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Essential requirement for sphingosine kinase activity in eNOS-dependent NO release and vasorelaxation

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ABSTRACT

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that acts both as an extracellular ligand for endothelial differentiation gene receptor family and as an intracellular second messenger. Cellular levels of S1P are low and tightly regulated by sphingosine kinase (SPK). Recent studies have suggested that eNOS pathway may function as a downstream target for the biological effects of receptor-mediated S1P. Here we have studied the possible interplay between intracellular SIP generation and the eNOS activation pathway. S1P causes an endothelium-dependent vasorelaxation in rat aorta that is PTX sensitive, inhibited by L-NAME that involves eNOS phosphorylation, and mainly dependent on hsp90. When rat aorta rings were incubated with the SPK inhibitor DL-threo-dihydrosphingosine (DTD), there was a concentration-dependent reduction of Ach-induced vasorelaxation, implying a consistent contribution of sphingolipid pathway through intracellular sphingosine release and phosphorylation. Co-immunoprecipitation experiments consistently showed increased association of hsp90 with eNOS after exposure of cells to S1P as well to BK or calcium ionophore A-23187. Interestingly, as opposite to A-23187, BK and S1P effect were significantly inhibited by pretreatment with the SPK inhibitor DTD. In conclusion, our data demonstrate that an interplay exists among eNOS, hsp90, and intracellularly generated S1P where eNOS coupling to hsp90 is a major determinant for NO release as confirmed by our functional and molecular studies.

Key words: hsp90 • sphingosine-1-phospate

Endothelium-derived nitric oxide is a critical regulator of cardiovascular homeostasis through its profound effects on blood pressure, vascular remodeling, platelet aggregation, and angiogenesis (1). NO production by endothelial nitric oxide synthase (eNOS) is regulated in a remarkably complex fashion. In general, in the basal state, eNOS produces a constant low amount of NO; when stimulated by a variety of stimuli, eNOS activity is enhanced and NO-concentration increases (2). eNOS is a Ca^{2+}/calmodulin (CaM)-dependent enzyme

localized to the caveolae of endothelial cells where it associates with caveolin-1 that maintains the enzyme in an inactive state. Ca^{2+}/CaM weakens the eNOS-caveolin-1 interaction, and thus the enzyme is activated by combined loss of caveolin-1 interaction and the direct effect of Ca^{2+}/CaM on the enzyme (3). Various extracellular signals such as shear stress, VEGF, estrogen, and bradykinin can increase cytoplasmic Ca^{2+} levels and activate CaM, which promotes NO release from endothelial cells. However, recent evidence demonstrates that subcellular localization; posttranslational modification, such as phosphorylation and dephosphorylation; and interactions with several regulatory proteins, including heat shock protein 90 (hsp90), all act as additional levels of regulation of eNOS activity. Interestingly, each of these regulatory processes seems to work by increasing the sensitivity of the enzyme to Ca^{2+}/CaM[4]. Recent studies have expanded the regulation of eNOS to sphingolipid signaling (5, 6). In particular, the sphingolipid metabolite sphingosine-1-phosphate (S1P) has emerged as a potent and versatile bioactive molecule (7). S1P is formed by removal of the amide-linked fatty acid side chain of ceramide through the action of the enzyme ceramidase. Production of S1P then occurs by phosphorylation of sphingosine at the 1-OH position by the enzyme sphingosine kinase (SPK) (8). S1P can act as an extracellular ligand, activating specific G-protein-coupled sphingolipid receptors in the plasma membrane (9). Alternatively, S1P produced after activation of various plasma membrane receptors can fulfill the role of a second messenger and stimulate Ca^{2+} channel on the endoplamic reticulum (10). To be considered as a significant intracellular messenger, levels of S1P need to be regulated by particular stimuli, and this occurs via the activation of the enzyme SPK. It is now well established that the extracellular effects of S1P are mediated via a recently identified family of plasma membrane G-protein-coupled receptors called endothelial differentiation gene (*EDG*) recently renamed S1P receptors (11), whereas specific intracellular sites of action remain to be defined. In addition, the role of intracellularly generated S1P in a physiological context has not been investigated. Recently, it has been shown that stimulation of $S1P_1$ receptor by S1P leads to activation of eNOS and induces an endothelium-dependent vasodilatation (12). Subsequent studies have begun to dissect the signaling pathway in cultured cells (13, 14). The majority of the studies, until now, explored the extracellular pathway (S1P/EDG-1) by which S1P regulates eNOS.

The aim of this study was to investigate on the potential intracellular second messenger role of S1P in eNOS activation in aortic vessel and on the contribution of this alternate pathway in the control of vascular tone. Herein we show that intracellularly generated SIP contributes to the eNOS activation process and therefore the vasodilatory effects induced by acetylcholine, bradykinin, and interestingly by S1P receptor activation. We also demonstrate that intracellularly generated S1P is important for hsp90 recruitment to eNOS thus participates in the activation

MATERIALS AND METHODS

Drugs

Acetylcholine (Ach), L-phenylephrine (PE), N^{ω}-nitro-L-arginine methyl ester (L-NAME), polyethylene glycol 400 (PEG), dimethyl sulphoxide (DMSO), pertussis toxin (from *Bordetella pertussis*), DL-threo-dihydrosphingosine (DTD), LY-290004, cyclopiazonic acid (CPA), and EGTA were purchased from Sigma (Milano, Italy), S1P were purchased from Tocris Cookson Ltd. (Bristol, UK) or Calbiochem (San Diego, CA), and geldanamycin (GA) was a generous gift of the drug Synthesis and Chemistry Branch, Development Therapeutics program, Division of

Cancer Treatment at the National Cancer Institute (Bethesda, MD). S1P, DTD, and LY-290004 were dissolved in DMSO. GA was dissolved in a solution of distilled water dH_2O/PEG (1/1); all others were dissolved in dH_2O.

Cell culture

Bovine aortic endothelial cells (BAEC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (HyClone, Logan, UT), 2.0 mmol/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were used between passages 5 and 8.

Tissue preparation

Male Wistar rats (250–300 g; Charles River, Milan, Italy) of 8–10 wk of age were killed, and the thoracic aorta was rapidly dissected and cleaned from fat and connective tissue. Rings of 2–3 mm length were cut and placed in organ baths (2–5 ml) filled with oxygenated (95% O_2-5% CO_2) Krebs solution at 37°C and mounted to isometric force transducers (type 7006, Ugo Basile, Comerio, Italy) and connected to a Graphtec linearecorder (WR 3310). The composition of the Krebs solution was as follows (mol/l): NaCl 0.118, KCl 0.0047, $MgCl_2$ 0.0012, KH_2PO_4 0.0012, $CaCl_2$ 0.0025, $NaHCO_3$ 0.025, and glucose 0.010. Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 min during which tension was adjusted, when necessary, to a 0.5 g, and bathing solution was periodically changed. In a preliminary study, a resting tension of 0.5 g was found to develop the optimal tension to stimulation with contracting agents. In each experiment aortic rings were first challenged with PE (10^{-6} mol/l) until the responses were reproducible. To verify the integrity of the endothelium, Ach cumulative concentration-response curve (10^{-8}–3×10^{-5} mol/l) was performed on PE-contracted rings. Vessels that relaxed <85% were discarded.

Role of endothelium-derived nitric oxide in S1P induced vasorelaxation

Aortic rings were contracted with PE (10^{-6} mol/l), once plateau was reached, a cumulative concentration-response curve to S1P (10^{-8}-3×10^{-5}mol/l) was performed. In another set of experiments, rings were denuded of the endothelium and S1P cumulative concentration-response curve was performed. To confirm the key role of eNOS-derived nitric oxide, GA (20 μmol/l) and L-NAME (100 μmol/l) were added in the organ baths where rat aorta rings had been contracted with PE. After 15 min of incubation, a cumulative concentration-response curve to S1P was performed.

LY-294002 was incubated with aortic rings using a protocol described previously (15). Briefly, thoracic aorta was dissected and rings, of the same size as described before, were cut and placed in a multiwell plate with 980 μl DMEM solution (containing penicillin 100 U/ml, streptomycin 100 μg/ml, and L-glutamine 2×10^{-3} mol/l) in the presence of LY-294002 (25 μmol/l) or vehicle (dH_2O 20 μl). Thereafter, the plate was placed at 37°C in an incubator (Mod. BB622, Heraeus Instruments) with humidified air (5% CO_2–95% O_2). After 16 h of incubation, rings were washed extensively with Krebs and then mounted in organ baths.

In a separate set of experiments, aortic rings were also incubated with activated PTX at the concentration of 1 μg/ml for 2 h (16). Rings were then stimulated with PE (10^{-6} mol/l) to assess

if there was any effect on the contractility. Immediately thereafter rings were contracted again with PE (10^{-6} mol/l), and a cumulative concentration-response curve to S1P was performed.

Western blot analysis

Thoracic aorta was dissected and cleaned from fat and connective tissue. Rings, of the same size as described before, were cut and placed in a multiwell plate with 980 μl DMEM solution (containing penicillin 100 U/ml, streptomycin 100 μg/ml and L-glutamine 2×10^{-3} mol/l) in the presence of S1P (1 μmol/l) or vehicle (dH_2O 20 μl). Thereafter, the plate was placed at 37°C in an incubator (Mod. BB622, Heraeus Instruments) with humidified air (5% CO_2-95% O_2). Since the time needed to build a cumulative dose response in the organ bath experiments was 15 min, we incubated the rings for 15 or 30 min. After the incubation period, rings were washed extensively with Krebs and then homogenated in lysis buffer (β-glicerophosphate 50 mmol/l, orthovanadate sodium 0.1 mmol/l, Mg Cl_2 2 mmol/l, EGTA 1 mmol/l, DTT 1 mmol/l, PMSF 1 mmol/l, aprotinin 10 μg/ml, leupeptin 20 μmol/l, 50 mmol/l NaF) using a Polytron homogenizer (2 cycles of 10 s at maximum speed). After centrifugation of homogenates at 10,000 rpm for 10 min, equal amounts of the denatured proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked by incubation in PBS containing 0.1% v/v Tween 20 and 5% nonfat dry milk for 2 h, followed by a overnight incubation at 4°C with rabbit S1P1 receptor polyclonal antibody (1:1000, BIOMOL Research Laboratories), rabbit anti-phospho-eNOS-Ser-1179 polyclonal antibody (1:1000, Cell Signaling), and mouse eNOS monoclonal antibody (1:2000, Transduction Laboratories). The filters were washed extensively in PBS containing 0.1% v/v Tween 20, before incubation for 2 h with anti-horseradish peroxidase-conjugate secondary antibody. Membranes were then washed and developed using enhanced chemiluminescence substrate (ECL, Amersham Pharmacia Biotech).

S1P determination

Rat aortic rings were incubated with Ach (10^{-6} M) or the vehicle (Krebs) and left in contact for 15 min. Immediately thereafter, the tissue were frozen. After being freeze-dried and the addition of 50 ng of S1P (C17 base) as internal standard, each sample was extracted in agreement with the procedure described by Van Veldhoven et al. (17). Sphingolipids were then exhaustively recovered from the organic phase (chloroform:methanol 2:1) by repetitive partition against 0.1 M aqueous ammonia. The water-soluble material was then evaporated at reduced pressure, the yellow residue was derivatized with 20 μl phenylisothiocyanate (PITC) in ethanol/triethylamine/pyridne/water (6:1:1:2), and the reaction mixture was evaporated with methanol at reduced pressure. The PTC-derivatives were then analyzed by negative ESI LC-MS (MicroMass Q-*tof* micro equipped wit a water HPLC alliance), by eluting the material with a gradient of methanol in water containing 0.3% of formic acid.

eNOS immunoprecipitation

Serum-starved BAEC (16 h) were washed twice with cold PBS after agonist stimulation (10 min). Cells were solubilized with a lysis buffer containing 1% Triton X-100, 50 mmol/l NaCl, 1 mmol/l EDTA, 0.1 mmol/l EGTA, 20 mmol/l sodium fluoride, 1 mmol/l sodium pyrophosphate, 1 mmol/l sodium orthovanadate, and 1 mmol/l Pefabloc and protease inhibitor cocktail (Roche

Diagnostics). Insoluble proteins were precipitated by centrifugation at 13,000 rpm for 10 min at 4°C, and the supernatants were then incubated overnight with the anti-eNOS antibody (2 μg) at 4°C. Protein A-Sepharose (Sigma; 50 μl of a 50% slurry) was then added and incubated for an additional hour. The immune complexes were precipitated by centrifugation, washed three times with lysis buffer, boiled in SDS sample buffer, separated by SDS-PAGE, and Western blotted as above.

Effect of SPK inhibition on Ach-induced vasorelaxation

Sphingosine to be active has to be phophorylated by SPK. The role of SPK in endothelium-derived nitric oxide production was assessed by using a specific SPK inhibitor, e.g., DTD. Aortic rings were incubated for 1 h in the organ baths with different concentration of DTD (10, 30, and 100 μmol/l) or the vehicle (DMSO). Afterward, rings were stimulated with PE (10^{-6} mol/l) to assess if there was an effect on the contractility. After being washed, rings were contracted again with PE 10^{-6} mol/l and cumulative concentration responses to Ach or SNP or A-23187 were performed.

Role of $S1P_1$ in Ach-induced vasorelaxation

Because specific pharmacological antagonists for S1P receptor subtypes are not available, we incubated aortic rings with activated PTX (17) (1 μg/ml for 2 h), which blocks G_i coupling and receptor activation ($S1P_1$). Rings were then stimulated twice with PE (10^{-6} mol/l) to assess if there was an effect on the contractility. Aortic rings were washed and contracted again with PE (10^{-6} mol/l), and a cumulative concentration-response curve to Ach (10^{-8}–3×10^{-5} mol/l) was performed.

Role of calcium in Ach and S1P-induced vasorelaxation

It is known that S1P directly releases calcium from intracellular calcium stores. To asses the role of extracellular and intracellular calcium in Ach- and S1P-induced vasorelaxation we used EGTA (2.5 mmlo/l) and CPA (10 μmol/l), an inhibitor of Ca^{2+}-ATPase in sarcoplasmic reticulum. After drug incubation, rings were stimulated with PE (10^{-6} mol/l) to assess if there was an effect on the contractility. Rings were then contracted with PE 10^{-6} mol/l and cumulative concentration responses to Ach and/or S1P performed.

Effect of sphingolipid biosynthesis inhibitors on Ach-induced vasorelaxation

Sphingosine is synthesized starting from sphingomyelin by the sequential action of sphingomyelinase and ceramidase and subsequently is phosphorylated. To address the involvement of sphingosine pathway, aortic rings were incubated for 1 h in the organ baths with different concentration of fumonisin (10 and 30 μmol/l), an inhibitor of dihydroceramide the enzyme that converts sphinganine in dihydroceramide, and MAPP (3 and 10 μmol/l) a specific inhibitor of the enzyme ceramidase that converts ceramide in sphingosine a ceramidase, or the vehicle (DMSO). After drug incubation, rings were stimulated with PE (10^{-6} mol/) to assess if there was an effect on the contractility. Rings were then contracted with PE 10^{-6} mol/l and cumulative concentration responses to Ach and/or SNP performed.

Effect of SPK inhibition on S1P induced vasorelaxation

Aortic rings were incubated with DTD a SPK inhibitor (1 h, 100 μmol/l) or vehicle (DMSO). Rings were then stimulated twice with PE (10^{-6} mol/l) to assess if there was an effect on the contractility. Rings were contracted again with PE 10^{-6} mol/l, and cumulative concentration responses to S1P were performed. At the end of experimental protocol, tissue functionality was assessed by using sodium nitroprusside

Statistical analysis

All results are reported as mean ± SE. To analyze the curve, ANOVA was used followed by Bonferroni's or Dunnett's test as posttests. A value of $P < 0.05$ was taken as significant.

RESULTS

S1P induces endothelium and receptor dependent vasorelaxation

In presence of endothelium, S1P induces a concentration-dependent vasorelaxant effect on PE precontracted rings (n=10; P<0.001; Fig. 1*A*). The onset of S1P relaxing effect is the same of Ach. When the endothelium is removed, S1P looses its vasorelaxant activity (Fig. 1*A*), implying a key role for nitric oxide in its vasorelaxant effect (n=10; P<0.001). This finding is confirmed by the fact that the NOS inhibitor L-NAME, at the concentration of 10^{-4} M after 15 min of incubation, significantly inhibits S1P-induced vasodilatation (Fig. 1*A*). It is known that S1P effects on EDG/S1P receptors can be explicated in a pertussis toxin dependent or independent manner (18, 19). S1P receptors 1, 3, and 5 are found in rat aorta; however, $S1P_1$ is the most abundantly expressed (20). The pertussis toxin sensitivity of $S1P_1$ receptor mediated effects suggests that this receptor is coupled to G_i. To verify if the effects of S1P are PTX sensitive, rat aortic rings were incubated with pertussis toxin followed by S1P stimulation. Indeed, the S1P vasorelaxant effect is abrogated after incubation with pertussis toxin (Fig. 1*B*, n=4; P<0.001).

S1P induced vasorelaxtion is coupled to an increase in eNOS activity

To further investigate on the role of eNOS-derived NO, we evaluated if S1P (1 μmol/l) incubated with rat aortic rings could increase eNOS phosphorylation on serine 1179. As shown in Fig. 1*C*, after treatment with S1P, a concentration- and time-dependent increase in eNOS phosphorylation was observed. To gain further insights into the cross talk between S1P and NO pathways, we evaluated the effect of GA (20 μ mol/l), an inhibitor that hinders eNOS activation by blocking its coupling to hsp90 (21), and LY-294002 (25 μmol/l), an inhibitor of PI-3 kinase activity that prevents downstream Akt-dependent eNOS phosphorylation (22), against S1P-induced vasorelaxation. GA treatment (Fig. 1*D*) virtually abolishes S1P-induced vasorelaxation (n=6; P<0.001), which is similar to endothelium removal or to NOS inhibition by L-NAME (for comparison see Fig. 1*A*). Conversely, LY-294002 (Fig. 1*D*) causes a significant (n=6; P<0.01) but less intense inhibition of S1P-stimulated vasorelaxation compared with the inhibitory effects observed with GA or L-NAME.

SPK activity is involved in endothelium-dependent vasodilatation

Incubation of aortic rings with Ach but not the vehicle caused a release of S1P 30 ng/ml/wet tissue S1P as determined by negative ESI LC-MS. Following this evidence of the involvement of S1P, we have operated a pharmacological modulation through specific inhibitors. Pretreatment of aortic rings with DTD, a specific SPK inhibitor, inhibits, in a concentration dependent manner, Ach-induced vasodilatation giving a significant inhibition at the concentration of 30 μmol/l (n=6; P<0.01) and 100 μmol/l (n=6; P<0.001; Fig. 2*A*). To confirm that DTD did not interfere with the ability of smooth muscle cells to respond to exogenous NO, vasodilatation stimulated by an NO donor, sodium nitroprusside (SNP), was performed in presence of the highest dose of DTD (100 μmol/l) used in this study. As seen in Fig. 2*B*, DTD does not modify SNP-induced vasorelaxation (n=6 NS). Equally, DTD has no effect on the calcium ionophore A-23187-induced vasorelaxation (n=6 NS; Fig. 2*C*).

Inhibition of sphingolipid biosynthesis reduces Ach-induced vasorelaxation

To assess the involvement of sphingolipid metabolism in endothelium-dependent vasorelaxation, inhibitors of S1P biosynthesis, which interfere at different levels of synthesis, were used. Fumonisin (10–30 μmol/l), an inhibitor of dihydroceramide, the enzyme that converts sphinganine to dihydroceramide (Fig. 3*A*), and MAPP (3–10 μmol/l), a specific inhibitor of the enzyme ceramidase that converts ceramide to sphingosine (Fig. 3*B*), were used. Fumonisin (n=6) and MAPP (n=6) both inhibit Ach-induced vasorelaxation, implying a consistent contribution of sphingolipid pathway, through both sphingosine biosynthesis and its phosphorylation by SPK, in Ach-induced vasorelaxation. To confirm the intracellular role of S1P in Ach-induced vasorelaxation, rat aortic rings were incubated with PTX followed by Ach stimulation. Relaxation induced by Ach was unaffected by pretreatment with PTX, excluding an extracellular and receptor-mediated role of S1P (Fig. 3*C*).

SPK inhibition abrogates receptor-dependent recruitment of hsp90 to eNOS and S1P-induced vasorelaxation

As mentioned previously, eNOS activation is correlated with an increased in the association of hsp90 and eNOS, which is necessary for eNOS activation. To assess the role of SPK in agonist-dependant hsp90 recruitment to eNOS, we performed eNOS immunoprecipitation from bovine aortic endothelial cell (BAEC) lysates and monitored the coprecipitation of the activating protein hsp90. As seen in Fig. 4*A* and *B*, S1P (5 μM), BK (10 μM), and A23187 (5 μM) stimulation of BAEC (10 min) all induce an increase in the amount of hsp90 detected in the eNOS immunocomplex (Fig. 4*A*, *B*, *C*; compare lanes 1 and 3). Inhibition of SPK by DTD (10 μM, 10 min) markedly reduces the ability of S1P and BK to promote hsp90 recruitment to eNOS (Fig. 4*A* and *B*; compare lanes 3 and 4). Interestingly, DTD fails to inhibit hsp90 recruitment by the receptor independent calcium-mobilizing agent A23187 (Fig. 4*C*; compare lanes 3 and 4). These results suggest that SPK activation is upstream of the receptor-induced increase in intracellular calcium levels necessary for hsp90 recruitment to eNOS and its activation. In addition, these data are in line with our functional studies (Fig. 4*D*), which show that SIP-induced vasodilatation of the rat aorta is significantly inhibited by DTD, while A23187-induced effects remained unaltered by SPK inhibition.

Intracellular calcium is involved in S1P-induced vasorelaxation

Pretreatment of aortic rings with EGTA, a calcium chelator, significantly inhibits Ach-induced vasodilatation (n=6; P<0.01) but has no effect on S1P-induced vasorelaxation, implying a contribution of extracellular calcium only in Ach-induced effect (Fig. 5). Conversely, CPA has a strong effect (70%), both on Ach (n=6; P<0.001) and S1P-induced relaxation (n=6; P<0.001), suggesting a key role for intracellular calcium.

DISCUSSION

Recent studies have suggested that eNOS pathway may function as a downstream target for the biological effects of S1P. This hypothesis is strongly supported by fact that S1P lipid precursors, receptor (EDG-1/S1P1), and eNOS all reside in the caveolae (13). The caveolae are characteristic flask-shaped invaginations of the plasma membrane present on many cell types, including endothelial and smooth muscle cells (23, 24). Importantly, caveolae have a distinct lipid composition; these structures termed "lipid rafts" are highly enriched in sphingolipids and cholesterol and relatively depleted of phospholipids; in this way the bilayer is more rigid and lipids are more confined in their movements (25).

In cultured endothelial cells, stimulation with S1P increases eNOS phosphorylation and in turn enhances nitric oxide production (6). eNOS-derived nitric oxide is a fundamental determinant of cardiovascular homeostasis; it regulates systemic blood pressure, vascular remodeling, and angiogenesis (26, 27). S1P receptors 1, 3, 5 are expressed in rat aorta, with $S1P_1$ being the most abundant (20). It has been shown that S1P causes an endothelium-dependent vasorelaxation. This response is mediated by a pertussis toxin sensitive G protein coupled receptor pathway that involves activation of PI-3 kinase, leading in turn to eNOS phosphorylation at Ser1179 (12). However, the possible link between S1P/eNOS signaling pathway and agonist-induced relaxation has not been clearly addressed. To be biologically active, sphingosine must be phosphorylated by SPK. Inversely, the dephosphorylation by S1P phosphatase leads to inactive metabolites (28–30). In this study, we first showed that S1P causes an endothelium-dependent vasorelaxation in rat aorta, which is PTX sensitive and is inhibited by L-NAME. Regulation of eNOS is a complex process; however, it is possible to specifically interfere with eNOS activity at different levels (22). In its basal state, eNOS is negatively regulated by several proteins and produces a low concentration of NO. After a specific stimulus, eNOS is shifted to a higher degree of activation. Among the posttranslational modifications that can positively regulate eNOS activity, the coupling of the enzyme to hsp90, and its phosphorylation on serine 1179 appear to be particularly critical. In our experimental conditions, we were able to show that SIP stimulation of rat aorta induces phosphorylation of eNOS. However, our in vitro experiments also show that the vasorelaxant effects of S1P are mainly dependent on hsp90 as revealed by the sensitivity to geldanamycin, which completely abrogated eNOS-dependent vasodilatation. This is in contrast to the partial inhibition exhibited by LY-294002, the PI3-kinase inhibitor. This evidence raised the possibility that an interplay may exist between eNOS, hsp90, and S1P. To understand this link, we performed specific experiments using Ach and inhibitors of sphingosine pathway. We first determined by negative ESI LC-MS that after incubation with Ach S1P was produced. When rat aortic rings were incubated with the SPKI inhibitor DTD, a concentration-dependent reduction of Ach-induced vasorelaxation that caused a shift in the maximal achievable relaxation was observed. These data imply that there is a consistent contribution of sphingolipid

pathway, through sphingosine release and its phosphorylation, in Ach-induced vasorelaxation. These data fit well with the molecular study performed by Meyer et al. (31) that has elegantly demonstrated that Ach receptors utilize the SPK/S1P pathway in addition to PLC-IP3 to mediate calcium mobilization. Furthermore, the contribution of this pathway to signaling appears to be relevant in vessel function since upon its blockage ~50% of the vasorelaxant effect is lost. This appears to be a selective phenomenon since SPKI inhibition by DTD does not directly interfere with eNOS-derived NO since it does not affect SNP- or A23187-induced vasodilatation.

Sphingosine has been shown to be secreted extracellularly and may generate biological responses through an autocrine/paracrine signaling response. Extracellular S1P, as first messenger, binds S1P receptors on the surface of endothelial cells, while the intracellular S1P acts as a second messenger leading to cytoskeleton changes and release of intracellular calcium. To understand whether S1P is produced intracellularly in response to extracellular agonists (Ach, BK) and then released from cells to act on cell surface, specific inhibitors of S1P synthesis and action were used, i.e., PTX or fumonisin and MAPP. PTX, which specifically uncouples the $S1P_1$ receptor from the small G protein G_i, had no effect on Ach-induced vasorelaxation excluding an extracellular and S1P-receptor-mediated role of S1P. MAPP is a specific inhibitor of the enzyme ceramidase that converts ceramide in sphingosine, while fumonisin is an inhibitor of dihydroceramide the enzyme that converts sphinganine in dihydroceramide. Both drugs significantly inhibited in a concentration-dependent manner Ach-induced vasorelaxation, confirming that there is a consistent contribution of intracellular S1P generation in endothelium dependent vasorelaxation.

To gain further insights in this mechanism, we sought to determine the effect of DTD on eNOS/hsp90 interaction. eNOS/hsp90 co-immunoprecipitation experiments consistently showed increased association of hsp90 with eNOS after exposure of cells to BK or calcium ionophore A-23187. These data imply a clear involvement of calcium as trigger of the eNOS/hsp90 coupling. Interestingly, in sharp contrast to A23187, BK effects were significantly inhibited by pretreatment of the cells with the SPK inhibitor DTD. This specificity in the response on BK-induced hsp90 recruitment suggests that SPK is a prerequisite for eNOS activation and further corroborates the in vitro data where similar functional effects were clearly evident. Thus, it was necessary to evaluate the role of SPK by using S1P and to evaluate the S1P-induced eNOS/hsp90 interaction. Exogenous S1P induced the co-immunoprecipitation of hsp90 with eNOS. This coupling was abrogated by pretreatment with DTD. Similarly, in vitro, DTD abrogated S1P-induced vasorelaxation, suggesting that S1P promotes via S1P-receptor 1 activation its own intracellular synthesis through SPK. Calcium modulation experiments, using CPA, further suggest that intracellular pool of S1P enhances hsp90/eNOS interaction possibly through intracellular calcium and in turn increases NO production.

In conclusion, here we confirm that S1P is an important determinant of endothelium-dependent relaxation and contributes to the modulation of vascular tone through a receptor-dependent pathway as well as through a novel metabolic intracellular pathway here shown. These data provide new biological insights and may pave the way for the development of novel therapeutic approaches for cardiovascular diseases.

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Fig. 1

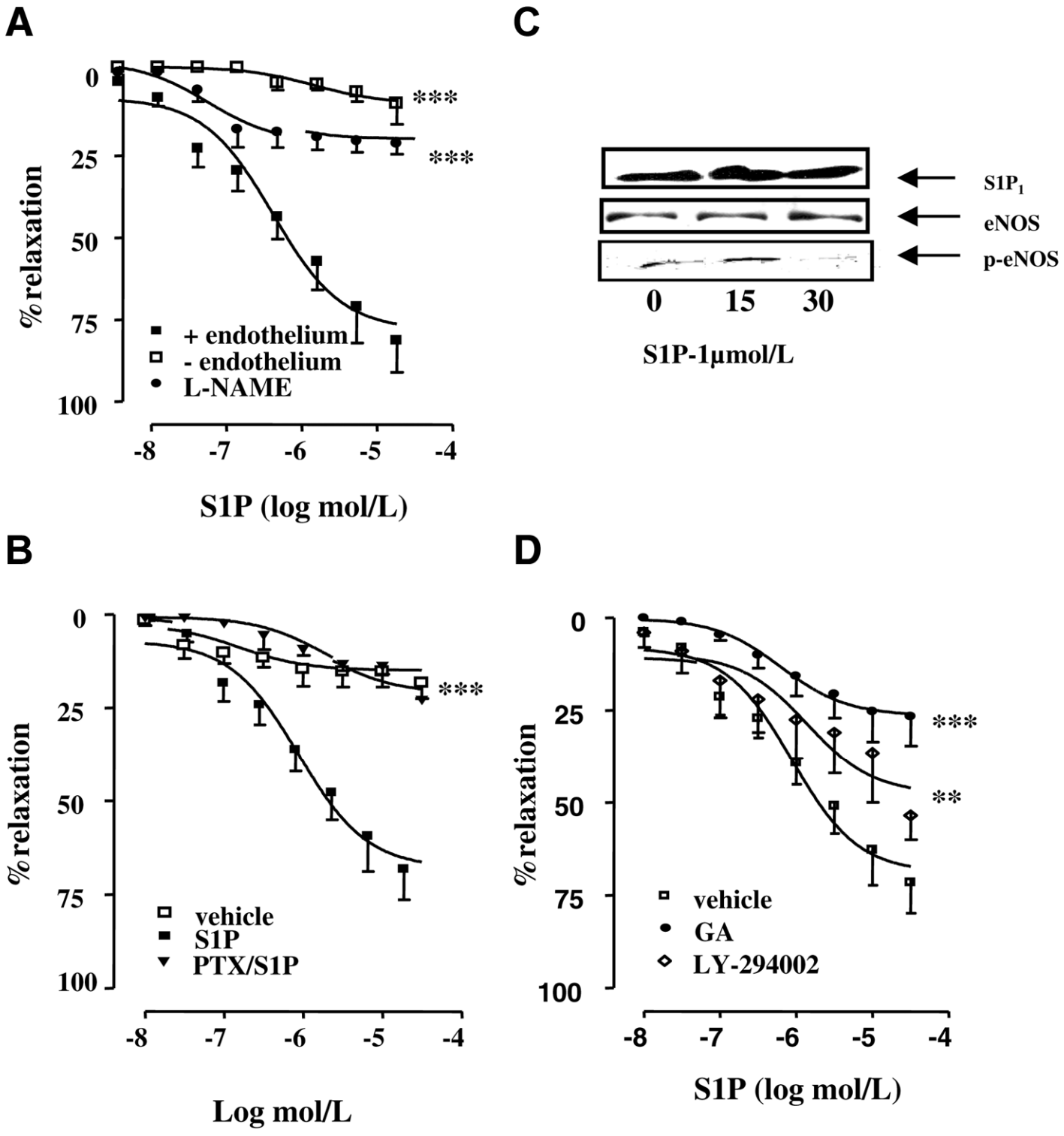

Figure 1. ***A)*** S1P induces a concentration-dependent vasorelaxant effect on PE precontracted rings (n=10; P<0.001) that is endothelium dependent (n=10; P<0.001) and inhibited by L-NAME (10^{-4} mol/l; n=6; P<0.001). Data are mean ± SE; ***P < 0.001 vs. +endothelium; ***B)*** S1P vasorelaxant effect is abrogated after incubation with pertussis toxin (1 µg/ml; n=4; P<0.001). Data are mean ± SE; ***P < 0.001 vs. S1P. ***C)*** S1P (1 µmol/l) incubated with rat aortic rings increases eNOS phosphorylation on serine^{1179} without affecting $S1P_1$ and eNOS expression (n=3). ***D)*** GA (20 µmol/l) treatment virtually abolishes S1P-induced vasorelaxation (n=6; P<0.001). Conversely, LY294002 (25 µmol/l) causes a significant (n=6; P<0.01) but less marked inhibition. Data are mean ± SE; ***P < 0.001 **P < 0.01 vs. vehicle.

Fig. 2

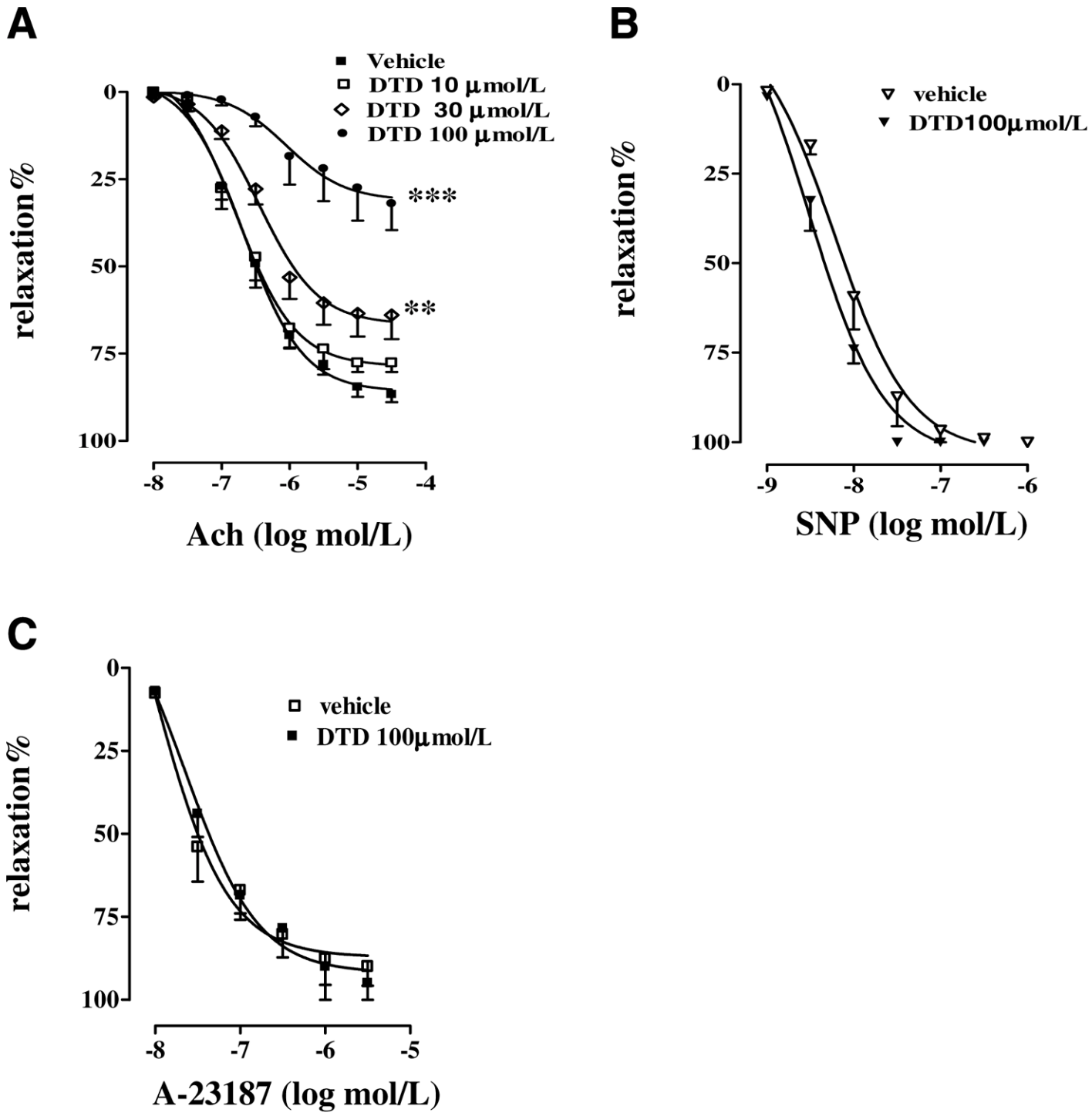

Figure 2. ***A***) Pretreatment of aortic rings with DTD, a specific sphingosine kinase inhibitor, reduces in a concentration-dependent manner Ach-induced vasodilatation at the concentration of 30 µmol/l (n=6; P<0.01) and 100 µmol/l (n=6; P<0.001). ***B***) DTD does not modify SNP induced vasorelaxation (n=6, NS). ***C***) DTD has no effect on the calcium ionophore A-23187-induced vasorelaxation (n=6, NS). Data are mean ± SE; ***P < 0.001 **P < 0.01 vs. vehicle.

Fig. 3

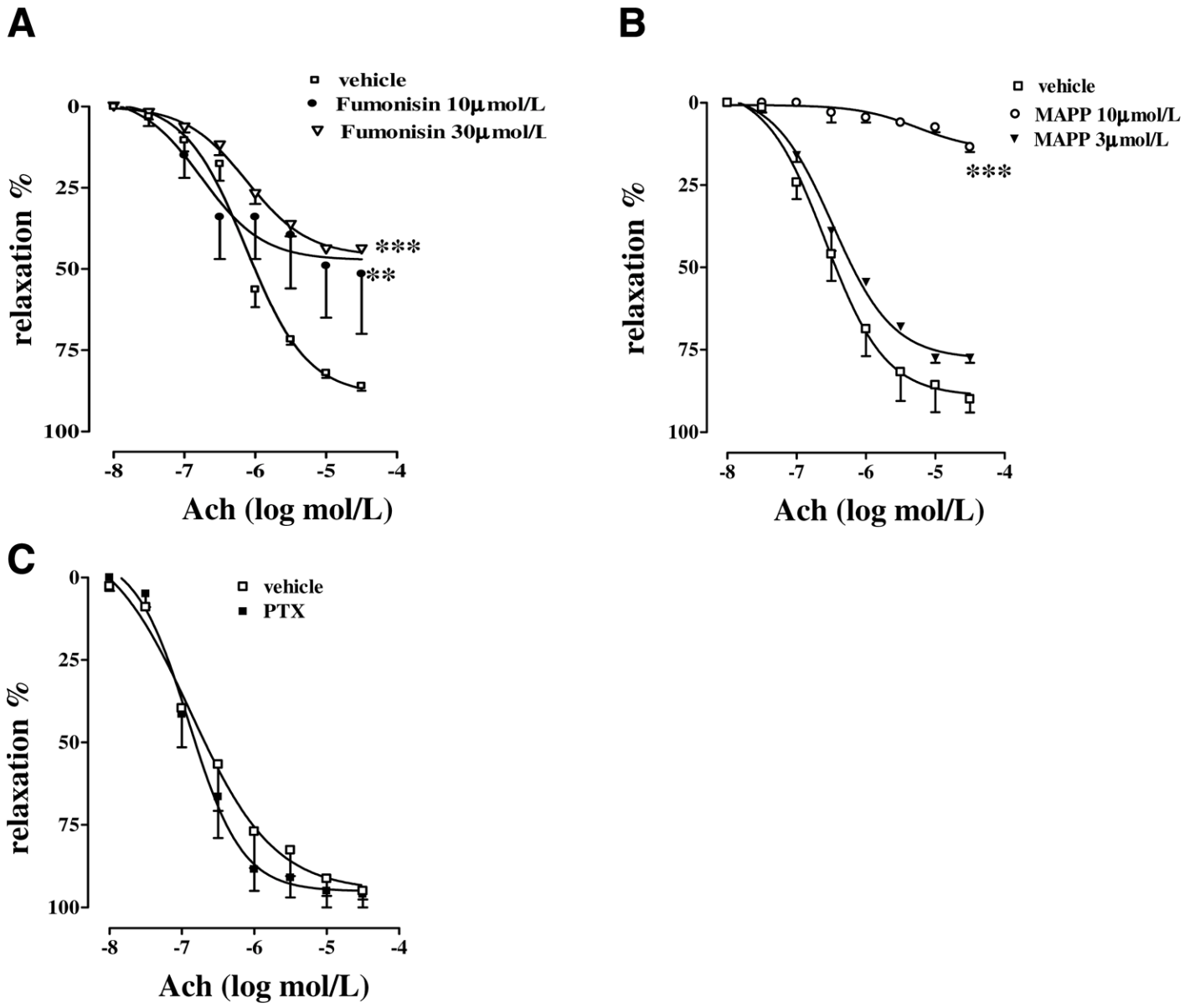

Figure 3. ***A***) Fumonisin, an inhibitor of dihydroceramide, the enzyme that converts sphinganine to dihydroceramide, at the doses of 10 µmol/l (n=6; P<0.01) and 30 µmol/l (n=6; P<0.001) reduces Ach-induced vasorelaxation. ***B***) MAPP, a specific inhibitor of the enzyme ceramidase that converts ceramide to sphingosine, at doses of 3 µmol/l (n=6, NS) and 10 µmol/l (n=6, P<0.001) reduces Ach-induced vasorelaxation. ***C***) Relaxation induced by Ach was unaffected by pretreatment with PTX excluding an extracellular and receptor mediated role of S1P (n=6; NS). Data are mean ± SE; ***P < 0.001 **P < 0.01 vs. vehicle.

Fig. 4

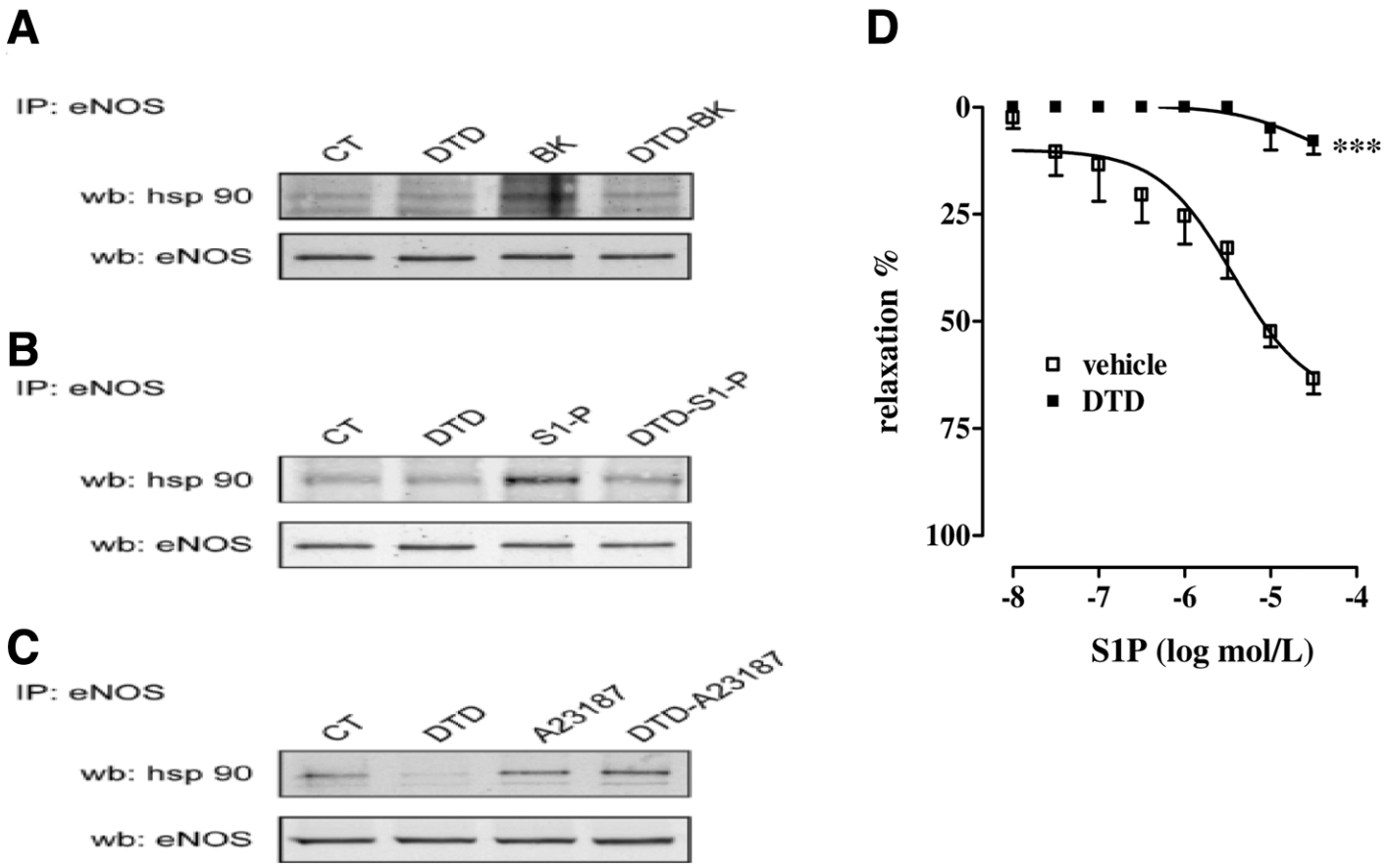

Figure 4. Stimulation of BAEC (10 min) with either BK (10 μmol/l, ***A***) or S1P (5 μmol/l; ***B***), or A23187 (5 μmol/l; ***C***) induces an increase in the amount of hsp90 detected in the eNOS immunocomplex (compare lanes 1 and 3). Inhibition of SPK by DTD (10 μmol/l, 10 min) markedly reduces the ability of S1P (***B***) or BK (***A***) to promote hsp90 recruitment to eNOS (compare lanes 3 and 4). Interestingly, DTD (***C***) fails to inhibit hsp90 recruitment by the receptor-independent calcium mobilizing agent A23187 (compare lanes 3 and 4). SIP-induced vasodilatation of the rat aorta is significantly inhibited by DTD (***D***). Data are mean ± SE; ***$P < 0.001$ vs. vehicle.

Fig. 5

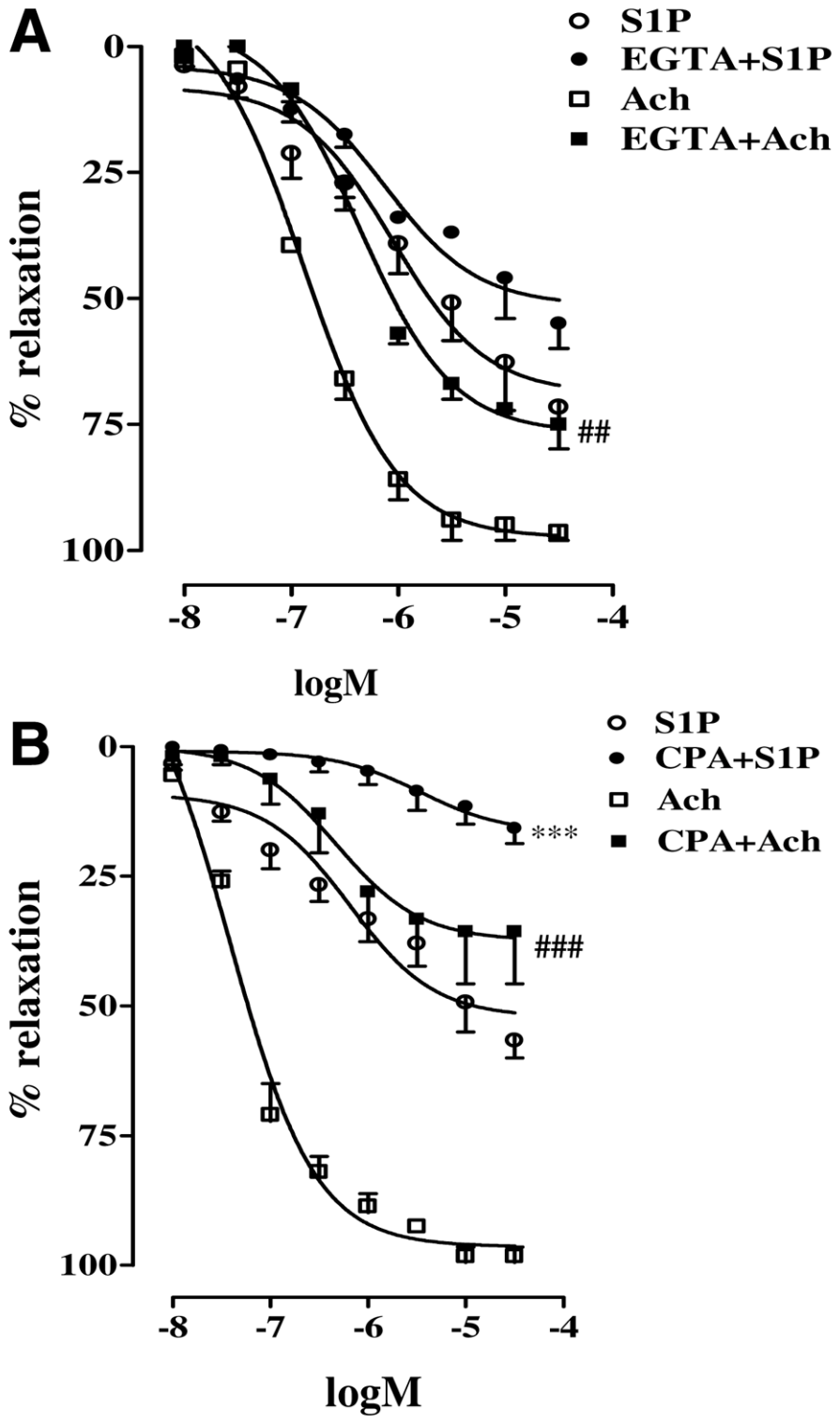

Figure 5. ***A*)** Pretreatment of aortic rings with EGTA (2.5 μmol/l), a calcium chelator, significantly inhibits Ach-induced vasodilatation (n=6; P<0.01) but has no effect on S1P-induced vasorelaxation. Data are mean ± SE; ##P < 0.001 vs. Ach. ***B*)** CPA (10 μmol/l) has a strong effect that inhibits both Ach- (n=6; P<0.001) and S1P-induced relaxation (n=6; P<0.001). Data are mean ± SE; ***P < 0.001 vs. S1P; ###P < 0.001 vs. Ach.