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S-nitrosothiols as potential therapeutics to induce a mobilizable vascular store of nitric oxide to counteract endothelial dysfunction

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Abstract:

Endothelial dysfunction predisposing to cardiovascular diseases is defined as an imbalance in the production of vasodilating factors, such as nitric oxide (NO), and vasoconstrictive factors. To insure its physiological role, NO, a radical with very short half-life, requires to be stored and transported to its action site. S-nitrosothiols (RSNOs) like S-nitrosoglutathione (GSNO) represent the main form of NO storage within the vasculature. The NO store formed by RSNOs is still bioavailable to trigger vasorelaxation. In this way, RSNOs are an emerging class of NO donors with a potential to restore NO bioavailability within cardiovascular disorders.

The aim of this study was to compare S-nitrosothiols ability, formed of peptide (glutathione) like the physiologic GSNO or derived from amino acids (cysteine, valine) like the synthetics S-nitroso-N-acetylcysteine (NACNO) and S-nitroso-N-acetylpenicillamine (SNAP), respectively, to produce a vascular store of NO either in endothelium-intact or endothelium-removed aortae in order to evaluate whether RSNOs can be used as therapeutics to compensate endothelial dysfunction. Sodium nitroprusside (SNP), a marketed drug already in clinics, was used as a non-RSNO NO-donor. Endothelium-intact or endothelium-removed

aortae, isolated from normotensive Wistar rats, were exposed to RSNOs or SNP. Then, NO-derived (NO_x) species, representing the NO store inside the vascular wall, were quantified using the diaminonaphthalene probe coupled to mercuric ions. The bioavailability of the NO store and its ability to induce vasodilation was tested using *N*-acetylcysteine, then its ability to counteract vasoconstriction was challenged using phenylephrine (PHE).

All the studied RSNOs were able to generate a NO store materialized by a three to five times increase in NO_x species inside aortae. NACNO was the most potent RSNO to produce a vascular NO store bioavailable for vasorelaxation and the most efficient to induce vascular hyporeactivity to PHE in endothelium-removed aortae. GSNO and SNAP were equivalent and more efficient than SNP. In endothelium-intact aortae, the NO store was also formed whereas it seemed less available for vasorelaxation and did not influence PHE-induced vasoconstriction. In conclusion, RSNOs - NACNO in a better extent - are able to restore NO bioavailability as a functional NO store within the vessel wall, especially when the endothelium is removed. This was associated with a hyporeactivity to the vasoconstrictive agent phenylephrine. Treatment with RSNOs could present a benefit to restore NO-dependent functions in pathological states associated with injured endothelium.

1. Introduction

Cardiovascular diseases (CVD) are an important leading cause of morbidity and mortality in the world and mainly originate from the disruption of endothelial homeostasis[1,2]. Endothelium impairment is a complex pathophysiological event that includes endothelial cells activation and dysfunction. Endothelial dysfunction is defined as an imbalance in the production of anti- and prothrombotic factors, anti- and proinflammatory factors as well as vasodilating and vasoconstrictive factors[3,4]. The decreased production and/or bioavailability of vasodilating factors like endothelium derived nitric oxide (NO) is the earliest and one of the most important events that characterize endothelial dysfunction [5,6] [7]. In endothelial cells, NO is responsible for vascular homeostasis maintenance through the coordination of the communication between endothelial and smooth muscle cells (SMC) in the vessel wall for example. The decrease in NO bioavailability results either from (i) a reduction of NO synthesis, linked to a decrease in eNOS protein expression [8] and/or eNOS activity impairment (uncoupling) [9], or from (ii) an increase in NO degradation. NO, as a gasotransmitter showing a very short half-life (less than 5 s [10]), is stored and transported as *S*-nitrosothiols (RSNOs), in relation with the *S*-nitrosation process (the formation of the S-NO bond). This physiological *S*-nitrosation process has been used to develop new NO donors, the RSNOs, with a potential use in cardiovascular disorders associated with endothelial dysfunction to restore NO bioavailability. Indeed, RSNOs are currently investigated (clinical trials) to reduce cerebral embolization after carotid angioplasty, recurrent stroke or systemic embolization for examples see [11]. Some RSNOs are peptides like the physiological *S*-nitrosoglutathione (GSNO) or are derived from amino acids (cysteine, valine) like the synthetics *S*-nitroso-*N*-acetylcysteine (NACNO) and *S*-nitroso-*N*-acetylpenicillamine (SNAP), respectively. Furthermore, GSNO is based on glutathione, the most abundant antioxidant in cells and tissues. NACNO is based on *N*-acetyl-*L*-cysteine, a mucolytic drug also used as a

glutathione precursor administered in acetaminophen-based intoxication, showing an antioxidant activity linked to the thiol function of the cysteine. SNAP is made of *N*-acetyl-*D*-penicillamine, an anti-inflammatory drug, used to treat rheumatoid polyarthritis, with an antioxidant thiol function. The *in vitro* stability of these RSNOs, in physiological media at physiological temperature, is 2 h for SNAP, 40 h for GSNO and 500 h for NACNO) [11]. The antioxidant property of the skeleton carrying NO as well as its ability for *S*-nitrosation will also help to limit the oxidative/nitrosative stress induced by NO oxidation into peroxynitrite ions (ONOO⁻) [12]. In *ex vivo* models, these RSNOs induce an immediate vasorelaxation (*e.g.* within 5 min following exposure in isolated aortae) with half maximal effective concentrations (EC₅₀) ranging from 0.1 μM for GSNO and NACNO to 1 μM for SNAP [11]. The huge difference between RSNOs stability *in vitro* and EC₅₀ *ex vivo* is linked to the fast release of NO induced by denitrosating enzymes such as protein disulfide isomerase (PDI) and gamma-glutamyl transferase (GGT) - present at the endothelial level - and subsequent activation of the soluble guanylate cyclase (sGC)/GMPc pathway within smooth muscle cells [13,14]. The presence of a functional endothelium is crucial for the immediate NO release from RSNOs as shown by a higher potency of RSNOs to induce vasodilation in the presence rather than in the absence of endothelium [13,15]. This was highlighted by a higher *ex vivo* vasorelaxant effects (1 log increase in pEC₅₀ values) of RSNOs on endothelium-intact aortic rings than on endothelium-denuded aortic rings [13].

Besides these immediate vasorelaxant effects related to sGC nitrosylation (NO coordination to heme iron), RSNOs are able to induce the vascular storage of NO either *ex vivo* or *in vivo* [16–18]. This NO store is mainly composed of NO-derived (NO_x) species like nitrite ions and *S*-nitrosated proteins or peptides made by transnitrosation reactions [12]. Those transnitrosation reactions with circulating or vascular peptides/proteins play important roles in NO transport and storage as well as NO signaling. The NO store can be mobilized for sGC

activation leading to vasorelaxation [16–19]. Transnitrosation processes depend on the physico-chemical properties of the molecules carrying SNO [10,20] and their metabolism by denitrosating enzymes [21]. So, we can hypothesize that the ability of GSNO, NACNO and SNAP to generate a NO store within the vascular wall will be different regarding the carrying thiol structure, which determines their handling by plasma membrane enzymes (GGT and PDI), surface thiols and amino acid transporters like the L-type amino acid transporter (L-AT) [22].

As endothelium plays an important role in the immediate vasorelaxant effect of RSNOs, we wonder whether it influences NO storage from RSNOs. We therefore compare the ability of three *S*-nitrosothiols, GSNO a physiological RSNO, and two synthetic RSNOs, NACNO and SNAP to generate NO storage within the vascular wall of aortae. Sodium nitroprusside (SNP) is evaluated as a non-RSNO NO-donor. The NO store is evaluated by quantifying NO_x species inside the vascular wall. Then, the bioavailability of such NO storage to regulate vasoactivity is revealed by measuring aortic vasoconstriction to phenylephrine (PHE) and the vasorelaxation induced in response to *N*-acetylcysteine (NAC). These two protocols (NAC and PHE) give functional proofs of the NO stored within the vascular wall, either in endothelium-intact or in endothelium removed aortae.

2. Material and methods

2.1. Chemicals

All reagents were of analytical grade. Mercuric chloride (HgCl₂) and sodium hydroxide (NaOH) were purchased from Prolabo (VWR). Carbachol, PHE, SNP and all other reagents were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Ultrapure deionized water (18.2 MΩ.cm) was used to prepare all solutions.

GSNO, SNAP and NACNO were synthesized extemporaneously at 10⁻² M final concentration by *S*-nitrosation of glutathione, *N*-acetylpenicillamine or *N*-acetylcysteine with sodium nitrite

(ratio 1:1) under acidic condition as previously described [23]. RSNO purity was assessed by ultraviolet spectrophotometry using the specific molar absorbance of the S-NO bond at 334 nm ($\epsilon_{\text{GSNO}} = 922 \text{ M}^{-1} \text{ cm}^{-1}$; $\epsilon_{\text{NACNO}} = 900 \text{ M}^{-1} \text{ cm}^{-1}$) and at 340 nm for SNAP ($\epsilon_{\text{SNAP}} = 1020 \text{ M}^{-1} \text{ cm}^{-1}$). All RSNO solutions were used in a concentration range >95% of the 10 mM initial concentration. All manipulations and assays involving NO donors were performed with subdued lighting, in order to minimize light-induced degradation of RSNOs.

2.2. Rats and ethical statements

All experiments were performed in accordance with the European Community guidelines (2010/63/EU) for experimental animals use in the respect of the 3 Rs' requirements for animal welfare. The projects untitled "Nitro-Vivo" and "BisNitro-Vivo" (n°APAFIS#1614-2015090216575422v2 and #15598-2018061619129620v3, respectively) were positively evaluated by the regional ethical committee for animal experiments and approved by the French Ministry of Research.

Twelve-week-old, male, normotensive Wistar rats (300-325 g) were purchased from Janvier Laboratories (Le Genest St Isle, France) and kept under standard conditions (temperature: $21 \pm 1^\circ\text{C}$, hygrometry $60 \pm 10\%$, light on 6 am to 6 pm). They ate standard diet (A04, Safe, Villemoisson-sur-Orge, France) and drank water (reverse osmosis system, Culligan, Brussels, Belgium) *ad libitum*.

Rats were anesthetized with sodium pentobarbitone (60 mg.kg^{-1} , intraperitoneal injection, Sanofi Santé Nutrition Animale, Libourne, France) and anesthesia adequacy was checked by testing the loss of the corneal and pinch paw withdrawal reflexes. If a change in the reflexes occurred, sodium pentobarbitone in bolus was immediately administered. After heparin (1000 IU.kg^{-1} heparine Choay, penis vein) administration, rats were sacrificed by exsanguination and segments (3 cm) of the descending thoracic aorta were removed, cleaned from surrounding connective tissues, cut into 2-mm long rings (8 rings per rat) and immediately

used for vasoactivity. In some rings, the endothelium was removed by gently rubbing the intimal surface with forceps. For NO_x species quantification, aortas (endothelium-intact or endothelium-removed) were frozen in liquid nitrogen immediately after collection and stored at -80 °C (less than 2 months) until the incubation with NO donors.

2.3. Quantification of NO derived species

The NO_x species (RSNO and nitrite ions) were quantified inside endothelium-intact and endothelium-removed aortae. Aortae were thawed at 37 °C for 2 min and cut into two pieces of 20 ± 2 mg each (one piece for control and one piece for RSNO incubation). All experiments were performed in the same condition as vasoactivity studies (section 2.4): each aorta piece was equilibrated during 60 min at 37 °C in 75 mL of Krebs' solution containing 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.6 mM CaCl₂, 24 mM NaHCO₃, 5.5 mM glucose, adjusted to pH 7.4 and under 95% O₂ and 5% CO₂ continuous bubbling. Then, GSNO, SNAP, NACNO or SNP (2 μM), or Krebs' solution as control, were incubated during 30 min (n = 3-15 per group, from 3-15 different rats in each group). Aortae were rinsed 3 times during 20 min with 75 mL of Krebs' solution to remove RSNO excess and were dried with a paper tissue. Dry aortae were then snap frozen in liquid nitrogen and crushed with a pestle in a mortar. To quantify NO_x species, aortae powder was mixed for 30 s with 600 μL of 0.105 mM 2,3-diaminonaphthalene (DAN) and 1.05 mM HgCl₂ prepared in HCl 0.6 M. The mixture was incubated 10 min at 37 °C under rotation. The reaction was stopped with 40 μL of 10 M NaOH to reach the maximum of naphthotriazole (NAT) fluorescence. After 15 min centrifugation at 18,000 g, 4 °C, the supernatant intensity of fluorescence was read in a black 96-wells plate at 415 nm after excitation at 375 nm (JASCO FP-8300, France). NO_x species quantity was calculated upon a standard curve built with GSNO (0.1 to 2 μM).

2.4. Vasoactivity studies

Vasoactivity was evaluated on endothelium-intact or endothelium-removed aortic rings using an isometric tension recording system in 10 mL organ chambers (EMKABATH, Emka Technology, France) [15]. The organ chambers were filled with Krebs' solution (10 mL, 37°C) and continuously bubbled with 95% O₂ and 5% CO₂. Following 60-min equilibration at a basal resting tension of 2 g, rings were exposed 2 times to KCl (60 mM, 5 min) to check viability.

Aortae were exposed for 30 min to GSNO, SNAP, NACNO or SNP (2 μM) [17,19], followed by 3 times washing (20 min each) with Krebs' solution to remove RSNO excess. Then, aortae were submitted to two different protocols to evaluate (i) NO storage in the aortic wall (NAC protocol) and (ii) vasoreactivity to a vasoconstrictor (PHE protocol):

1. NAC at 10⁻⁵ M (n = 8-10 per group, from 4-8 different rats in each group) was added on pre-constricted aortae (10⁻⁶ M PHE) in order to displace NO from cysteine-NO residue and mobilize NO for nitrosylation of soluble guanylate cyclase, cGMP production and vasodilation (Fig. 1). Indeed, NAC enters inside the cells and displaces NO from RSNO by transnitrosation process to produce *S*-nitrosocysteine. This instable *S*-nitrosocysteine immediately releases NO, which become available to activate the sGC inducing vasorelaxation (Fig. 1A). In the absence of any NO store, the aortic ring does not relax under this NAC protocol (Fig. 1B).
2. Cumulative concentration response curves to PHE from 3×10⁻¹⁰ M to 3×10⁻⁵ M (n = 6-17 per group, from 3-7 different rats in each group) were performed in order to evaluate whether the NO storage decreases vasoconstrictive capacities.

The role of the endothelium was investigated by performing experiments in either endothelium-intact or endothelium-removed aortae. Endothelium integrity or removal was assessed on 10⁻⁶ M PHE pre-constricted aortae using 10 μM carbachol, a muscarinic

acetylcholine receptor agonist inducing vasorelaxation when the endothelium is intact. Aortic rings showing less than $17 \pm 1\%$ of relaxation were considered as endothelium-removed.

2.5. Data analysis and statistical tests

All the results were presented as means \pm standard deviation and analyzed (Graph Pad prism® software version 6.0); $p < 0.05$ was considered as significant.

Results of NO_x species quantification were analyzed using the Kruskal Wallis test for non-parametric (as some groups contained only 3 aortic rings) values followed by a Dunn's post-test. NAC vasorelaxant effect (Fig. 1) was calculated using the following equation (Eq. 1):

$$\% \text{ Vasorelaxation} = 100 - \left(\frac{\Delta \text{ Tension}_{NAC}}{\text{ Tension of PHE precontraction}} \right) * 100$$

The contractile effect of PHE was calculated as followed (Eq.2):

$$\text{Delta T (g)} = \text{ Tension Phenylephrine} - \text{ Tension at Baseline}$$

For PHE response curves, half maximal effective concentrations (EC₅₀) and maximal responses (E_{max}) were calculated by fitting each concentration response curve with the Hill logistic equation (Eq. 2):

$$\% \text{ Contraction} = E_{min} + \left(\frac{E_{max} - E_{min}}{1 + 10^{(\log EC_{50} - \text{Concentration}) * \text{Hill slope}}} \right)$$

The pEC₅₀ values were calculated as $-\log EC_{50}$.

Analysis and comparisons of different treatments effects on NAC vasorelaxant effects, pEC₅₀ and E_{max} of PHE were performed by the One-Way ANOVA test followed by a Bonferroni's multiple comparisons test.

Analysis of endothelium impact on the effect of each treatment on NO_x species production, NAC vasorelaxant effect, pEC₅₀ and E_{max} of PHE were performed using Student's *t*-test.

3. Results

Endothelium-intact (Fig. 2A) and endothelium-removed (Fig. 2B) aortae showed an increase in NO_x species content following each NO-donor treatment compared to control. The control

represents the physiological NO store inside of the aortic wall, which was two times higher in endothelium-intact aorta compared to endothelium-removed aorta, confirming the role of endothelium in NO synthesis. The amount of NO_x species was greater for NACNO treatments in both types of aortae compared to other NO-donor treatments, and reached similar values whatever the presence or the absence of the endothelium. However, for GSNO, SNAP and SNP treatments, the amount of NO_x species tended to be higher in endothelium-intact compared to endothelium-removed aortae confirming the importance of the endothelium in their metabolism.

To assess whether the NO store formed following NO-donors incubation is available for vasorelaxation, aortae were submitted to the NAC protocol, which allows the release of NO from *S*-nitrosated peptides or proteins (Fig. 3). In endothelium-intact aortae, NAC-induced vasorelaxation was only shown for NACNO treatment with 13% of vasorelaxation from PHE-precontracted aortic rings (Fig. 3A). In endothelium-removed aortae, NAC-induced vasorelaxation was shown for GSNO, NACNO and SNAP treatments (Fig. 3B). The highest values of NAC-induced vasorelaxation were reached for NACNO treatment in both endothelium-intact and endothelium-removed aortae. The vasorelaxation induced by NAC following SNP treatment did not lead to significant vasorelaxation compared to the control (Fig. 3B). The results were also statistically analyzed for each treatment in endothelium-intact *versus* endothelium-removed aortae to highlight the impact of endothelium presence on NO bioavailability from the NO store. The endothelium increased the bioavailability of NO for vasorelaxation only for *S*-nitrosothiols (GSNO, NACNO and SNAP) treatments (Fig. 3). The functional availability of this mobilized NO store was challenged by comparing PHE-induced vasoconstriction curves with or without NO-donor pretreatments (Fig. 4). Concentration-dependent response curves to PHE were not modified by any NO-donor treatment compared to the control condition in endothelium-intact aortae (Fig. 4A). However,

in endothelium-removed aortae the curves were shifted to the right (one log unit) for GSNO, NACNO, SNAP and SNP treatments compared to control (Fig. 4B). The curves shifting to the right was materialized by a decrease of pEC₅₀ values, without any change in E_{max} values (Table 1). Moreover, the treatment of endothelium-removed aortae with GSNO, NACNO, SNAP and SNP decreased the pEC₅₀ values close to the pEC₅₀ values of endothelium-intact aortae. As expected, E_{max} values of PHE were higher in endothelium-removed compared to endothelium-intact aortae. The results were also statistically analyzed for each treatment in endothelium-intact *versus* endothelium-removed aortae to highlight the impact of endothelium presence on PHE-induced vasoconstriction. Endothelium removal increased pEC₅₀ as well as E_{max} values of PHE whatever the treatment (except E_{max} for SNP treatment, Table 1).

4. Discussion

RSNOs as a physiological storage and transport form of NO may be potential therapeutics to restore NO bioavailability. In this study, we evaluated whether (i) a RSNO treatment is able to produce NO store in vascular tissues - this was measured as the NO_x species content - and whether (ii) this NO store is available to achieve the physiological roles of NO. For the first time, the NO store produced by RSNO treatment was quantified in both endothelium-intact and endothelium-removed aortae. We used endothelium-removed aortae as a model of endothelial dysfunction. The three RSNO treatments studied here increased NO_x content, and thus NO store in the aortic wall. This NO store is available for NAC-induced vasodilation, as well as for the opposition to PHE-induced vasoconstriction. However, while endothelium-intact and endothelium-removed aortae seemed to accumulate NO store in the same quantities (a little bit higher in endothelium-intact aortae certainly due to the endogenous production of NO by the endothelium), the physiological responses to its mobilization did not follow the same profile.

This might be explained by a higher catabolism of RSNOs and SNP by enzymes present both at the endothelial and smooth muscle cell levels in endothelium-intact aortae. Gamma glutamyl transferase (GGT, responsible for GSNO catabolism) and redoxins like PDI and thioredoxin (Trx) are distributed on endothelial cells and smooth muscle cells [12,13,21] (Fig. 4). In previous studies, we showed that GGT activity is higher on endothelial cells (2.46 ± 0.20 nmol/min/mg proteins) than on smooth muscle cells (1.35 ± 0.20 nmol/min/mg of proteins) [12,13,21]. That could explain the small increase in NO_x species in endothelium-intact aortae. Concerning SNP, its transfer of NO has been shown to be catalyzed by thiols like glutathione [24]. As our system does not include GSH in the medium surrounding aortae, the transfer was assumed by thiols present at the cell membrane level (Fig. 5). However, this type of transfer seems to transfer different type of NO_x species or to be less efficient than those catalyzed by GGT and redoxins for GSNO, NACNO or SNAP (Fig. 5). Indeed, the NAC protocol applied to aortae pre-incubated with SNP did not induce any vasorelaxation. NAC enters inside the cells where it is deacetylated into cysteine (Fig. 6). Then, the cysteine residue, in the presence of NO storage, displaces NO from *S*-nitrosated proteins (Pr-SNO) by transnitrosation process to produce *S*-nitrosocysteine [16]. This instable *S*-nitrosocysteine immediately releases NO, which become available to stimulate the sGC/GMPc pathway and induce vasorelaxation. As NAC only reveals a NO store formed of Pr-SNO [16], we can conclude that the NO store formed by SNP pre-incubation is mainly formed of nitrite ions. So, even if the quantity of NO_x species formed by SNP inside aortae is not different from the one formed by GSNO, NACNO and SNAP, the nature of the store, either nitrite ions or Pr-SNO, is dominating for NO bioavailability for vasorelaxation. In addition, as the existence of NOS uncoupling (NOS itself can generate superoxide under certain conditions)[25], endothelium may negatively regulate the vasodilation induced by NO donors (especially for SNP). This effect may be decreased by the reductant effects of peptides released during the transportation

and degradation of RSNOs. The NACNO treatment produced a similar amount of NO store either in endothelium-intact and in endothelium-removed aortae. This phenomenon was probably due to the mechanism of NACNO incorporation into the cells using the L-AT present either on endothelial cells [26] or on smooth muscle cells [22] without any catabolism. In order to prove NO bioavailability following treatments, NAC was applied to displace NO from the NO store and induce vasorelaxation. The NO store produced by NACNO treatment was shown to be more available for vasorelaxation compared to GSNO, SNAP and SNP either in endothelium-intact and endothelium-removed aortae. However, the NAC-induced vasorelaxation was higher in endothelium-removed than in endothelium-intact aortae. This might be explained the formation of a bigger NO store in the endothelium due to the presence of more active enzymes than the ones present on smooth muscle cells. The NAC-induced release of NO from this endothelial store can either create a new store through protein S-nitrosation process or diffuse in smooth muscle cells before inducing the vasorelaxation (Fig. 6A). In the presence of endothelium, NAC direct access to smooth muscle cells is weaker (Stated in grey in Fig. 6A) so the direct release of NO from the NO store present in smooth muscle cells is less implicated in the vasorelaxation. In endothelium-removed aortae, the NO store is made directly in smooth muscle cells when upon release induced by NAC can produce directly the vasorelaxation (Fig. 6B). This kind of hypothesis was also stated by Sarr and coworkers showing that the vasorelaxation induced by the releasable NO store is unmasked by endothelial dysfunction[27].

Concerning the responses to alpha-1 adrenergic receptor agonists, it is well known that the vasoconstriction induced by PHE in endothelium-intact aortae is a balance between alpha-1 adrenergic receptors activation on either smooth muscle cells (vasoconstriction) and endothelial cells (leading to the activation of the NO/sGC signaling pathway and then attenuation of smooth muscle cells vasoconstriction) [28,29]. In the present study, as in others

[16–19], the vasoconstriction induced by agonists of alpha-1 adrenergic receptors was modified by RSNOs treatment only in endothelium-removed aortae. Furthermore, as pEC_{50} values of PHE are equivalent in control endothelium-intact aortae and endothelium-removed aortae treated with NO-donors, we may hypothesize that these treatments, through the storage of NO in smooth muscle cells, might mimic the role of the (lacking) endogenous NO production and endothelial function. However, as E_{max} was not modified, the vasoconstriction efficiency of PHE is retained even after NO-donor treatments. This phenomenon was already described – but only for GSNO - in a model of induced endothelial dysfunction [27].

In conclusion, our study proposes NO-donors as a potential treatment for endothelial dysfunction using endothelium-removed aortae in direct comparison with endothelium-intact aortae. These NO-donors were shown to induce the formation of a NO store, which was quantified and assessed for vasoactivity. The bioavailability of the NO store was challenged with NAC-induced vasorelaxation and PHE-induced vasoconstriction. The NO store formed by NO-donor, mainly by NACNO, was available for vasorelaxation and produced a hyporeactivity to PHE. However, these functional results (PHE and NAC protocols) were not in total agreement with the NO store formed by NO-donors pretreatments. Indeed, on one side, increased quantities of NO_x species in both endothelium-intact and removed aortae was highlighted and on the other side, higher functional mobilization and vasoactivity capacities were observed only in endothelium-removed aortae. NACNO treatment was more potent than other treatments regarding the quantity of NO stored in the aortic wall and the amplitude of the functional impact especially for NO store mobilization by NAC. We also showed that the bioavailability of the NO store for vasorelaxation is not only a matter of quantity, but is also dependent from the presence of the endothelium. And finally, we suggested that NO-donors,

mainly NACNO, could be regarded as promising candidate to compensate endothelium function lost along cardiovascular diseases.

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FIGURE CAPTIONS

Fig. 1. Descriptive scheme of the *N*-acetylcysteine (NAC) protocol applied on aortae and expected results if a NO store is formed (A) or if no NO store is formed (B) after pre-incubation with each treatment. PHE: Phenylephrine, ΔT : Variation of tension induced by NAC from PHE-precontracted aorta.

Fig. 2. Quantity of nitric oxide-derived (NO_x) species in endothelium-intact (A) or endothelium-removed (B) rat aortae after incubation or not (control) with 2 μ M *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetylcysteine (NACNO), *S*-nitroso-*N*-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP) for 30 min. Results are presented as mean \pm SD of n = 3-15 per group, from 3-15 different rats in each group and compared with a Kruskal-Wallis test; * p < 0.05 *versus* control (Dunn's post-test) and with Student's *t*-test; \$ p < 0.05 *versus* « endothelium-intact corresponding group ».

Fig. 3. Vasorelaxant effects of *N*-acetylcysteine (10⁻⁵ M) in endothelium-intact (A) and endothelium-removed (B) rat aortae after incubation or not (control) with 2 μ M *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetylcysteine (NACNO), *S*-nitroso-*N*-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP) for 30 min, followed by one hour of washing with Krebs' solution. Results are expressed as the percentage of 10⁻⁶ M phenylephrine precontraction, presented as mean \pm SD of n = 8-10 per group, from 4-8 different rats in each group and compared with one-way ANOVA; *p < 0.05 *versus* control, #p < 0.05 *versus* NACNO (Bonferroni's multiple comparisons test) and with Student's *t*-test; \$ p < 0.05 *versus* « endothelium-intact corresponding group ».

Fig. 4. Concentration-dependent response curves to phenylephrine of endothelium-intact (A) and endothelium-removed (B) rat aortae after incubation or not (control) with 2 μ M *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetylcysteine (NACNO), *S*-nitroso-*N*-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP) for 30 min. Results are presented as mean \pm SD of n = 6-17 per group, from 3-7 different rats in each group and analyzed using the Hill equation.

Fig. 5. Schematic representation of *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetylcysteine (NACNO), *S*-nitroso-*N*-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP) metabolism to produce a NO store of *S*-nitrosated proteins (Pr-SNO) in cells. Protein disulfide

isomerase (PDI), gamma-glutamyltransferase (GGT), L-type amino acid transporter (L-AT), *S*-nitrosocysteine (Cys-NO).

Fig. 6. Schematic representation of *N*-acetylcysteine (NAC)-induced vasorelaxation either in endothelium-intact (A) and in endothelium-removed aortae (B). NAC enters inside endothelial cells (A) or smooth muscle cells (B) using the L-type amino acid transporter (L-AT). Then, NAC is deacetylated in cysteine residues that, in the presence of a NO store (PrS-NO), forms Cys-NO by transnitrosation process. The unstable Cys-NO immediately releases NO that either diffuse to smooth muscle cells to activate the soluble guanylate cyclase (sGC) (A) or activate directly the sGC in the case of endothelium-removed aortae (B)

Figure 1

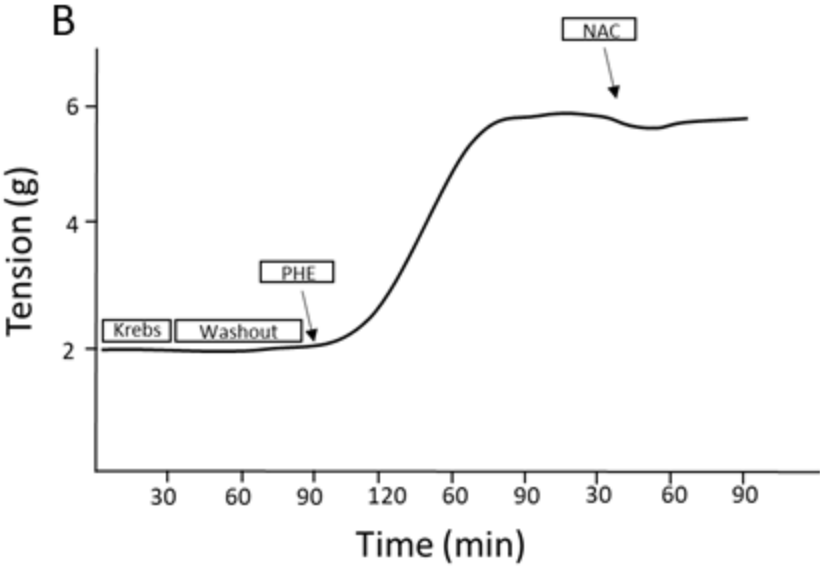
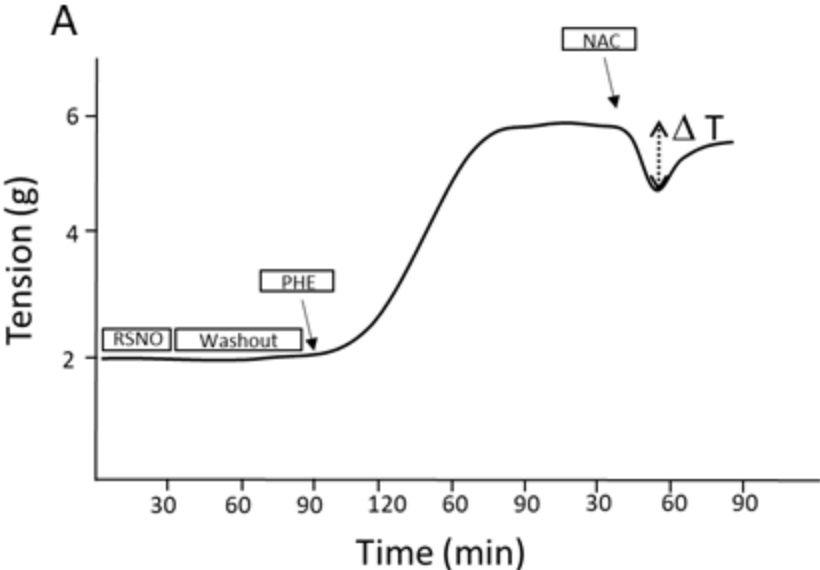


Figure 2

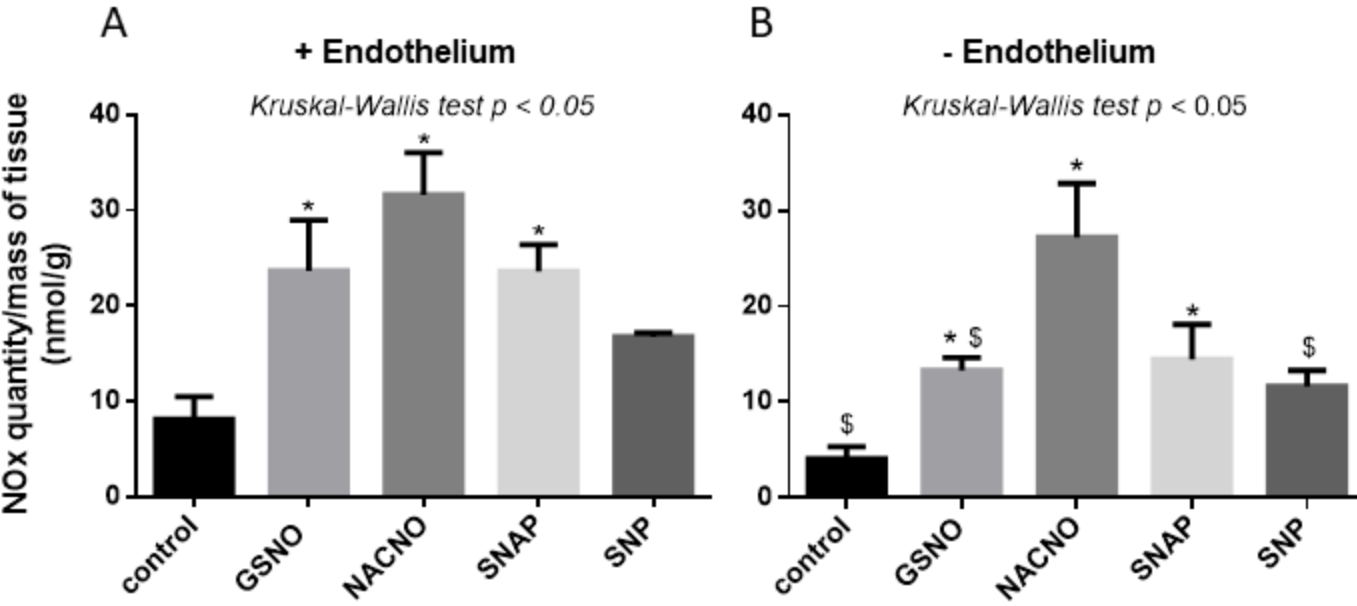


Figure 3

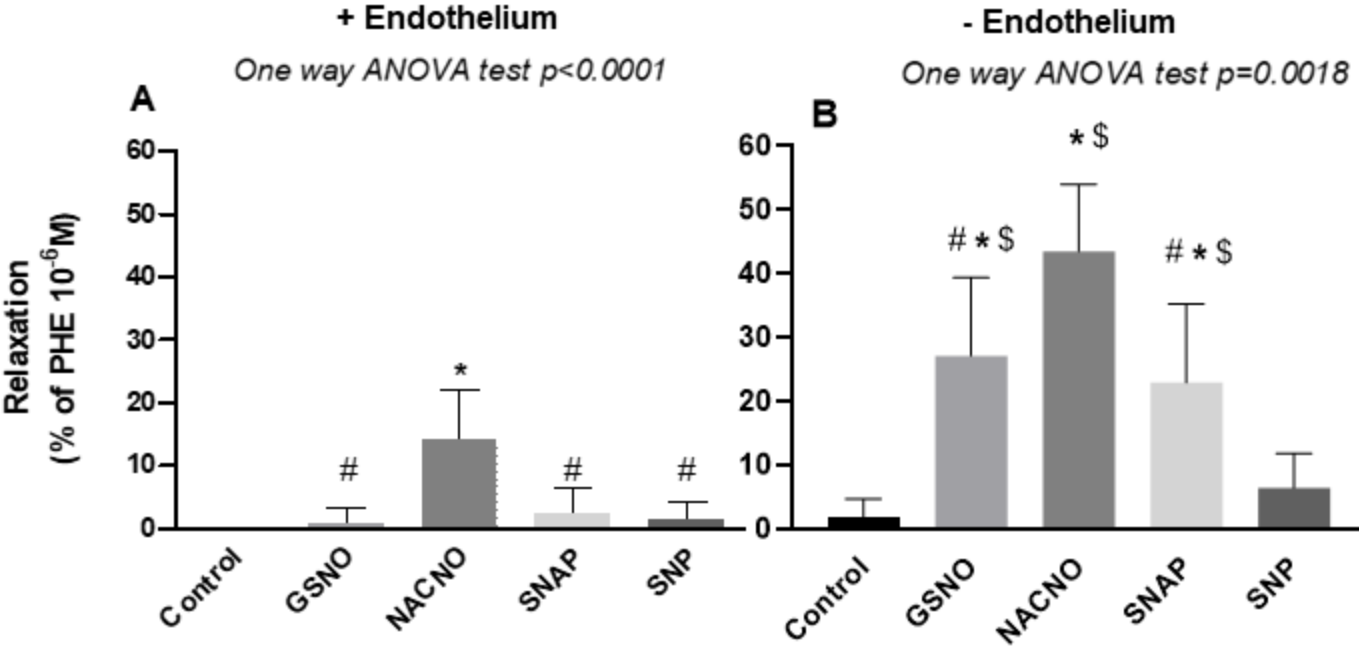


Figure 4

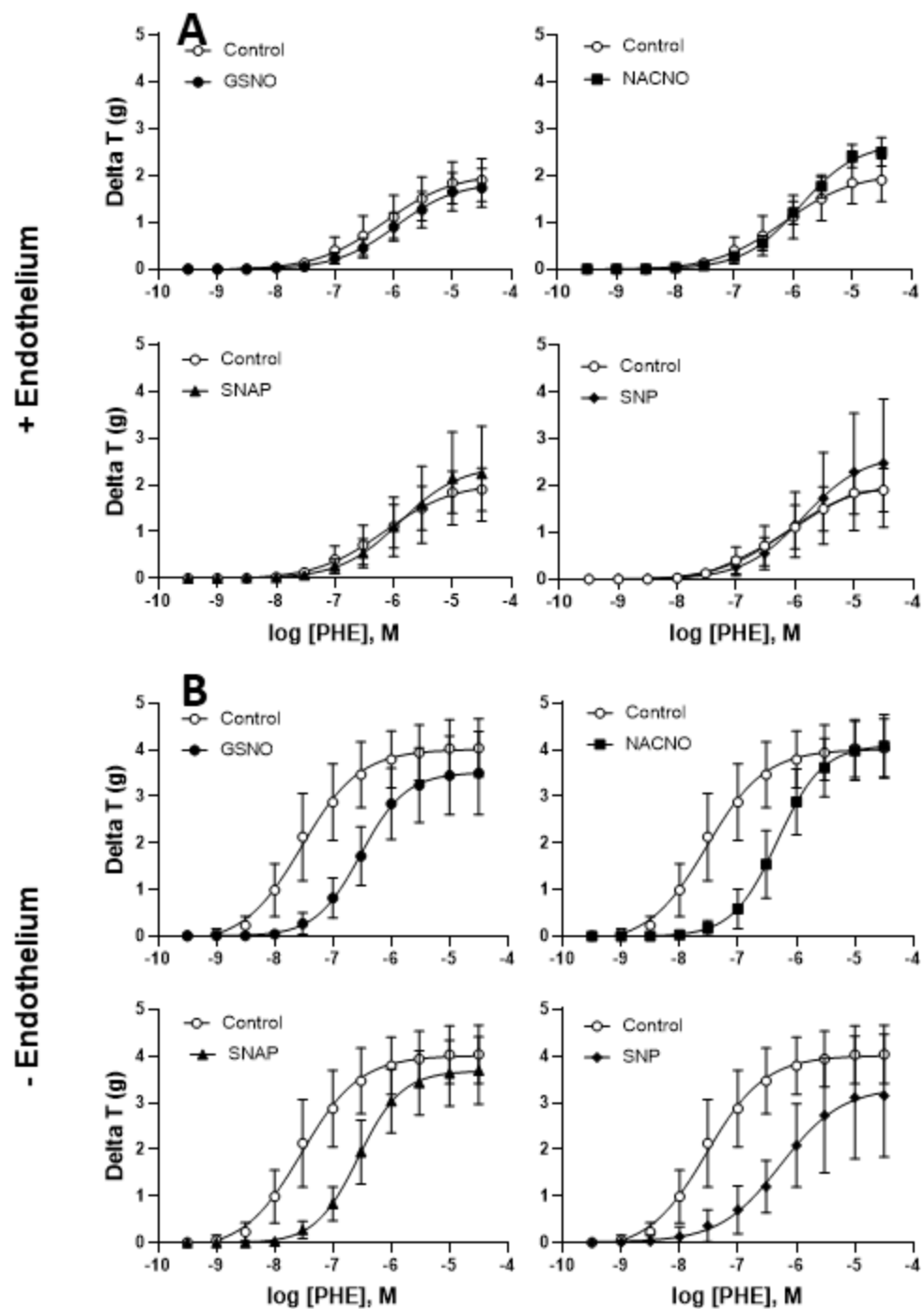


Figure 5

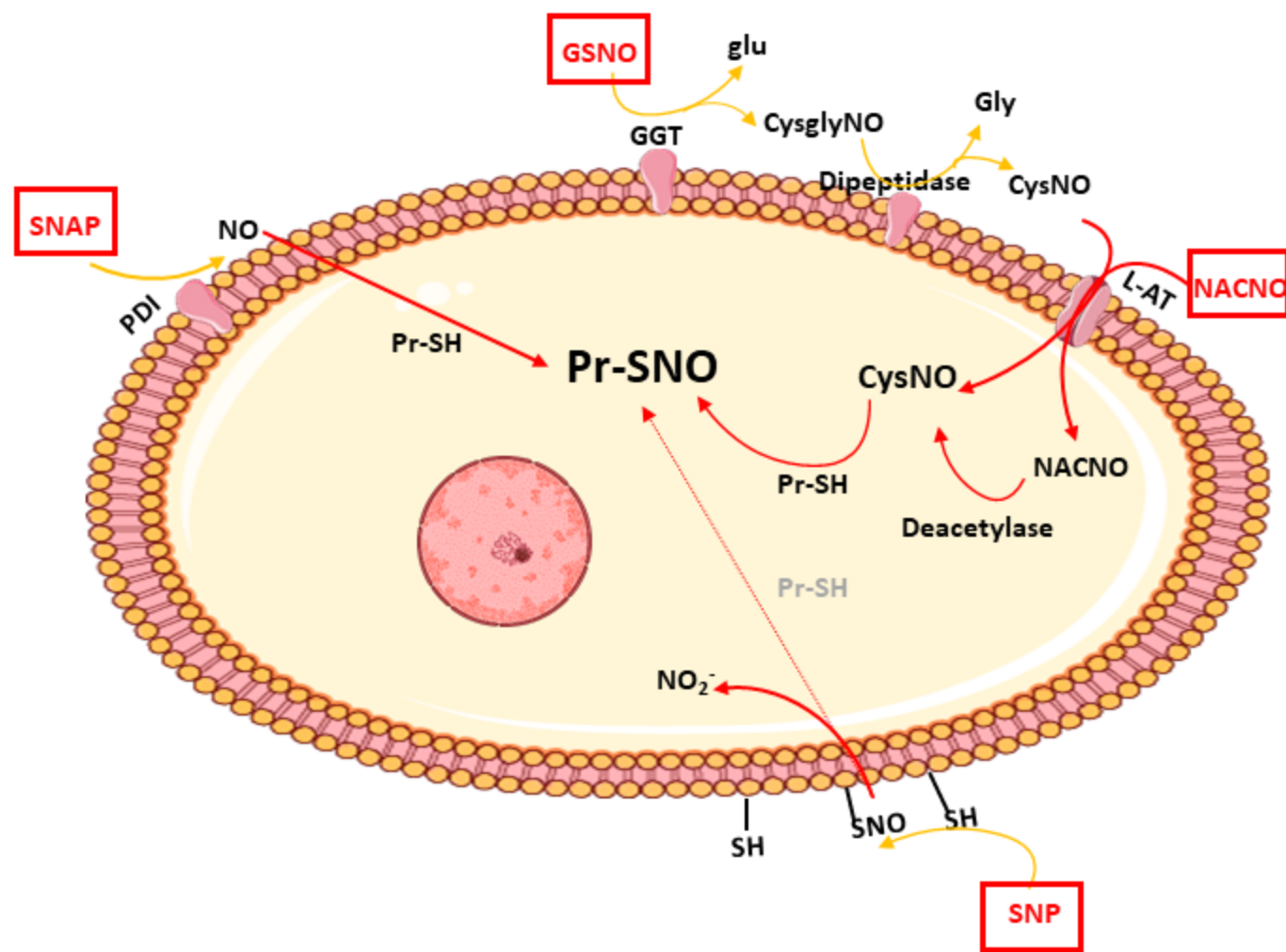


Figure 6

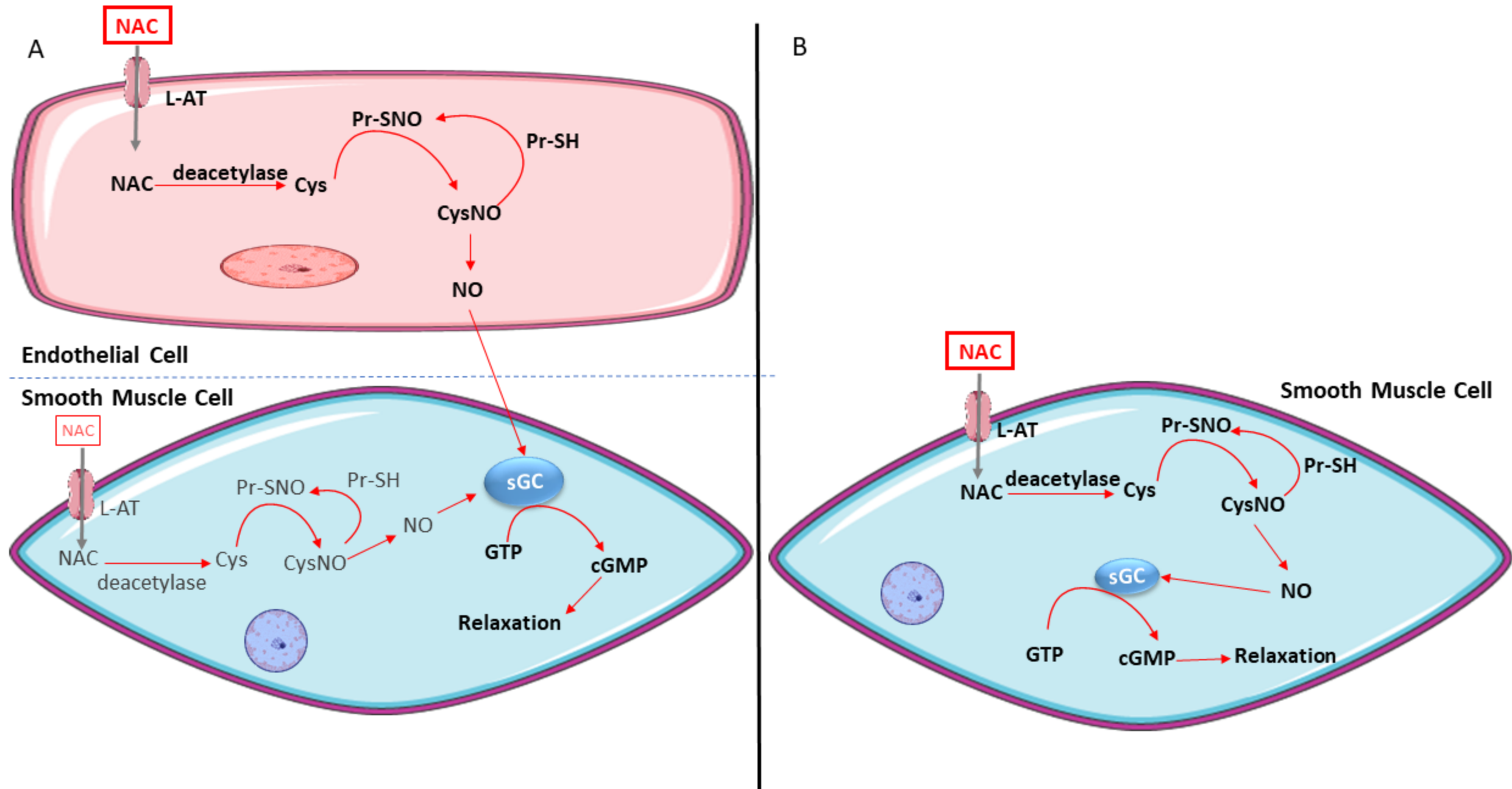


Table 1. Pharmacodynamic parameters calculated from phenylephrine concentration curves established on endothelium-intact and endothelium-removed aortae subjected or not to 2 μ M of *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetylcysteine (NACNO), *S*-nitroso-*N*-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP) treatment. Results are presented as mean \pm SD of n = 6-17 per group, from 3-7 different rats in each group and compared with one-way ANOVA; * p< 0.05 *versus* control (Bonferroni's multiple comparisons test), and with Student's *t*-test; \$ p<0.05 *versus* « endothelium-intact corresponding group ».

		Control	GSNO	NACNO	SNAP	SNP
Endothelium-intact	pEC₅₀	6.1 \pm 0.4	5.9 \pm 0.2	5.9 \pm 0.2	5.9 \pm 0.2	5.8 \pm 0.1
	E_{max} (g)	2.0 \pm 0.5	1.9 \pm 0.4	2.7 \pm 0.3	2 \pm 1	2 \pm 1
Endothelium-removed	pEC₅₀	7.5 \pm 0.4^{\$}	6.5 \pm 0.2*^{\$}	6.3 \pm 0.2*^{\$}	6.5 \pm 0.2*^{\$}	6.3 \pm 0.3*^{\$}
	E_{max} (g)	4.0 \pm 0.6^{\$}	3.5 \pm 0.7^{\$}	4.1 \pm 0.7^{\$}	3.7 \pm 0.7^{\$}	3 \pm 1

