitrite; SIN-1,3-morpholino-sydnonimine; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; GC, guanylyl cyclase; and SCA, sickle cell anemia.

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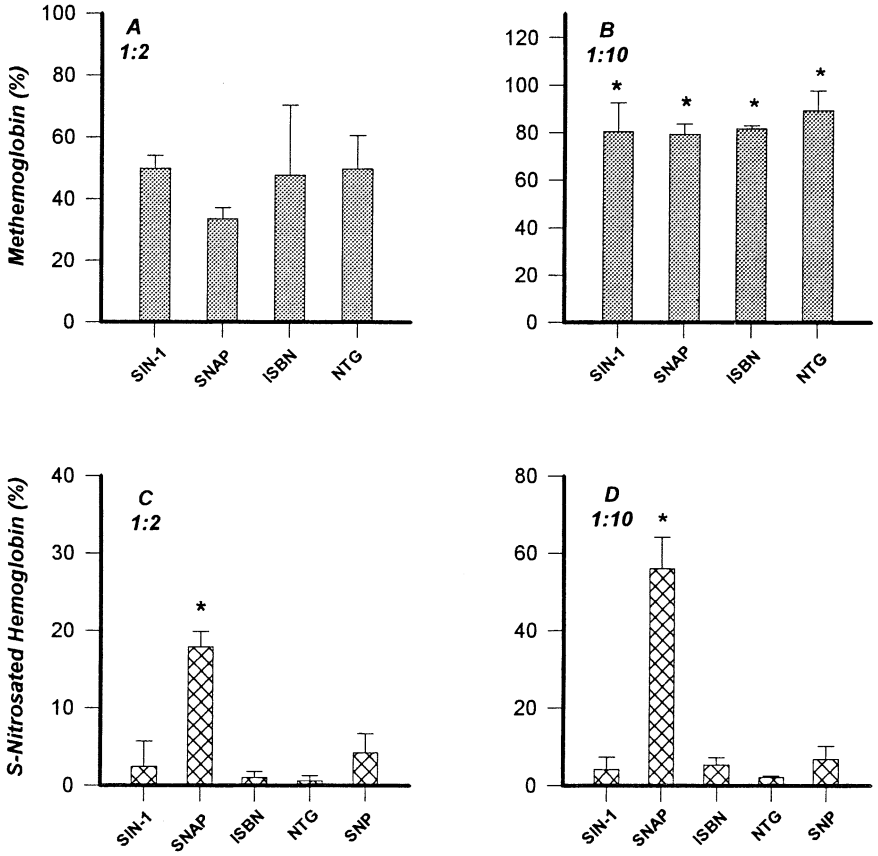

FIG. 1. Interaction of different NO donors with oxyHb at different molar ratios (1:2 and 1:10) of oxyHb: NO donor. Panels A and B show the percent of metHb formation (*, $P < 0.05$, by ANOVA, vs control). Panels C and D show the percent of *S*-nitrosated Hb formation, expressed as a percentage of the estimated –SH content (*, $P < 0.05$, by ANOVA, between SNAP vs all other NO donors). Data are expressed as means ± SD of three replicates.

Preparation of oxyHb

OxyHb solution was prepared daily [5]. Briefly, crystallized rat Hb was dissolved in phosphate buffer (0.1 M, pH 7.4) by gentle swirling. Solid sodium dithionite was added to the solution, and then oxygen was blown gently over the surface of the solution for approximately 30 min. Purification and desalting of oxyHb were accomplished by passing the resulting solution over a Sephadex G-25 column. The concentration of the desalted oxyHb stock solution was determined by measuring the maximal Soret absorbance ($A_{415\ nm}$) with a Cary spectrophotometer (model 118) and the molar extinction coefficient of 131 $mM^{-1}\ cm^{-1}$ [5].

Incubation of Different NO Donors with oxyHb

NO donors were incubated with oxyHb (0.8 mM) solution at molar ratios of 1:2 and 1:10 (oxyHb:NO donor, in a final volume of 300 μL) at room temperature for 60 min. Control samples were incubated with buffer and treated in the same fashion. At the end of the incubation interval, a 200-μL aliquot of the sample was passed over a Sephadex G-25 column to remove the low molecular weight thiols that were not associated with oxyHb as well as any nitrate/nitrite ions formed. Quantification of the *S*-nitrosated protein was carried out according to the method of Saville [11]. To correct for dilution of the samples after passing over the column, protein concentrations of the samples were measured before and after by the Bradford assay [12]. The extent of conversion of oxyHb to metHb was obtained by measuring the absorbance of the control and the sample at $A_{540\ nm}$ (the absorption maxima of the β-band) as well as at $A_{610\ nm}$ (the isosbestic point). Changes in the total concentration of oxyHb among different samples were normalized by determining the ratio of the absorbances at the two wavelengths ($A_{540\ nm}/A_{610\ nm}$). The extent of metHb formation was estimated from the algebraic difference of the absorbance ratio between the control and the NO donor sample and was expressed as the percent change from the control. In separate experiments, the metHb content in the control samples, in the absence of the NO donors, after a 60-min incubation (30 ± 12.2%, N = 13) was determined according to a method described previously [13], and was subtracted from the total metHb content obtained after incubation with various NO donors. Due to the reaction of the heme site of oxyHb with cyanide, metHb formation after incubation with SNP could not be determined readily by spectrophotometry.

TABLE 1. Extent of *S*-nitrosation of oxyHb by various NO donors

NO donor studied	Chemical class	*S*-Nitrosated Hb formed (μM) (Molar ratio–oxyHb:NO donor) 1:0	1:2	1:10
NTG	Organic nitrate	25.3 ± 13.7	27.4 ± 8.4	28.0 ± 14.5
ISBN	Organic nitrite	18.3 ± 4.2	29.6 ± 7.1	59.7 ± 14.5*
SNAP	*S*-Nitrosothiol	46.3 ± 26.1	190 ± 24*	493 ± 71*
SIN-1	Sydnonimine	33.2 ± 41,4	51.6 ± 68.0	66.0 ± 67.3
SNP	Ferrous–nitrosyl complex	35.5 ± 20.5	46.9 ± 34.5	90.1 ± 27.1

Control experiments were conducted in the absence of the NO donors (N = 3 for each set). Data are expressed as means ± SD of three replicates for each NO donor.
*Denotes statistical significance when compared with its control (ANOVA, $P < 0.05$).

RESULTS AND DISCUSSION

Under the conditions employed, we observed that the NO donors examined differed in their interactions with rat oxyHb. All the NO donors caused concentration-dependent formation of metHb versus control, by a decrease in the absorption maxima of the β-band of oxyHb ($P < 0.05$ at the ratio of 1:10, Fig. 1, A and B). This reaction, i.e. formation of metHb, has been ascribed to a reaction between oxyHb and the free radical form of NO, viz. $NO^{\bullet}$ [4, 5].

In the absence of NO donors (control experiments), the apparent content of *S*-nitrosated Hb ranged from 18.3 to 46.3 μM, representing 2.3 to 5.8% of the total Hb added. The extent of *S*-nitrosation of oxyHb by various NO donors was substantially different (Fig. 1, C and D). SIN-1 and NTG were shown to react predominantly with the heme center, and not with the sulfhydryl site of oxyHb ($P > 0.05$). In contrast, SNAP exhibited substantial *S*-nitrosation of oxyHb at all concentrations examined (Table 1, $P < 0.05$). Reaction of ISBN with oxyHb at the higher ratio resulted in formation of some nitrosated protein (Table 1, $P < 0.05$). The nitrosated Hb content of the samples incubated with SNP was not different from the control (Table 1, $P > 0.05$).

The NO donors chosen for this study represented different chemical classes, viz. organic nitrates (NTG), organic nitrites (ISBN), nitrosothiols (SNAP), sydnonimines (SIN-1), and a ferrous-nitrosyl complex (SNP). All NO donors employed in our study have been shown to release NO chemically, with the exception of NTG, which releases NO enzymatically [14]. The present study also indicated that oxyHb could facilitate the production of $NO^{\bullet}$ from NTG. These results are in agreement with the shorter biological half-life of NTG in blood versus plasma [15].

Similar to RSNOs, metal-nitrosyl complexes such as SNP have been viewed as NO^{+} carriers [16]. Our results indicate that although substantial *S*-nitrosation of oxyHb by SNAP was observed, SNP did not produce significant amounts of *S*-nitrosated Hb under the conditions studied. These observations suggest that the intrinsic NO^{+}-transferring activity of SNP may be weaker that that of RSNO, when directed to rat oxyHb.

The interactions of NO donors with different Hb binding sites offer insights into the dissimilar pharmacological actions observed, not infrequently, among these compounds. Similar to Hb, the enzyme soluble GC, which generally is recognized as the principal target enzyme for NO, contains both the sulfhydryl sites (–SH) and a heme center that can be activated by various NO donors. Ignarro *et al.* [17] demonstrated that in a heme-deficient preparation of soluble GC, the enzymatic activity was dependent on the oxidation-reduction state of the –SH group located at the catalytic site. These authors further demonstrated that RSNOs such as *S*-nitrosocysteine were capable of activating soluble GC through the –SH group [18]. In addition, covalent exchange of NO^{+} from RSNOs to the –SH group of the cell membranes such as NMDA receptors or calcium channels can result in direct activation of signal transduction pathways that are independent of cyclic GMP production [3, 19]. The activation of different signaling pathways by various NO metabolic forms indeed may account for the diversity observed in the effects elicited by NO donors. Our results further underscore the view that NO donors are not identical to each other, either biochemically or pharmacologically.

The interactions between Hb and different NO donors may be of importance in the therapeutic applications of these agents in diseases such as SCA and α-thalassemia [20, 21]. It has been demonstrated recently that low concentrations of NO can augment the oxygen affinity of sickle erythrocytes both *in vivo* and *in vitro*, thus offering a new alternative approach for the treatment of SCA [20]. Recent work by Stamler and colleagues [8, 9] also demonstrated the importance of the interactions between NO and Hb in the regulation of blood flow. The differential interaction between NO donors and oxyHb that we observed here may, therefore, have important implications regarding the choice of appropriate NO donors for use in therapy.

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