Free Radical Biology and Medicine 82 (2015) 13–21

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Diazeniumdiolation of protamine sulfate reverses mitogenic effects on smooth muscle cells and fibroblasts

Robert van Lith [a], Jian Yang [a], Guillermo A. Ameer [a,b,c,d,*]

[a] Biomedical Engineering Department, Northwestern University, Evanston, IL 60208, USA
[b] Department of Surgery, Feinberg School of Medicine, Chicago, IL 60611, USA
[c] Chemistry of Life Processes Institute, Northwestern University, Evanston, IL 60208, USA
[d] Simpson Querrey Institute, Northwestern University, Evanston, IL 60208, USA

ARTICLE INFO

Article history:
Received 22 August 2014
Received in revised form
16 January 2015
Accepted 26 January 2015
Available online 2 February 2015

Keywords:
Protamine sulfate
Nitric oxide
Oxidative stress
Peptide
Diazeniumdiolate
Free radicals

ABSTRACT

After vascular interventions, endothelial cells are typically injured or lacking, resulting in decreased NO synthesis to maintain vascular health. Moreover, inflammation as a result of the tissue injury and/or the presence of an implanted foreign polymer such as a vascular graft causes excessive generation of reactive oxygen species (ROS) (e.g., superoxide), which can react with NO. The combination of the above creates a general decline in NO bioavailability, as well as oxidative stress due to less available NO to scavenge ROS. Localized NO delivery is an attractive solution to alleviate these issues; however, NO donors typically exhibit unpredictable NO payload release when using nitrosothiols or the risk of nitrosamine formation for synthetic diazeniumdiolates. The objective of this study was therefore to synthesize an NO donor from a biological peptide that could revert to its native form upon NO release. To this effect, protamine sulfate (PS), an FDA-approved peptide with reported vasodilator and anticoagulant properties, was diazeniumdiolated to form PS/NO. PS/NO showed diazeniumdiolate-characteristic UV peaks and NO release in physiological solutions and was capable of scavenging radicals to decrease oxidative stress. Furthermore, PS/NO selectively inhibits the proliferation of smooth muscle cells and adventitial fibroblasts, thereby reversing reported mitogenic properties of PS. Endothelial cell growth, on the other hand, was promoted by PS/NO. Finally, PS retained its anticoagulant properties upon diazeniumdiolation at clinically relevant concentrations. In conclusion, we have synthesized an NO prodrug from a biological peptide, PS/NO, that selectively inhibits proliferation of smooth muscle cells and fibroblasts, retains anticoagulant properties, and reverts back to its native PS form upon NO payload release.

Nitric oxide (NO) is a small, easily diffusible gaseous molecule that plays numerous roles in human physiology. NO is soluble in both hydrophilic and hydrophobic environments and has an in situ half-life of just a few seconds [1]. NO is secreted at low levels constitutively by endothelial or neuronal nitric oxide synthase (NOS), whereas under certain conditions, e.g., pathogen invasion or severe inflammation, it can be secreted in larger quantities by inducible NOS. In the body, NO acts as a signaling molecule in neurological, cytotoxic, and cardiovascular functions [2]. For example, NO, continuously secreted by the endothelium lining blood vessels through endothelial NOS, is considered vital for maintaining vascular homeostasis. NO has been shown to promote endothelial health, prevent platelet adhesion, and inhibit smooth muscle cell proliferation and migration. After interventions as a result of, e.g., atherosclerosis, such as coronary bypass surgery or percutaneous coronary interventions, endothelial cells are typically injured or lacking, resulting in decreased NO synthesis to maintain vascular health. Moreover, inflammation as a result of tissue injury and/or the presence of an implanted foreign polymer such as a vascular graft causes generation of excessive reactive oxygen species (ROS) (e.g., superoxide), which can react with NO to form peroxynitrite. The combination of the above creates a general decline in NO bioavailability, as well as oxidative stress due to less available NO to scavenge ROS. In this sense, NO acts as an antioxidant compound by scavenging the ROS superoxide. Although $ONOO^-$ can also act as a pro-oxidant, it is considered less harmful than superoxide and its downstream reaction product hydroxyl ($OH^\bullet$); therefore the overall net effect of NO scavenging superoxide is considered oxidative stress lowering [3]. The resulting lack of NO bioavailability, though, is considered the main cause of high thrombosis risk and the development of neointimal hyperplasia, ultimately resulting in repeated interventions. Although NO delivery to alleviate the above-mentioned issues has been recognized as a potential therapy, systemic methods (e.g., through inhalation) are impractical and lead to unwanted side effects (e.g., hypotension as a result of NO-triggered vasodilation), and NO is ineffective at reaching the target

* Corresponding author at: Biomedical Engineering Department, Northwestern University, Evanston, IL 60208, USA. Fax: +1 847 491 4928.
E-mail address: g-ameer@northwestern.edu (G.A. Ameer).

http://dx.doi.org/10.1016/j.freeradbiomed.2015.01.022
0891-5849/

site because of its labile nature and rapid reactions with other molecules. Local delivery of NO at the site of injury is therefore required, and a variety of NO donors have been developed for effective treatment of such conditions, because NO gas itself has a short half-life and therefore limited utility as a therapeutic agent [4,5]. NO donors are characterized in several categories, with the most important being *S*-nitrosothiol and diazeniumdiolate compounds owing to their respective unique properties. *S*-nitrosothiols, though interesting as biological compounds, typically require catalysts to release their NO moieties, and kinetics can be highly unpredictable, and release at a specific site and rate is often not possible [6].

Diazeniumdiolates, also called NONOates, on the other hand, are a class of NO donors that have a diolate group (NONO) bound to an amine group [7]. NO reacts with the amine to form a radical, which then reacts with another NO molecule to form the NO-donating complex [8]. These compounds can generate two molecules of NO per diazeniumdiolate moiety in acidic or neutral solutions, whereas in the basic, frozen, or lyophilized state, diazeniumdiolates are generally stable [9]. Release kinetics vary with temperature, pH, and the molecular structure of the donor [8]. The by-product of the reaction ideally is the original amine compound [10]; this is of great importance as many NO donors, e.g., sodium nitroprusside, produce toxic by-products after release of NO. Synthetic diazeniumdiolate prodrugs may be prone to formation of nitrosamines, which are considered carcinogens, and long-term effects are unclear. Diazeniumdiolates, however, can also potentially be designed from peptides and/or proteins to leave an innocuous and/or naturally occurring molecule as the remaining product. Though previously the diazeniumdiolation of bovine serum albumin was demonstrated by conjugating NONO-carrying groups [11], our objective is to convert naturally occurring peptides to diazeniumdiolates without making significant alterations to the protein or peptide structure, utilizing naturally occurring amine and amide groups.

We therefore chose to derivatize a naturally occurring small peptide with beneficial properties, protamine sulfate (PS) (Fig. 1). PS may exert a dual benefit because of its reported properties, especially if, upon release of its NONO moieties, it regains its natural structure. PS has been in use as an FDA-approved drug for many years as a heparin antidote and is routinely used during cardiovascular surgery for this purpose. It has a molecular weight of approximately 4–5 kDa, consists of 60–70% L-arginine residues, and is highly basic, with a net charge of +21 [12]. The richness in L-arginine groups is thought to provide PS with a stimulatory effect on the endothelial endogenous NO production machinery, resulting in increased NO secretion [13,14]. Evidence from endothelial cells (ECs) suggests that PS increases intracellular Ca^{2+} levels [15], induces EC-dependent vasodilation [16], and stimulates NOS activity [13,17]. In smooth muscle cells (SMCs), on the other hand, EC-independent vasodilation [18] was shown. Furthermore, recently it was shown that PS induces NO release in kidney [19] and pulmonary cells [16,20]. These effects may be mediated through intracellularization of protamine [19,21,22]. This suggests possible intracellular utilization of L-arginine for NOS-mediated NO production. Indeed, guanidinium functionalities of arginine residue repeats have been shown to be essential for cellular uptake of polypeptides [23]. Finally, PS has proline groups, as well as numerous guanidinium moieties that may be functionalized to diazeniumdiolate groups [24]. These characteristics make PS a promising candidate for delivering exogenous NO through diazeniumdiolation of the peptide, whereas PS may also have the benefit of NO-like effects simultaneously [25]. Moreover, PS is known for its potent antibacterial properties [26–28] and has a known mild anticoagulant and fibrinolytic effect [29,30], favorable properties for the anticipated applications.

Herein, we report the synthesis of dually functional PS by converting free amines into diazeniumdiolates. We hypothesized that the native structure could be modified to carry NONO groups and that upon liberation of its NO load, native PS would be recovered. Moreover, we hypothesized that NO-releasing PS/NO would have NO-specific effects on vascular cells. By combining the native properties of PS with NO-releasing capabilities, we anticipate a potentially more efficient and readily applicable drug.

Materials and methods

Materials

Nitric oxide was obtained from Matheson Gas Products (Montgomeryville, PA, USA) and used as received at 99.5% UHP grade. Protamine sulfate from salmon was used for all reactions (Catalog No. P4020, Sigma). Unless noted otherwise, reagents and solvents were obtained from Sigma–Aldrich Chemical Co. (Milwaukee, WI, USA) and used without further modification. A suitable apparatus for NO reactions was custom-built as has been described previously [31]. Human umbilical vein endothelial cells (HUVECs), human aortic smooth muscle cells (HASMCs), human aortic adventitial fibroblasts (HAoAFs), endothelial growth medium, stromal cell growth medium, and smooth muscle cell growth medium were acquired from Lonza.

Synthesis and characterization of diazeniumdiolated protamine sulfate

To convert PS into the diazeniumdiolated compound PS/NO, PS was suspended in a 0.5 M solution of sodium methoxide in methanol at room temperature (100 mg/ml). The solution was then placed in a pressure bottle, flushed 10 times with argon gas to limit the presence of oxygen, and treated with 5 atm pressurized NO gas for 3 days. The resulting yellow residue was washed with methanol twice and with diethyl ether thrice and subsequently vacuum-dried and stored in a vacuum desiccator, light-protected, at room temperature. Control PS was formed by following identical procedures, but exposing PS to argon gas instead of NO. For in vitro cell experiments, peptides were gas sterilized with ethylene oxide (Anprolene AN74i, Andersen Products, Haw River, NC, USA) for 12 h. Successful conversion to a diazeniumdiolate compound was assessed by UV–Vis by dissolving 1 mg of PS/NO in 1 ml phosphate-buffered saline (PBS) and monitoring the decrease in the characteristic UV peak. Ultraviolet spectra were recorded on

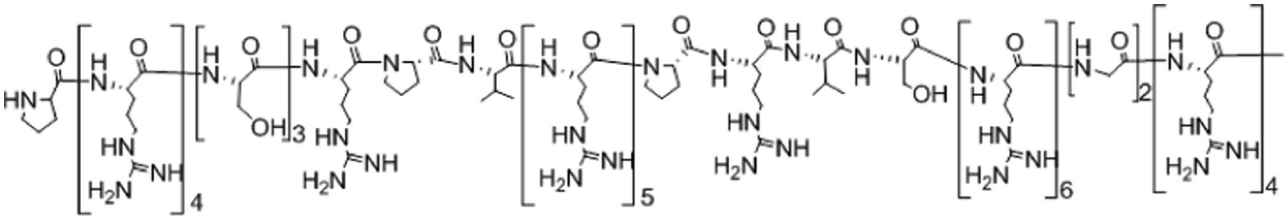

Fig. 1. Structure of protamine sulfate, including approximately 60–70% arginine residues.