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SPHINGOSINE-1-PHOSPHATE INDUCES ENDOTHELIAL NOS ACTIVATION THROUGH PHOSPHORYLATION IN HUMAN CORPUS CAVERNOSUM

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Running title: Sphingosine -1- Phosphate and human corpus cavernosum

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ABBREVIATIONS: ED, erectile dysfunction, S1P, sphingosine-1-phosphate, GPCRs, G protein-coupled receptors, eNOS, endothelial nitric oxide synthase, HCC, human corpus cavernosum, NO, nitric oxide, EDG, endothelial differentiation gene, PI3k, phosphatidyl-inositol 3-kinase, Akt, serin/threonine kinase or protein kinase B, nNOS, neuronal nitric oxide synthase.

ABSTRACT

Sphingosine-1-phosphate (S1P) is the natural ligand for a specific G protein-coupled receptors. In endothelial cells, S1P has been shown to modulate the activity of the eNOS, through phosphorylation operated by Akt. Nitric oxide (NO) produced by nNOS and eNOS plays a central role in triggering and maintaining penile erection. This study has assessed the possibility of a similar cross talk between eNOS and S1P in human corpus cavernosum and if this interaction is connected to penile vascular response. Quantitative RT- PCR demonstrated the presence of $S1P_1$, $S1P_2$, $S1P_3$ receptors in both the HCC and the penile artery. S1P, on its own, did not relax or contract HCC strips but on the other hand, incubation with S1P (0.1μM) caused a 6 fold increase in relaxation induced by a subliminal dose of acetylcholine. This effect is dependent upon eNOS activation through an Akt-dependent phosphorylation, as demonstrated by pharmacological modulation with L-NAME and wortmannin and by western blot studies. In human tissue S1P appears to be the possible candidate for the activation of the eNOS calcium-independent pathway. This pathway may represent a new therapeutic area of intervention in ED in order to develop a way to selectively promote NO production at endothelial level. This approach could also be used to enhance PDE5 therapy in patients with ED that are poor responders such as in the case of diabetes.

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INTRODUCTION

Penile erection is one component of a complex series of integrated physiological processes and biochemical events coordinated at the level of the peripheral and central nervous system (Moreland et al., 2001). Erectile dysfunction (ED) can be thought of as a result of a number of haemodynamic events, where changes in the blood flow to the erectile tissue is the most common cause (Broderick, 1998). Basically, upon sexual stimulation, penile erection is significantly dependent on adequate blood flow to the erectile tissue. This requires coordinated arterial endothelium-dependent vasodilation and sinusoidal endothelium-dependent corporal smooth muscle relaxation. Nitric oxide (NO) is the principal peripheral pro-erectile neurotransmitter which is released by both non-adrenergic non-cholinergic neurons and the sinusoidal endothelium to relax corporal smooth muscle through the cGMP pathway. This leads to corporal smooth muscle relaxation. NO synthase (NOS) catalyses production of NO from L-arginine. Both isoforms, namely neuronal NOS (nNOS) and endothelial NOS (eNOS), expressed constitutively, mediate penile erection. The binding of a Ca^{+2} / calmodulin complex is essential for the activation of the constitutive enzymes. Studies have shown that eNOS can be activated in a calcium independent manner. The calcium independent activation relies on the phosphorylation of the enzyme at serine 1177 operated by Akt (protein kinase B). It is known that blood-flow-induced fluid shear stress in the penile vasculature, stimulates phosphatidyl-inositl-3-kinase (PI3k) to phosphorylate protein kinase B which in turn, phosphorylates eNOS to generate NO. The importance of this pathway has also been demonstrated by in vivo studies in rats. Electrical stimulation of the cavernous nerve or intracavernosum injection of a vasorelaxant agent has been shown to cause Akt phosphorylation and eNOS activation giving an erectile response, that had been abolished by Akt inhibitors pre-treatment (Burt et al., 2002). Thus, nNOS may initiate cavernosal tissue relaxation, while the shifting eNOS to a higher level of activation (Fulton et al., 2001) contributes to the attainment and the maintenance of full erection (Burnett, 2004). Recently much attention has been focused on membrane phospholipids, in particular sphingosine-1-phosphate (S1P). S1P is a bioactive lipid

produced by the metabolism of sphingomyelin (Watterson et al., 2003; Pyne and Pyne, 2000). S1P exhibits its action by binding to a family of G-protein-coupled receptors (GPCR) named S1P (1-5), originally referred to as the endothelial differentiation gene (EDG) family. To date, five members, EDG-1/$S1P_1$, EDG-5/$S1P_2$, EDG-3/$S1P_3$, EDG-6/$S1P_4$, and EDG-8/$S1P_5$ have been identified (Anliker and Chun, 2004). These receptors are coupled to different intracellular second messenger systems, including adenylate cyclase, phospholipase C, phosphatidylinositol 3-kinase/protein kinase Akt, mitogen-activated protein kinases, as well as Rho- and Ras-dependent pathways (Hla, 2003; Waeber et al., 2004). S1P potently and robustly activates eNOS in cultured vascular endothelial cells in a pathway that involves G-protein dependent activation of Akt (Igarashi et al., 2001). Since the penile tissue vasculature is a perfect example of microcirculation here we have evaluated if in human corpus cavernosum, exists a similar cross talk between eNOS and S1P. Our study shows that i) human corpus cavernosum possesses S1P receptors ii) S1P strongly potentiates acetylcholine-induced relaxation of human corpus cavernosum strips iii) this effect is related to an increased phosphorylation of eNOS as demonstrated by pharmacological modulation studies.

METHODS

Human tissue

A male –to-female transsexual surgical procedure was performed by amputating the penis and testicles and creating a neovagina to simulate female external genitalia. Patients were prescribed appropriate pre-surgery hormone treatment, with antiandrogens and estrogens, so as to facilitate a female appearance. The therapy was discontinued two months prior to surgery. The corpora cavernosa was carefully cut out of the amputated penis and placed in ice-cold oxygenated Krebs solution and washed thoroughly with heparinized Krebs solution. After the lavage, the corpora cavernosa was placed in an ice-cold Krebs solution and kept on ice until experimentation (Mirone et al., 2000). All patients were informed of procedures and signed their written consent. The protocol was approved by the Ethics Committee of the Medical School of the University of Naples.

HCC Strips.

Longitudinal strips (2cm) of trabecular tissue were dissected from the penis and immediately placed in Krebs solution and kept at 4°C until use (Mirone et al.,2000). Krebs solution, composed of the following: (mM) 115.3 NaCl, 4.9 KCl, 1.46 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 11.1 glucose (Carlo Erba, Italy). HCC strips were mounted in a 2 ml organ bath containing oxygenated (95% O_2 and 5% CO_2) Krebs solution at 37°C. HCC strips were connected to isometric force transducers (Model 7002, Ugo Basile, Comerio, Italy) and changes in tension were recorded continuously by using a polygraph linearcorder (WR3310,Graphtec, Yokohama, Japan). Tissues were preloaded with 2g of tension and allowed to equilibrate for 90 min in Krebs solution that was replaced at 15 min intervals. After equilibration, tissues were standardized by using repeated phenylephrine (PE; 3 μM; Sigma, Italy), eliciting contractile responses until three equal responses were obtained. After standardization, endothelial integrity was assessed by testing the responses to

acetylcholine (ACh; 0.01μM-10μM; Sigma, Italy). Strips without endothelium were obtained by incubating with distilled water for 15s. A curve concentration-effect, induced by S1P (0.01μM-10μM; Tebu-bio, Italy) was performed in presence or absence of endothelium using HCC strips precontracted with PE (3μM). HCC strips were challenged with S1P (0. 01μM-10μM) directly on basal tone to assess any contractile response. A curve-concentration effect was performed to evaluate the concentration of Ach needed to reach a 15% relaxation result. Once the subliminal dose was determined, the HCC strips were washed twice and contracted with PE (3μM) and left to incubate in the organ bath with various doses of S1P (0.1 μM-3μM) for 10 min. They were then challenged with previously determined Ach doses (ranging between 10 – 300nM depending on sensitivity of the human strips). The dose of S1P that gave the best potentiating effect was 0.1 μM and this dose was used throughout the study. In another set of experiments, HCC strips were incubated with L-NAME (100μM, Sigma, Italy) or with Wortmannin (WT, 2μM; Tocris, UK). The inhibitors were added to the organ bath 30 minutes prior to S1P (0.1μM). The optimal incubation time was previously determined in this study's experimental conditions (data not shown). A curve concentration-effect of Ach (0.01μM-10μM) was performed on HCC strips in the presence of WT (2μM). In order to demonstrate the specificity of S1P on Ach-induced relaxation, we used alprostadil (PGE_1, 100ng/ml; Caverjet, Pharmacia Italia S.p.A., Milan, Italy) or sodium nitroprusside (SNP, Sigma, Italy) to elicit the relaxant response.

Quantitative RT-PCR analysis

Presence of S1PRs was previously screened by PCR and we assessed that only $S1P_1$, $S1P_2$ and $S1P_3$ were present (data not shown). Total mRNA from human corpus spongiosum or penile artery was extracted using TRIZOL reagent (Gibco, Invitrogen, Milan, Italy), according to manufacturer's recommendations. Reverse transcription was performed and 100 ng of the above RNA samples were used for quantitative PCR. Samples were run in triplicate in 50μl reactions using an ABI PRISM 5700 Sequence Detector System (Applied Biosystems, Foster City,

California). Samples were incubated at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles at 95°C for 15s and 60°C for 1 min. SYBR Green oligonucleotides to detect human $S1P_1$, $S1P_2$, $S1P_3$ receptors were specifically designed using Primer Express Software (Applied Biosystem, Foster City, California, USA) and validated for their specificity. Oligonucleotides used were H-$S1P_1$-F, 5'-gggccaccacctacaagct-3', H-$S1P_1$-R, 5'-gctgacagggccacaaacat-3', H-$S1P_2$-F, 5'-gcgccattgtggtggaaaa-3', H-$S1P_2$-R, 5'-cattgccgagtggaacttgct-3', H-$S1P_3$-F, 5'-ggtgattgtggtgagcgtgtt-3', H-$S1P_3$-R, 5'-aggccacatcaatgaggaaga-3'. Relative quantification of target cDNA was determined by arbitrarily setting the control value to 100 and changes in cDNA content of a sample were expressed , thereafter, as a multiple . Differences in cDNA input were corrected by normalizing signals obtained with primers specific for GAPDH. To exclude non-specific amplification and/or the formation of primer dimers, control reactions were performed in the absence of target cDNA. All the experiments were run in triplicate.

Western blot analysis

HCC strips were placed in 24-well plates containing 1 ml of Krebs solution at 37°C and incubated for 60 min. Following the stabilization of HCC strips incubated in the wells, Ach (3μM) or S1P (0.1μM) were added. S1P was also incubated with wortmannin (2μM), which was added 30 min prior to the addition of S1P (0.1μM), and left to incubate for an additional 10min. At the end of each specific treatment period, HCC strips were quickly frozen in liquid nitrogen and then used for western blot studies. HCC strips were homogenized in lysis buffer (ß-glycerophosphate, 0.5 mM, sodium orthovanadate, 0.1 mM, $MgCl_2$, 2 mM, EGTA, 1 mM, DTT, 1 mM) and protease inhibitors by using an Ultra-Turrax homogenizer (Ika-labortech-nik, Staufen, Germany). Protein concentrations were determined by using the Bradford assay (Bio-Rad, Milan, Italy). Protein samples (50 μg) were subjected to electrophoresis on an SDS 10% polyacrylamide gel and electrophoretically transferred onto a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Germany). The immunoblots were developed with 1:500 dilutions of the indicated

antibodies, and the signal was detected with the enhanced chemiluminescence system according to the manufacturer's instructions (Amersham Biosciences, UK). Anti-eNOS and anti-peNOS-1177 were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA), and Alpha Diagnostic International Inc (San Antonio, USA). All salts used for Western blot analysis were purchased from ICN Biochemical (Eschwege, Germany).

Calculation and Statistical Analysis

Data are expressed as mean±S.E.M. from nine separate human specimens and at least nine strips were used for each experiment. The responses are expressed as relaxation percent of phenylephrine contraction (3μM). For multiple comparisons, results were analyzed by ANOVA, followed by the Bonferroni's post test. For comparison between two values, the unpaired Student's t test was used. A value of $P<0.05$ was considered significant.

RESULTS

PCR analysis

Quantitative RT-PCR study showed that spongiosum tissue (figure 1a) and the penile artery (figure 1b) possess mRNA S1P receptors. In both tissue, $S1P_3$ mRNA was significantly ($P<0.001$) higher than $S1P_2$ and $S1P_1$ respectively. $S1P_3$ was about 2000 fold more abundant than $S1P_1$ and 4 fold more abundant than $S1P_2$ in spongiousum tissue (Fig. 1a). In penile artery, $S1P_3$ was 300 and 15 fold more abundant than $S1P_1$ and $S1P_2$, respectively (Fig. 1b).

In vitro study

S1P (0.01-10µM) had no effect on pre-contracted HCC strips with or without endothelium (Fig. 2). Similarly, S1P (0.01-10µM) did not contract HCC strips by itself (data not shown). The endothelial removal by distilled water did notmodify the phenylephrine induced contraction in HCC strips. Indeed, the contraction elicited by PE (3µM) before treatment, was 80±7.1 dyne/mg tissue, while after treatment 85±6.2 dyne/mg tissue (n=10; NS). Tests were performed to assess, if S1P could enhance submaximal Ach effect. The response to the concentration of Ach that gave 15% of tissue relaxation was strongly enhanced by incubating the strips with S1P (0.1µM) for 10 min (Fig. 3a) but not with the vehicle. During this experiment, S1P caused a powerful significant increase in Ach-induced relaxation (Fig. 3a, $P<0.01$) that corresponded to a 6 fold increase in relaxation percent ($P<0.005$). In order to assess the specificity of the action of S1P against Ach, we performed experiments with PGE_1 or SNP. Alprostadil (PGE_1, 100ng/ml)-induced relaxation was not modified by either S1P (Fig. 3c) or its vehicle (Fig. 3b). The same effect was observed when SNP was used (data not shown).

Pharmacological modulation

Two different agents capable of interfering in eNOS activity e.g. L-NAME, as false substrate or wortmannin, as inhibitor of eNOS phosphorylation were used to operate a pharmacological modulation of eNOS. Incubation of HCC strips with L-NAME reverted the response (Fig. 4a, $P<0.001$) indicating that the potentiating effect observed is mainly driven through eNOS activation. In order to evaluate if the effect observed was linked to an increased eNOS phosphorylation we incubated the HCC strip with wortmannin. Wortmannin, similarly to L-NAME, abolished the S1P-induced Ach potentiation (Fig.4a, $P<0.001$). Wortmannin by itself caused a significant reduction of Ach-induced relaxation ($P<0.0001$, Fig. 4b). Next, in order to confirm that the observed effect was due to a modulation of eNOS phosphorylation, we performed a western blot study. Incubation of HCC strips with S1P caused a significant increase in eNOS phosphorylation (p-eNOS) that was significantly higher than that caused by the vehicle (Fig. 6, $P<0.01$). Incubation with higher doses of S1P (1μM) did not cause further increase in eNOS phosphrylation (data not shown). Incubation of HCC strips with wortmannin prevented this effect (Fig. 6) confirming that S1P selectively activates eNOS phosphorylation. Interestingly in our experimental conditions Ach (3μM) by itself induced an increase in eNOS phosphorylation that was significantly lower than S1P.

DISCUSSION

A substantial body of evidence implicates NO in normal erection function. NO from nerves, and possibly endothelia, plays a crucial role in initiating and maintaining intracavernous pressure increase, necessary for penile erection. This haemodynamic event is dependent upon cyclic GMP synthesized by soluble guanylyl cyclase via NO in smooth muscle cells. The constitutive NOS isoforms, neuronal (nNOS) and endothelial (eNOS), are regulated by cofactors such as calcium, calcium binding protein calmodulin, oxygen and reduced NADPH. However, eNOS can be shifted by phosphorylation to a higher level of activation through a calcium independent pathway. This pathway of activation involves phosphatidyl-inositol 3-kinase (PI3k) that activates the serine/threonine protein kinase (Akt), causing eNOS phosphorylation (Dimmler et al., 1999; Michell et al., 1999; Morales-Ruiz et al., 2001). In order to evaluate if a similar regulation exists in human tissue we used corpus cavernosum obtained from patients that received an appropriate pre-surgery hormone treatment, with antiandrogens and estrogens that was suspended two months before the surgery. A preliminary qualitative PCR study demonstrate the presence in human corpus cavernosum and penile artery of mRNA for $S1P_1$, $S1P_2$ and $S1P_3$ receptors. Data from the quantitative RT-PCR indicates that the most abundant receptor is $S1P_3$, both in corpus spongiosum and in the penile artery. S1P induces a dose-dependent vasorelaxation of isolated, pressurized mesenteric arterioles in wild-type but not in eNOS-/- mice and this effect is endothelium dependent (Dantas et al., 2003). However, when we added S1P to strips contracted with PE we did not observe any relaxant or contracting effect in the human tissue. No contracting effect was present on basal tone. On the other hand, S1P activates eNOS in cultured vascular endothelial cells, by promoting eNOS phosphorylation (Igarashi et al., 2001). In addition, it has been demonstrated in vivo that the PI3k/Akt pathway physiologically mediates erection. Both electrical stimulation of the cavernous nerve and direct intracavernosal injection of papaverine cause an erectile response coupled to a rapid increase in phosphorylated Akt and eNOS (Hurt et al., 2002). Erection and phosphorylation

are both diminished by Wortmannin and LY294002, inhibitors of PI3-kinase, the upstream activator of Akt (Hurt et al., 2002). Overall, these data indicate that a stimulus is necessary to trigger the calcium independent activation of eNOS e.g. the phosphorylating mechanism. For this reason we sought to investigate if S1P could synergize with a physiological stimulus that uses eNOS activation to transduce the signal downstream receptor activation e.g. acetylcholine. Tissue incubation with S1P caused a marked increase in the relaxant response of HCC strips to a subliminal dose of acetylcholine. Strikingly, S1P at a dose of 0.1μM, caused an increase of acetylcholine relaxant effect of about 6 times, indicating that S1P can give an important contribute to the peripheral machinery involved in supporting the smooth muscle relaxation necessary to achieve and maintain erection. The possibility that this mechanism is acting in vivo in man, is supported by the fact that S1P concentration in serum is 0.5 μM (Yatomi et al., 2001). Interestingly, this potentiating effect was not present when were used other relaxant agents such as PGE_1 or sodium nitroprusside, indicating that the effect observed in HCC is not linked to either the eicosanoid pathway or to a direct interaction with the endogenously generated NO. Pharmacological modulation of the response further demonstrated that the effect observed was mediated through eNOS activation. Indeed, eNOS inhibition by L-NAME reverted the S1P potentiating action and wortmannin, an inhibitor of the eNOS phosphorylation pathway, mimicked this effect. Indeed, wortmannin significantly reduced the relaxation elicited by acetylcholine indicating that the calcium independent pathway also plays a role in the dynamic physiological vasodilatory mechanisms. These functional data strongly suggest that the interaction responsible of the effect observed takes part at the eNOS level and that eNOS phosphorylation, e.g. the calcium independent activation, is the main mechanism involved. This hypothesis is further strengthened by the western blot studies where, S1P caused a significant increase of the eNOS phosphorylated protein within the time frame used in the in vitro functional study that was prevented by wortmaninn.

In conclusion, we have demonstrated that human cavernous tissue as well as the penile artery has

receptors for S1P and that activation by S1P does not cause directly either, relaxation or contraction of HCC strips. Conversely, S1P strongly and selectively potentiates acetylcholine response mostly by increasing NO release and promoting eNOS phosphorylation. Our finding suggests S1P as the possible endogenous mediator, responsible for the calcium-independent eNOS activation through phosphorylation in human corpus cavernosum playing a “boosting role” in the haemodynamic events involved in erection. The mechanism described here fits with the physiological requirements for penile erection being, a gradual haemodynamic phenomenon.. It is feasible that NO released by nerve endings is sufficient to initiate the complex haemodynamic events involved in erection, but it is not enough to maintain and sustain the vasodilatation necessary in order to achieve a full erection. Both the eNOS phosphorylation pathway and the calcium dependent pathway, essential for shifting eNOS from a basal state to a higher state of activation, contribute to maintenance of the vasodilatory state (Fulton et al., 2001). Thus, the phosphorylated eNOS activation pathway may be explored as a new therapeutic area of intervention in ED in order to develop a way of promoting NO production at endothelial level. This approach could also be used to enhance PDE5 therapy in patients with ED who are poor responders such as in the case of diabetes.

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FIGURE LEGENDS

Figure1. panel a) Quantitative RT-PCR for S1P1, S1P3 and S1P2 in spongiosum tissue ***P<0.001 *vs* S1P-1 and S1P-2, °°°P<0.001 *vs* S1P-2. panel b) penile artery ***P<0.001 *vs* S1P-1 and S1P-2, °°°P<0.001 *vs* S1P-2. The data represent the mean ± SEM from three different human specimens.

Figure2. Effect of S1P (0.001-10μM) on HCC strips precontracted with phenylephrine (3μM) in presence or in absence of endothelium. Ach (0.01-10μM) or SNP (0.01-10μM) were used as positive control. The data represent the mean±SEM from nine separate human specimens (nine strips for each experiment).

Figure 3. Potentiation of Ach response by S1P (0.1μM) or vehicle added 10 min prior to Ach. **P<0.01 *vs* Ach and vehicle (a). Relaxant effect of PGE_1 (100ng/ml) in presence of vehicle (b) or (c) in presence of S1P (0.1μM). . The data represent the mean±SEM from nine separate human specimens (nine strips for each experiment).

Figure 4**.** L-NAME (100μM) or wortmannin (WT, 2μM) reverts S1P (0.1μM)-induced potentiation of Ach-induced relaxation, ***P<0.001 *vs* Ach, L-NAME+S1P and WT+S1P (a).
WT (2μM;***P<0.001) reduces Ach-induced relaxation (b) . The data represent the mean±SEM from nine separate human specimens (nine strips for each experiment).

Figure 5. Panel a) Representative western blot for total content of eNOS and phosho-eNOS-S^{1177} in HCC strips after S1P (0.1μM), Ach (3μM), vehicle or wortamannin (WT, 2μM) incubation for 30 min followed by S1P (0.1μM) challenge. Panel b) Densitometric analysis of changes in percent of phospho-eNOS in tissue incubated as described above, **P<0.01 *vs* S1P. Bands were quantified in arbitrary units by densitometry. Each bar represents the mean±SEM of percent ratio in phospho-eNOS/eNOS from three different human specimens.

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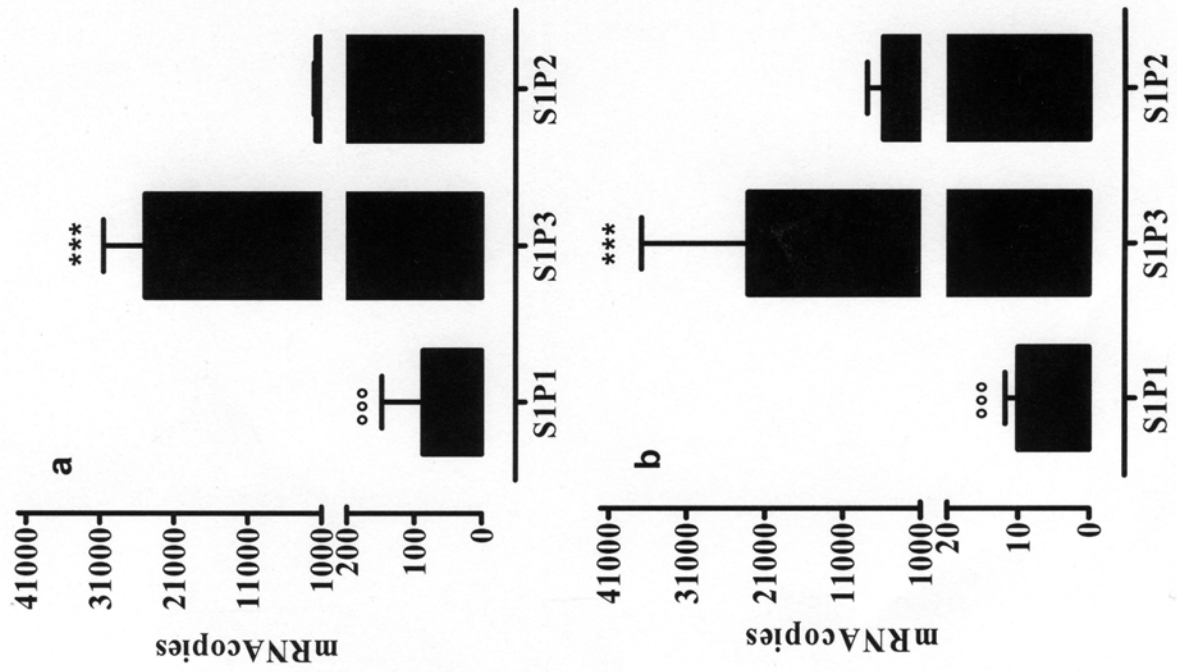

Fig.1

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Fig.2

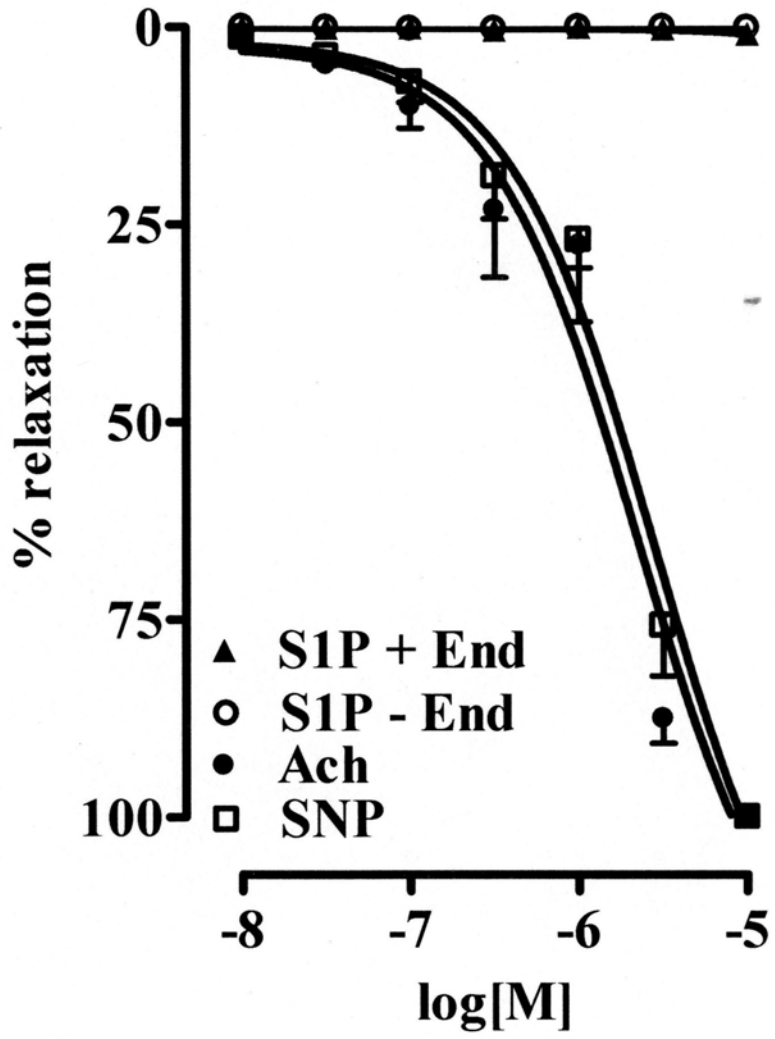

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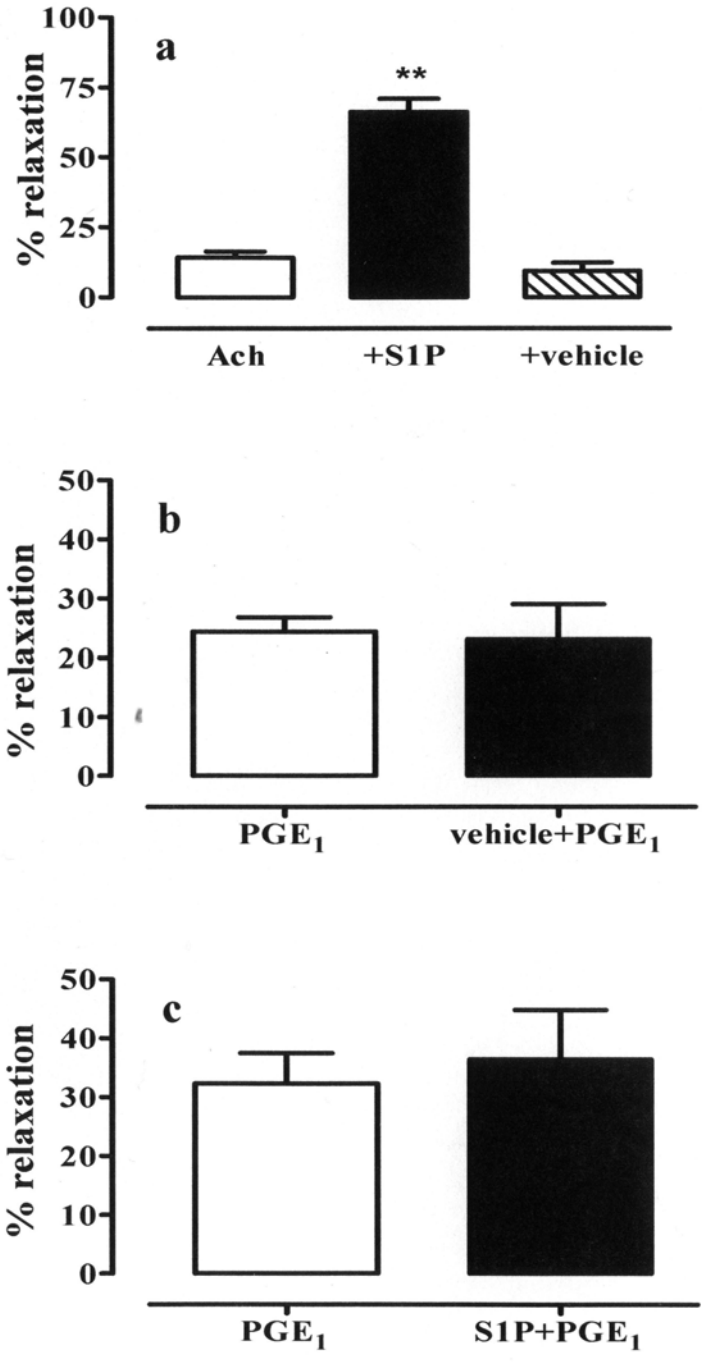
Fig.3



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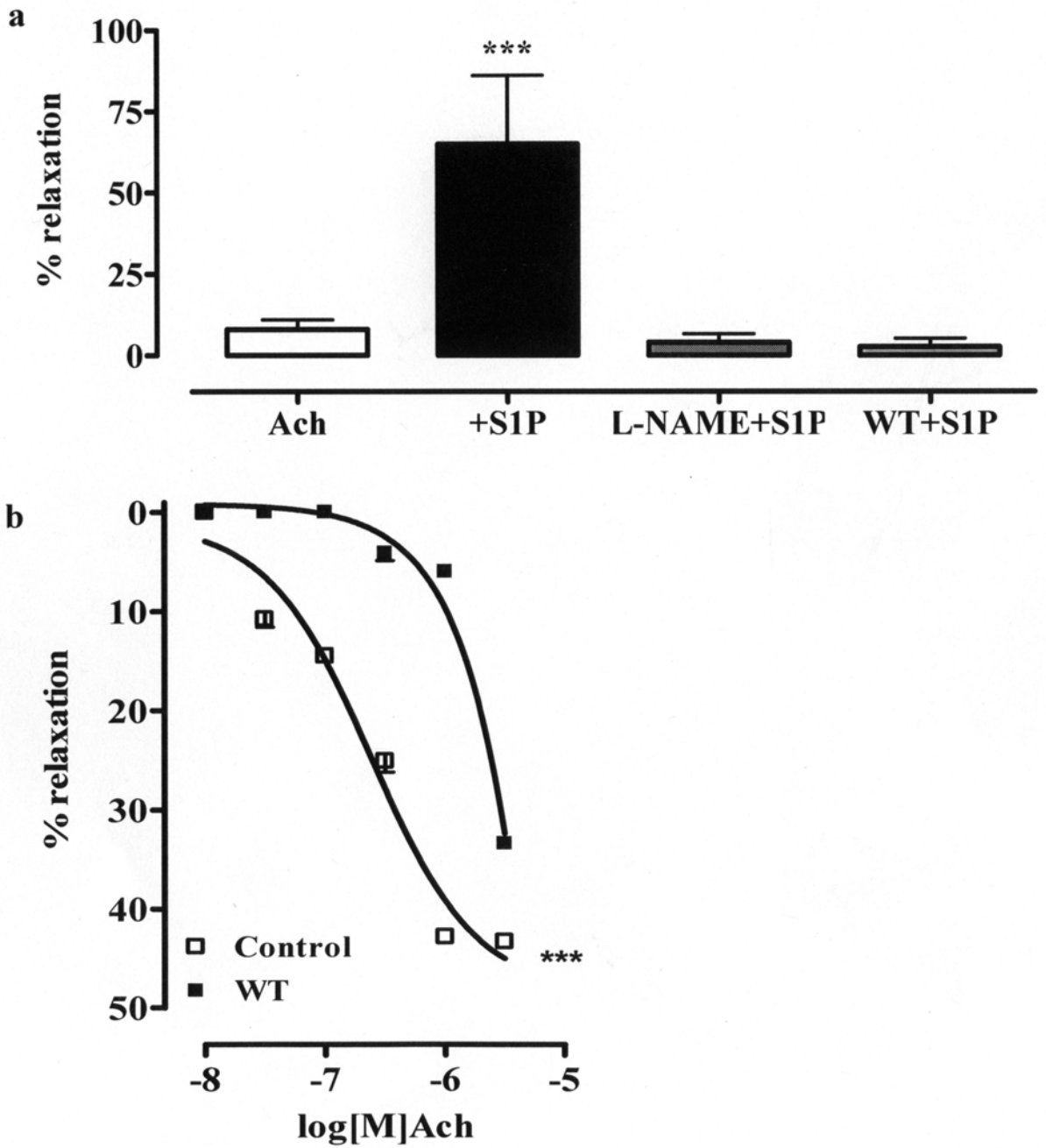

Fig.5