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Aging and hypertension decrease endothelial NO-related dilating function and gamma-glutamyltransferase activity but not *S*-nitrosoglutathione-induced aortic vasodilation

Running title: GGT and GSNO in aged SHR

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ABSTRACT

S-nitrosoglutathione (GSNO), which is involved in the transport and the storage of NO, induces vasorelaxation. It requires gamma-glutamyl-transferase (GGT), an enzyme present on the endothelium, to transfer NO into the cell. We evaluated whether aging and hypertension, which induce NO-related dilating dysfunction, are associated with decreased vascular GGT activity and modify the vasorelaxant effect of GSNO.

Thoracic aortic rings isolated from male Spontaneous Hypertensive Rats (SHR) and Wistar Kyoto Rats (WKY) aged 20-22 (adult) or 57-60 weeks (mature) were preconstricted with phenylephrine, then submitted to concentration-vasorelaxant response curves (maximal response: E_{max}; pD_2) to GSNO and carbachol (the latter to measure NO-related dilating function). GGT activity was measured using chromogenic substrate.

Both aging and hypertension lowered E_{max} values for carbachol (E_{max} -8% in adult SHR, -42% in mature SHR *versus* age-matched WKY, p_{age} and $p_{hypertension} < 0.05$) demonstrating NO-related dilating dysfunction. Aortic GGT activity also decreased with aging and hypertension (-22% in adult and -75%, reaching 3 nmol/min/g of tissue, in mature SHR *versus* 12 in age-matched WKY and 23 in adult WKY,

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p_{age} and $p_{hypertension} < 0.05$). The pD_2 values of GSNO were similar in mature SHR and WKY but higher in adult SHR ($p_{interaction} < 0.05$).

Aging in hypertensive rats decreased NO-related vasorelaxant function and vascular GGT activity, but did not lower the vasorelaxant response to GSNO. This opens perspectives for GSNO-based therapeutics restoring nitric oxide bioavailability and vascular protection in a context of endothelial dysfunction.

Keywords: NO-dependent vasorelaxation, Spontaneous Hypertensive Rat, *S*-nitrosoglutathione, gamma-glutamyltransferase

INTRODUCTION

S-nitrosoglutathione (GSNO), the nitrosated form of glutathione, is an endogenous low molecular weight *S*-nitrosothiol involved in the storage and transport of NO. Several studies reported the vasorelaxant properties [1,2] and/or hypotensive effects of GSNO [3], as its protective effects against platelet aggregation [4]. GSNO is an interesting candidate for therapeutics as it mimics endogenous GSNO-related functions. It is currently investigated as NO-donor to restore the decreased NO homeostasis occurring in many cardiovascular diseases such as hypertension or atherosclerosis, the latter mainly concerning large conductance arteries [5]. In the present study, we used aortic rings as model for studying integrative functions of GSNO.

Uptake of NO from GSNO into the cells requires the action of membrane enzymes [6]. Gamma-glutamyl transferase (GGT) is one of the enzyme implicated in the release of NO from GSNO and its uptake into the cell [7, 8]. GGT specifically catalyzes endogenous as exogenous GSNO breakdown producing cysteinylglycine and NO in endothelial cells. There, either NO diffuses into smooth muscle cells to activate the soluble guanylyl cyclase/cyclic guanosine monophosphate pathway and induce vasorelaxation, or it reacts with endothelial glutathione or proteins cysteine residue to form *S*-nitrosothiols [1, 2, 9].

We have previously documented that endothelial GGT is critical for GSNO-dependent NO-delivery and vasorelaxation in aortic rings isolated from normotensive rats [10]. More recently, we showed that hypertension in adult rats is associated with a slight endothelial NO-dependent dysfunction and a moderate decrease in aortic GGT activity. However, at this age, this does not impair GSNO-induced vasodilation and aortic rings of hypertensive rats even show enhanced sensitivity to GSNO [11].

In the present study, we evaluated GSNO-induced vasorelaxation in older animals, the mature spontaneous hypertensive rat (SHR), which represents a suitable model for aging and hypertension associated with NO-related dilating dysfunction. Our hypothesis was that endothelial dysfunction

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may heavily impair GGT activity of the vessel wall, and thus, the bioactivity of exogenous treatment with GSNO would be modified. To this purpose we analyzed: *(i)* NO-related dilating function of SHR; *(ii)* the corresponding vascular GGT activities; *(iii)* the corresponding exogenous GSNO-inducible vasorelaxation.

MATERIAL AND METHODS

Chemicals

All reagents were of analytical grade and obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Ultrapure deionized water (18.2 MΩ.cm) was used to prepare solutions. Standard solutions of GSNO were prepared by nitrosation of glutathione after mixing glutathione with sodium nitrite (ratio 1:1) in acidic medium according to the method previously described [12].

The purity of GSNO was assessed by ultraviolet spectrophotometry using its molar absorbance at 334 nm (ε = 922 $M^{-1}.cm^{-1}$).

Rats and ethical statements

All experiments were performed in accordance with the European Parliament guidelines (2010/63/EU) for the use of experimental animals and the respect of the 3 Rs' requirements for Animal Welfare. The protocols and procedures were approved by the advisory regional ethical committee on animal experiments: Comité d'Ethique Lorrain en Matière d'Expérimentation Animale, CELMEA (project "NitroVivo", APAFIS#1614-2015090216575422 v2).

Male young adult normotensive Wistar-Kyoto rats (WKY) or SHR (11 weeks-old, 300-325 g) were purchased from Janvier Laboratories (Le Genest St Isle, France), kept under standard conditions (temperature: 21 ± 1°C, hygrometry 60 ± 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*, until 20-22 weeks of age (adult rats; mean body weight 436 ± 3 and 430 ± 12 g; mean systolic blood pressure, tail-cuff method, 141 ± 10 and 211 ± 23 mmHg in WKY and SHR, respectively) and until 57-60 weeks of age (mature rats; mean body weight 598 ± 9 and 411 ± 20 g; mean systolic blood pressure, tail-cuff method, 146 ± 7 and 207 ± 8 mmHg in WKY and SHR, respectively).

Rats were anesthetized with sodium pentobarbitone (60 $mg.kg^{-1}$, intraperitoneal injection, Sanofi Santé Nutrition Animale, Libourne, France) and the adequacy of anesthesia was checked by testing the loss of the corneal and pinch paw withdrawal reflexes. If a change in the reflexes occurred, a bolus of sodium pentobarbitone was immediately administered. After administration of heparin

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(1000 IU.kg^{-1} heparine Choay, penis vein), rats were sacrificed by exsanguination and segments (3 cm) of the descending thoracic aorta were removed. Vessels were cleaned from surrounding connective tissues, cut into 2-mm long rings (8 rings per rat) and immediately used for vasoactivity. Some samples of aortic rings were frozen in liquid nitrogen and kept at -80°C until biochemical studies were analyzed.

Vasorelaxation studies

Vasorelaxation was evaluated on endothelium-intact aortic rings [10]. Aortic vasoactivity was measured using an isometric tension recording system in 10 mL organ chambers (EMKABATH, Emka Technology, France). All manipulations and assays involving GSNO were performed under conditions of subdued light, in order to minimize light-induced degradation. The bath was filled with Krebs' solution containing 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.6 mM $CaCl_2$, 24 mM $NaHCO_3$, 5.5 mM glucose, adjusted to pH 7.4 (10 mL, 37°C) and continuously bubbled with 95% O_2 and 5% CO_2. Following 60-min equilibration with a basal resting tension determined at 2 g, rings were exposed 2 times to KCl (60 mM, 5 min). Aortic rings (n = 5 - 13 per group, from 3 - 8 different rats in each group) were then preconstricted with 10^{-6} M phenylephrine. At the plateau of contraction, concentration-relaxation response curves to increasing concentrations of GSNO (10^{-10} to 3.10^{-5} M) were performed. The role of GGT was assessed in adult rats by stimulating GGT activity with the exogenous γ-glutamyl acceptor glycylglycine (20 mM), as well as by inhibition with the serine-borate complex (SBC, 20 mM), a competitive reversible inhibitor [10].

NO-related dilating dysfunction was evaluated by measuring the ability of carbachol, a muscarinic acetylcholine receptors agonist, to relax preconstricted aortic rings. Decreases in maximal response (E_{max}) to carbachol (10^{-10} to 10^{-5} M response curves) witness NO-related dilating dysfunction [13, 14].

GGT activity in aorta

GGT activity was measured spectrophotometrically after hydrolysis of the synthetic GGT substrate *L*-γ-glutamyl-3-carboxy-4-nitroanilide as previously described [15]. Briefly, aortic rings were homogenized and incubated for 2 h at 37°C in Tris buffer (100 mM, pH 7.4) containing 1 mM *L*-γ-glutamyl-3-carboxy-4-nitroanilide, 20 mM glycylglycine and 10 mM $MgCl_2$. After centrifugation at 42,000 × *g* for 10 min at 4°C, supernatant absorbance was read at 405 nm to monitor the release of 5-amino-2-nitrobenzoate (ε = 9500 M^{-1}.cm^{-1}) from *L*-γ-glutamyl-3-carboxy-4-nitroanilide. Enzyme activities are expressed in nmol of 5-amino-2-nitrobenzoate per min per g of tissue.

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Data analysis and statistical tests

Relaxant responses to GSNO or carbachol were given as the percentage of 10^{-6} M phenylephrine precontraction and calculated as:

% of relaxation = [Tension (PHE 10^{-6} M, g) – Tension (GSNO or carbachol, g)] / Tension (PHE 10^{-6} M, g) – Tension (BASELINE, g)] x 100.

The half maximal effective concentration (EC_{50}) and maximal response (E_{max}) were calculated by fitting each individual concentration response curve using the Hill logistic equation (Graph Pad prism® software version 5.0):

$$\% \text{ relaxation} = E_{min} + ((E_{max} - E_{min}) / (1+10^{((\log EC_{50} - \text{concentration}) \times \text{Hill slope})})$$

where E_{min} and E_{max} = minimal and maximal response reached in each concentration-response curve.

The pD_2 was calculated as -log EC_{50}.

After modelling individual concentration response curve, means ± S.E.M. of E_{max} and pD_2 were analyzed by a 2-ways (age, hypertension) ANOVA followed by a post-hoc Bonferroni test. The null hypothesis was rejected at $p < 0.05$. A 2-ways (GGT modulators, hypertension) ANOVA was also performed to analyze GSNO pD_2 values in 20-22 weeks SHR and WKY rats.

All data are shown as means ± S.E.M

RESULTS

All the concentration response curves to carbachol fitted the Hill model and gave similar pD_2 values, around 7.1-7.4 in all groups (Figure 1). Both aging and hypertension lowered E_{max} values for carbachol (E_{max} -8% in adult SHR, -42% in mature SHR *versus* age-matched WKY, p_{age} and $p_{hypertension} < 0.05$) demonstrating NO-related dilating dysfunction.

Thoracic aortic GGT activity followed similar trends (p_{age} and $p_{hypertension} < 0.05$) with a -22% decrease in adult and -75% in mature SHR, *versus* age-matched WKY (Figure 2).

Concentration-response curves to GSNO reached similar E_{max} in all groups (Figure 3). The values for pD_2 were similar in mature SHR and WKY and higher in adult SHR ($p_{interaction} < 0.05$).

The influence of GGT modutators to GSNO-induced vasorelaxation in adult SHR was similar to that of age-matched WKY, with higher responses to GSNO in adult SHR in all circumstances (Figure 4, $p_{GGT\ modulators}$ and $p_{hypertension} < 0.05$). Indeed, inhibition of GGT activity with SBC was associated with a decrease in the vasorelaxant effect of GSNO (decrease in its pD_2 value) both in adult WKY and SHR group. On the opposite, the pD_2 value for GSNO increased after activation of the enzyme with glycylglycine.

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DISCUSSION

Our previous work on adult SHR, submitted (or not) to a high salt diet showed that hypertension is accompanied by a decreased activity of GGT, the main enzyme catalyzing the release of bioactive NO from GSNO [11]. Nevertheless, vasorelaxation induced by GSNO was slightly improved. In the present study, we evaluated GSNO-induced vasorelaxation in older animals, the mature SHR, with a marked NO-related dilating dysfunction, and showed strong decline in aortic wall GGT activity. Despite, bioactivity of an exogenous treatment with GSNO remained stable (while, again, it increased in adult SHR).

The degree of severity of endothelial dysfunction is commonly evaluated on the basis of a decrease in either pD_2 and/or E_{max} of the concentration-response curves to carbachol or acetylcholine (endothelial muscarinic receptor agonists). This mainly reflects NO-related dilating dysfunction. We and Isabelle et al. [16] showed strong NO-dependent dysfunction in mature SHR (E_{max} -60% in Isabelle et al. 2012; -42% present Figure 1). It has however to be noticed that global endothelial dysfunction in SHR involves not only decreased NO bioavailability but also decreased production of endothelium-hyperpolarizing factor and prostacyclin [17, 18], higher production of endothelium-dependent vasoconstrictive agents such as endothelin-1 [19, 20]. However, endothelial-dependent hyperpolarisation is a mechanism of relaxation more important in resistance vessels than in larger calibre vessels as aorta. Furthermore, there are structural changes (*e.g.*, vascular wall thickening, smooth muscle hypertrophy, vascular inflammation inducing alteration of the extracellular matrix, vascular infiltration and activation of immune cells leading to vascular remodeling), especially with aging, that can additionally impede the endothelium's ability to relax vessels [19, 21].

Several studies showed that serum GGT levels was increased in cardiovascular diseases [22]. Besides the soluble enzyme found in serum, GGT is also present at cellular level in vascular tissues and is highly expressed in arterial endothelium [23]. We have previously shown that such GGT activity is involved in the utilization of GSNO, in that it promotes the local release of NO from GSNO thus mediating its vasorelaxant effect [10]. Thus, damages of the endothelial cells occurring during aging in SHR affect endothelial GGT enzymatic function. Contrary to our hypothesis, this decline in aortic GGT activity did not induce any decrease in the vasorelaxant effect of GSNO. In adult SHR, GSNO-induced vasodilation still depends on GGT, as shown by our experiments performed in the presence of either an activator or an inhibitor of the enzyme. At this age, GSNO is even more potent in SHR than in WKY rats (present higher pD_2 values, and [11]). In the latter paper, we suggested that (i) even if it decreases, the GGT activity remains sufficient to maintain GSNO vasorelaxant effect and/or (ii) other pathways involved in the denitrosation process of GSNO are substituting for GGT. Membrane

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PDI, a membrane enzyme from the redoxins family, which is involved in the release of NO from GSNO and in GSNO vasorelaxant effect [9], increased its expression under oxidative stress [24, 25]. Moreover, hypertension and ageing present oxidative stress, *e.g.* through upregulation of NOX [11, 26, 27, 28]. Moreover, we previously showed [11] that inhibition of PDI with bacitracin did decrease the pD_2 values of GSNO in adult SHR. Therefore, the decreased GGT activity occurring during hypertension and/or endothelial dysfunction may be balanced by an oxidative stress-related increase in PDI expression and/or activity, maintaining the vasorelaxant effect of GSNO in mature SHR, and even slightly improving it in adult SHR.

CONCLUSION

In conclusion, NO-related dilating dysfunction in mature SHR is accompanied by a 75% decline of GGT aortic wall bioactivity, one of the main enzymes catalyzing the release of bioactive NO from GSNO. Nevertheless, vasorelaxation induced by GSNO is unaffected. As many cardiovascular diseases are associated with a decreased bioavailability of NO, leading to impaired vasodilation, pro-inflammatory/oxidative, pro-proliferative and pro-thrombotic status [29, 30], our results open new perspectives to further development of GSNO-based therapeutics for restoring nitric oxide bioavailability and vascular protection in a context of endothelial dysfunction. Atherosclerosis, for example, which mainly concerns large conductance arteries where vasoactive functions depend specifically on the bioavailability of nitric oxide NO [5, 31] leads to the use of several NO donors (organic nitrates) for therapeutics. However, these treatments are known to provide fast NO release concomitant with induction of oxidative stress and tolerance [32, 33] and new NO-donors, such as *S*-nitrosothiols are interesting therapeutic alternatives as they do not present the drawbacks of organic nitrates.

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FIGURE LEGENDS

Figure 1: Influence of hypertension and ageing on the vasorelaxant responses to carbachol

Cumulative concentration-vasorelaxation response curves to carbachol of pre-constricted endothelium-intact aortic rings isolated from adult (A) and mature (B) SHR and WKY rats (pD_2, E_{max} and p values from the 2-ways ANOVA and post-hoc Bonferroni test. n number of aortic rings per group. $^{\#}p<0.05$ versus adult WKY, *$p<0.05$ versus adult SHR, $^{\$}p<0.05$ versus mature SHR

Figure 2: Influence of hypertension and ageing on aortic GGT activity

GGT activity measured in endothelium-intact thoracic aorta in adult and mature SHR and WKY rats $p_{age} = 0.0005$; $p_{hypertension} = 0.0284$, $p_{interaction} = 0.5225$; p values from the 2-ways ANOVA; n number of aortic rings per group

Figure 3: Influence of hypertension and ageing on the vasorelaxant responses to GSNO

Cumulative concentration-vasorelaxation response curves to GSNO of pre-constricted endothelium-intact aortic rings isolated from adult (A) and mature (B) SHR and WKY rats (pD_2, E_{max} and p values from the 2-ways ANOVA and post-hoc Bonferroni test, *$p<0.05$ versus adult SHR).

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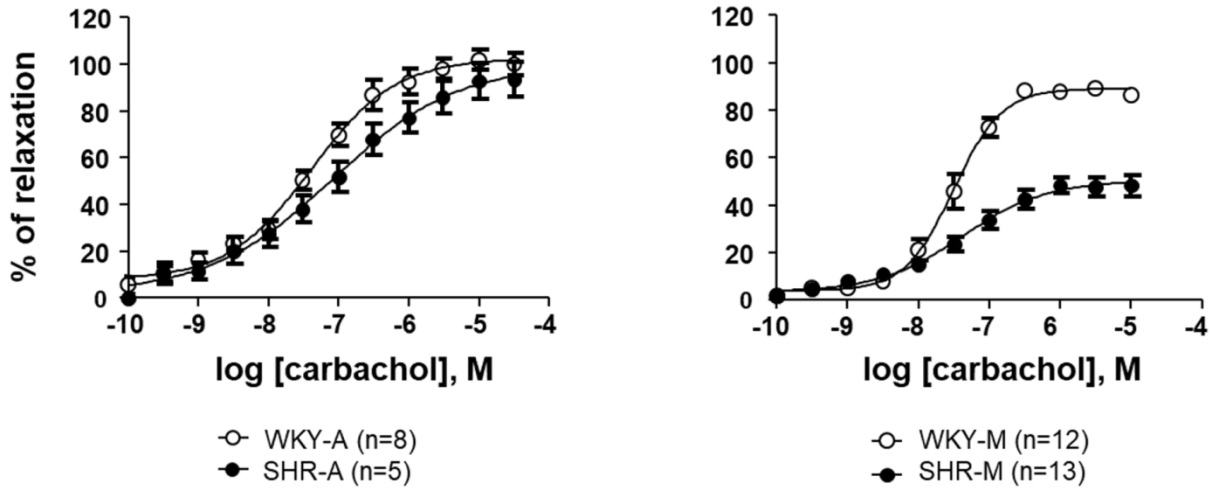
Figure 4: Impact of GGT activator and inhibitor on the vasorelaxant response to GSNO

pD_2 obtained from cumulative concentration response curves with GSNO in the presence or not of glycylglycine (20 mM) or serine-borate complex (SBC, 20 mM) in pre-constricted endothelium-intact aortic rings isolated from adult SHR and WKY rats. $p_{GGT\ modulator}$ <0.0001; $p_{hypertension}$ <0.0001, $p_{interaction}$ = 0.7172; p values from the 2-ways ANOVA; n number of aortic rings per group



	Adult WKY (n = 8)	Adult SHR (n = 5)	Mature WKY (n = 12)	Mature SHR (n = 13)	p_{age}	p_{HTA}	$p_{interaction}$
pD_2	7.3 ± 0.2	7.1 ± 0.5	7.4 ± 0.1	7.4 ± 0.1	0.0523	0.1274	0.6799
E_{max} (%)	106 ± 3	98 ± 10	90 ± 2	52 ± 4 *, #, $	<0.0001	0.0032	0.031

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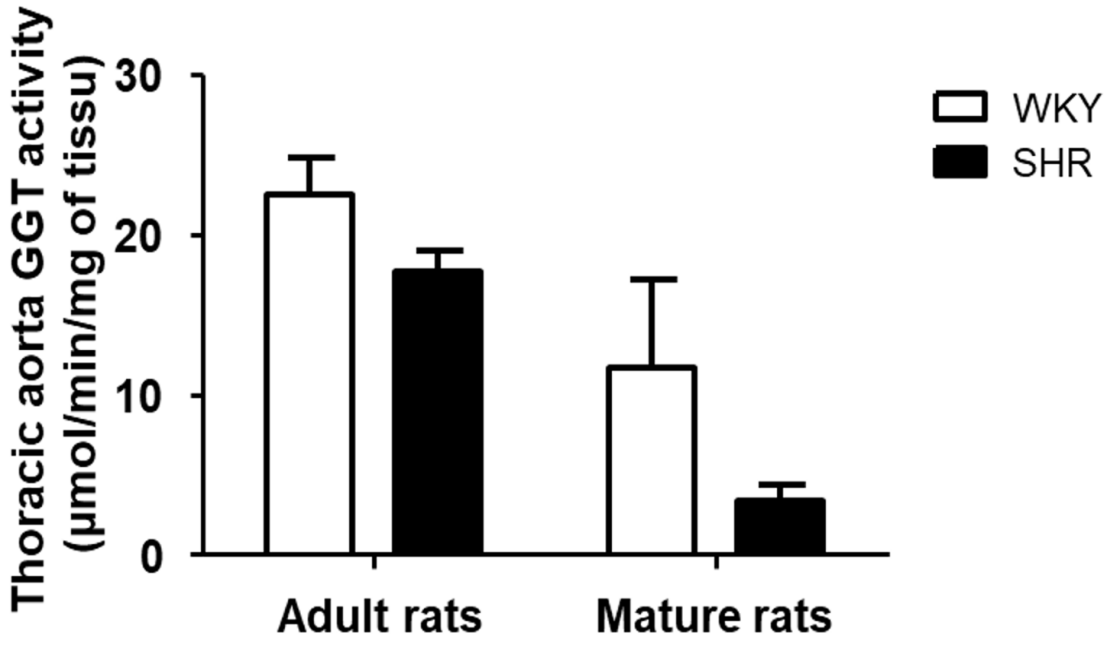

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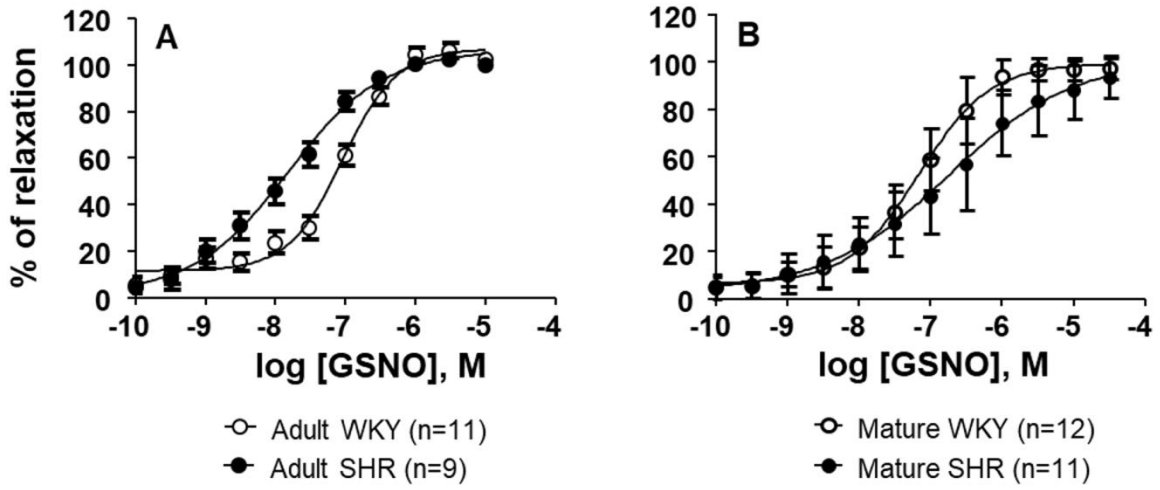
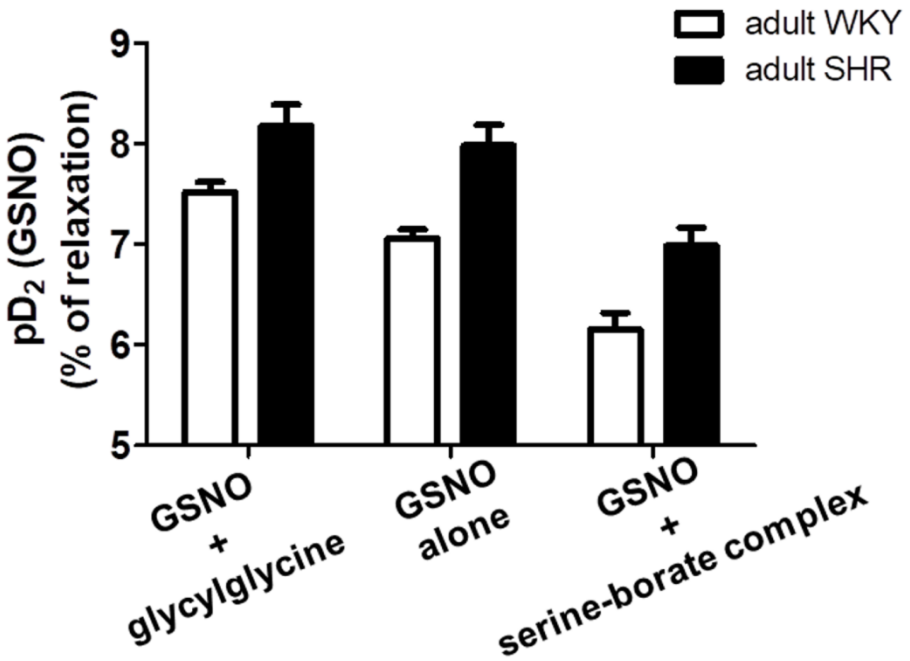


	Adult WKY (n = 11)	Adult SHR (n = 9)	Mature WKY (n = 12)	Mature SHR (n = 11)	p_{age}	p_{HTA}	$p_{interaction}$
pD_2	7.1 ± 0.3*	8.0 ± 0.2	7.1 ± 0.1*	6.8 ± 0.2*	0.0011	0.0664	0.0001
E_{max} (%)	104 ± 2	104 ± 2	100 ± 1	99 ± 3	0.0623	0.7627	0.9676



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