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Title page

Opposing Roles of Endothelial and Smooth Muscle Phosphatidylinositol 3-Kinase in Vasoconstriction: Effects of Rho-Kinase and Hypertension

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Running Title page

Running title: Endothelial PI3-kinase, rho-kinase and vasoconstriction

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Non-standard abbreviations:

DMSO: dimethyl sulfoxide

eNOS: endothelial nitric oxide synthase

EI: endothelium-intact

EX: endothelium-denuded

GPCR: G protein-coupled receptor

KCl: potassium chloride

KPSS: high potassium (K^+)-containing physiological saline solution

L-NAME: N^G-nitro-L-arginine methyl ester

NO: nitric oxide

PI3K: phosphatidylinositol 3-kinase

VSM: vascular smooth muscle

Y-27632: R-[+]-trans-N-[4-pyridyl]-4-[1-aminoethyl]-cycloheaxanecarboxamide

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Abstract

Phosphatidylinositol 3-kinase (PI3K) can activate endothelial nitric oxide synthase (eNOS), leading to production of the vasodilator nitric oxide (NO). By contrast, vascular smooth muscle (VSM) PI3K may partially mediate vascular contraction, particularly during hypertension. We tested whether endothelial and VSM PI3K may have opposing functional roles in regulating vascular contraction. Secondly, we tested whether the pro-contractile protein rho-kinase can suppress endothelial PI3K/eNOS activity in intact arteries, thus contributing to vasoconstriction by G protein-coupled receptor (GPCR) agonists. We studied contractile responses to the GPCR agonist phenylephrine, and the receptor-independent vasoconstrictor potassium chloride (KCl), in aortic rings from Sprague-Dawley rats. In endothelium-intact rings, the PI3K inhibitor wortmannin (0.1 μmol/L), markedly augmented responses to phenylephrine ($P<0.05$) by~50%, but not to KCl. However, in endothelium-denuded or L-NAME (100 μmol/L)-treated rings, wortmannin reduced responses to phenylephrine and KCl ($P<0.05$). Furthermore, the rho-kinase inhibitor Y-27632 (1 μmol/L) abolished responses to phenylephrine, and this effect was partially reversed by wortmannin or L-NAME. The ability of wortmannin to oppose the effect of rho-kinase inhibition on contractions to phenylephrine was L-NAME-sensitive. In aortas from angiotensin II-induced hypertensive rats, relaxation to acetylcholine (10 μmol/L) was impaired in normotensive controls ($P<0.05$), and vasoconstriction by phenylephrine was markedly enhanced and not further augmented by wortmannin. These data suggest that endothelial PI3K-induced NO production can modulate GPCR agonist-induced vascular contraction, and that this effect is impaired in hypertension in association with endothelial dysfunction. In addition, endothelial rho-

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kinase may act to suppress PI3K activity, and hence attenuate NO-mediated relaxation and augment GPCR-dependent contraction.

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Introduction

Endothelial phosphatidylinositol 3-kinase (PI3K) can be activated by diverse stimuli such as fluid shear stress (Huang et al., 2004), estrogen (Hisamoto et al., 2001) and growth factors (Gerber et al., 1998; Zeng et al., 2000). The PI3K signalling pathway stimulates the protein kinase Akt, leading to phosphorylation and activation of endothelial nitric oxide synthase (eNOS), resulting in increased production of nitric oxide (NO) (Dimmeler et al., 1999; Michell et al., 1999). Conversely, in vascular smooth muscle, the PI3K pathway has been reported to contribute to vascular contraction both under physiological conditions (Su et al., 2004), and in a model of hypertension (Northcott et al., 2002; Northcott et al., 2004). In addition, a recent study in cultured human endothelial cells provided novel evidence for an interaction between PI3K and the pro-contractile protein rho-kinase within the endothelium (Wolfrum et al., 2004). The findings suggest that rho-kinase may suppress PI3K activity and consequent NO production by endothelial cells (Wolfrum et al., 2004), in addition to its direct contractile effect on vascular smooth muscle in response to G protein-coupled-receptor (GPCR) agonists (Gohla et al., 2000; Miao et al., 2002). However, no functional evidence for this phenomenon has been demonstrated in intact arteries. Moreover, upregulation of the rhoA/rho-kinase signalling pathway in several vascular disorders (Kandabashi et al., 2000; Sato et al., 2000b) including hypertension (Chrissobolis and Sobey, 2001; Mukai et al., 2001) and angiotensin II-related vascular dysfunction (Yamakawa et al., 2000; Funakoshi et al., 2001; Takeda et al., 2001) is now widely recognized, but there is currently no information on the functional importance of endothelial rho-kinase in vascular disease.

Thus, the first aim of this study was to test whether endothelial and vascular smooth muscle PI3K have opposing roles in mediating vascular contraction following

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GPCR activation. Secondly, we tested for functional evidence of an interaction between endothelial rho-kinase and PI3K. Thirdly, we examined whether modulation of vascular contraction by endothelial or vascular smooth muscle PI3K is altered in angiotensin II-induced hypertension.

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Methods

All experimental procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee and complied with National Health and Medical Research Council of Australia guidelines. Adult male Sprague-Dawley rats (296±10 g, *n*=61) were studied.

Experimental protocol

Rats were euthanased by inhalation of 80% CO_2:20% O_2. The thoracic aorta was removed, cleaned of connective tissue and cut into four segments of equal length (4-5 mm). Ring segments were mounted at 0.5 g in 10 mL organ chambers containing Krebs-bicarbonate solution bubbled with 5% CO_2 in O_2 at 37°C. Isometric tension was continuously recorded using a Grass FT03 force transducer and MacLab4 Chart computer software (Version 3.5.4). Following equilibration for 45 min, each ring was exposed to isotonic high K^+-containing physiological saline solution (KPSS), in which Na^+ in Krebs solution was replaced by K^+ ($[K^+]_{KPSS}$=124 mmol/L). The KPSS-induced contraction was allowed to reach a stable level over 10-15 min. Following several washouts and return to stable baseline (~0.5 g), each ring was precontracted to ~50% of its KPSS response with serotonin (1-3 μmol/L) or phenylephrine (0.1-0.3 μmol/L). Sustained relaxation (>70% of precontracted tone) of aortic rings in response to acetylcholine (10 μmol/L) was taken to confirm the endothelium to be functionally intact. In some experiments, the endothelium was removed by gentle rubbing with a wooden stick and this was confirmed by failure to relax in response to acetylcholine. Smooth muscle viability of these rings was verified by a complete relaxation response to the NO donor, sodium nitroprusside (10 μmol/L). Following several washouts and return to stable baseline, concentration-response curves were established for two vasoconstrictor agents: the GPCR agonist

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phenylephrine, and the receptor-independent vasoconstrictor, potassium chloride (KCl).

Effect of PI3K and rho-kinase inhibition on contractile responses

The effect of PI3K inhibition was assessed by pre-treating aortic rings with wortmannin (0.1 μmol/L). Experiments were carried out in endothelium-intact and -denuded rings, as well as in endothelium-intact rings pre-treated with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L). In all sets of experiments with wortmannin, vehicle (dimethyl sulfoxide, DMSO)-treated rings served as controls.

The effect of the rho-kinase inhibitor Y-27632 (1 μmol/L) on contractile responses to phenylephrine or KCl was examined either alone or in the presence of wortmannin and/or L-NAME. Inhibitors were added 30 min before commencing concentration-response curves.

Short-term hypertension

Adult male Sprague-Dawley rats (*n*=5) were treated with angiotensin II (0.7 mg/kg per d s.c.) for 14 d via a surgically implanted osmotic minipump (Alzet Model 2001, Alza Corporation, CA, USA). A further three rats were implanted with minipumps containing vehicle (sterile water), thus serving as controls for the angiotensin II-treated rats. On Days 0 and 14, rats were anesthetized, a femoral artery was cannulated and arterial pressure was measured and recorded using a pressure transducer. On Day 14, rats were euthanased by intravenous anesthetic overdose. The thoracic aorta was isolated as described and concentration-response curves to phenylephrine were established in the presence or absence of wortmannin (0.1 μmol/L). Contractile responses of aortic rings from vehicle-treated rats did not differ

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from responses of non-operated controls. These data were therefore combined for analyses, as appropriate.

Drugs

Acetylcholine chloride was obtained from Research Organics (Cleveland, OH, USA) and potassium chloride was obtained from Ajax Finechem (Seven Hills, NSW, Australia). Y-27632 (R-[+]-trans-N-[4-pyridyl]-4-[1-aminoethyl]-cyclohexanecarboxamide) was generously provided by Welfide Corporation (Osaka, Japan). All other drugs were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Wortmannin was dissolved in DMSO and diluted in deionized water. All other drugs were dissolved and diluted in deionized water or saline. At the final bath concentration used (0.005%), DMSO vehicle had no effect on contractile responses.

Statistics

In each ring, contractile responses were normalized as a percentage of the KPSS response. Relaxation responses to acetylcholine are presented as percentage inhibition of precontractile tone. Each *n* represents the number of animals used. Comparisons were made using Student's paired or unpaired *t* tests or ANOVA, as appropriate. Newman-Keuls test was used for post-hoc comparisons. $P<0.05$ was considered significant.

Results

Effect of PI3K inhibition on contractile responses

In endothelium-intact aortic rings, the PI3K inhibitor wortmannin augmented the maximum response to phenylephrine by ~50% ($P<0.05$; Figure 1A), but reduced the maximum response to KCl ($P<0.05$; Figure 1B). Wortmannin also augmented the maximum response to a second GPCR agonist serotonin (data not shown). By contrast, in either endothelium-denuded or L-NAME-treated rings, wortmannin slightly reduced (by <10%) maximum responses to both phenylephrine and KCl ($P<0.05$; Figures 1C-F).

Effects of rho-kinase inhibition alone or combined with PI3K inhibition

In endothelium-intact aortic rings, the rho-kinase inhibitor Y-27632 effectively abolished contractions to phenylephrine ($P<0.05$; Figure 2A). In endothelium-denuded and L-NAME-treated rings, Y-27632 also reduced maximum responses to phenylephrine ($P<0.05$; Figures 2C and E), but by substantially less than in endothelium-intact rings (see Figure 2A). Co-treatment with wortmannin partially reversed the inhibitory effect of Y-27632 in endothelium-intact rings ($P<0.05$; Figure 2A), but not in endothelium-denuded or L-NAME-treated rings (Figures 2C and E). In contrast, Y-27632 only modestly attenuated responses to KCl, and to a similar degree, in all rings (Figure 2B, D and F). Moreover, co-treatment with wortmannin did not reverse the inhibitory effect of Y-27632 on KCl, but in fact further reduced the contractions to KCl in all rings (Figure 2B, D and F).

Effect of PI3K inhibition on contractile responses of hypertensive vessels

After 14 days, angiotensin II treatment caused a marked increase in mean arterial pressure to 169±2 mmHg (n=5, $P<0.05$; Figure 3A), compared with 97±5 mmHg in vehicle-treated rats (n=3). Relaxations to acetylcholine were markedly

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impaired in the hypertensive vessels (*P*<0.05; Figure 3B) while contractions to phenylephrine were enhanced in hypertension by ~30% (*P*<0.05; Figure 3C). Importantly, maximum responses to phenylephrine were unaffected by wortmannin in hypertension (Figure 3D). Furthermore, responses to serotonin were similarly augmented in hypertensive arteries and were also unaffected by wortmannin (data not shown).

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Discussion

The present study provides functional evidence for several roles of PI3K in the regulation of vascular tone. First, NO generated as a result of endothelial PI3K basal activity may modulate GPCR-mediated vascular smooth muscle contraction. Second, rho-kinase within endothelium may suppress PI3K activity and consequently reduce NO activity and augment GPCR- mediated vasoconstriction. Third, vascular smooth muscle PI3K may have a minor role in mediating GPCR-dependent and –independent vascular contraction. Fourth, the ability of endothelial PI3K to offset vasoconstriction appears to be impaired in hypertension in association with endothelial dysfunction, leading to augmented vascular contractility.

Physiological roles of vascular PI3K

Endothelial PI3K can stimulate production of NO via activation of the protein kinase Akt, and consequent phosphorylation and activation of eNOS in response to various stimuli (Zeng et al., 2000; Hisamoto et al., 2001). Little is known regarding the functional roles of endothelial PI3K in modulating contractile responses in intact arteries. In the present study, wortmannin markedly augmented vasoconstriction by phenylephrine, but attenuated responses to KCl. We observed a similar potentiation of responses to a second GPCR agonist, serotonin, following PI3K inhibition (data not shown). In contrast, in endothelium-denuded or L-NAME-treated rings, wortmannin attenuated contractile responses to both phenylephrine and KCl. These findings suggest that endothelial PI3K can counteract GPCR-mediated vascular contractions via eNOS/NO.

The effect of PI3K inhibition to attenuate all contractile responses in endothelium-denuded or L-NAME-treated aorta suggests a direct role for vascular smooth muscle PI3K in vascular contraction, consistent with two recent studies (Yang

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et al., 2001; Su et al., 2004). The precise mechanism(s) by which vascular smooth muscle PI3K might promote contraction is still unclear, but could include antagonism of cyclic nucleotide signaling pathways (Komalavilas et al., 2001), interactions with protein kinase C (Su et al., 2004) or regulation of voltage-gated calcium channels (Macrez et al., 2001).

Interaction between endothelial rho-kinase and PI3K

The small G protein rhoA and its downstream effector rho-kinase contribute to contraction of vascular smooth muscle via "calcium sensitization"(Somlyo and Somlyo, 2000), and activity of both proteins is upregulated in hypercontractile vascular diseases such as hypertension (Chrissobolis and Sobey, 2001; Mukai et al., 2001), atherosclerosis (Miyata et al., 2000), and coronary and cerebral vasospasm (Katsumata et al., 1997; Sato et al., 2000a). Although the functional roles of rhoA/rho-kinase in vascular smooth muscle have been studied extensively, there is very little known about the functional importance of endothelial rho-kinase in modulation of vascular tone. In cultured endothelial cells, rhoA can negatively regulate eNOS protein expression by destabilising eNOS mRNA (Laufs and Liao, 1998), and possibly regulate eNOS phosphorylation via inhibitory effects on PI3K and Akt (Ming et al., 2002). A more recent study has provided evidence that rho-kinase may attenuate NO production in cultured endothelial cells via inhibition of endothelial PI3K activity (Wolfrum et al., 2004). In the present study, we found functional evidence for such an effect of endothelial rho-kinase. Treatment with the rho-kinase inhibitor Y-27632 effectively abolished contractile responses to phenylephrine, whereas responses to KCl were only modestly reduced, consistent with previous findings that responses to GPCR agonists are particularly sensitive to rho-kinase inhibition (Uehata et al., 1997; Budzyn et al., 2004). We now provide two

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novel findings in relation to the functional importance of endothelial rho-kinase. Firstly, inhibition of GPCR-mediated contraction by Y-27632 is substantially endothelium- and eNOS-dependent, indicating that endothelial rho-kinase normally suppresses relaxant effects of eNOS-derived NO. Secondly, additional treatment with wortmannin selectively attenuated the inhibitory effect of Y-27632 on responses to phenylephrine in a strictly endothelium- and eNOS-dependent manner. Thus, these findings provide the first functional evidence for an interaction between endothelial rho-kinase and PI3K in mediating vasoconstriction by a GPCR agonist. Interestingly, an additional pro-contractile effect of rho-kinase as an inhibitor of endothelial PI3K activity is consistent with observations that vasoconstrictor sensitivity to rho-kinase inhibition is markedly diminished in the absence of eNOS function (Chitaley and Webb, 2002; Budzyn et al., 2004). Thus, a novel implication of our study is that inhibition of GPCR-dependent vascular contraction by Y-27632, and other rho-kinase inhibitors, is partially endothelium- and PI3K/eNOS-dependent.

Role of PI3K during hypertension

In association with profound hypertension and endothelial dysfunction following angiotensin II infusion for 14 days, we observed markedly enhanced responses to the rho-kinase-dependent GPCR agonist phenylephrine. Importantly, PI3K inhibition did not augment contractile responses to phenylephrine (or serotonin; data not shown) in these arteries. This is consistent with (i) a vasoconstrictor response unopposed by PI3K/eNOS-derived NO, perhaps due to the lack of functional PI3K in endothelium; but (ii) normal levels of PI3K activity in vascular smooth muscle of hypertensive vessels, unlike in the DOCA-salt model in which plasma levels of angiotensin II are not elevated (Northcott et al., 2002; Northcott et al., 2004).

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Therapeutic implications

This study has provided functional evidence compatible with a potentially important role of endothelial PI3K in modulating vascular tone, and for an inhibitory effect on this enzyme by endothelial rho-kinase. In many cardiovascular disease states, diminished NO levels are paralleled by increased expression and/or activity of rhoA/rho-kinase (Harrison, 1997; Shimokawa, 2002). Thus, redressing the imbalance between these two opposing systems could be an attractive therapeutic approach. Our data are therefore compatible with a new concept that excessive stimulation of the rhoA/rho-kinase pathway exacerbates endothelial dysfunction by reducing PI3K/eNOS activity. Consistent with this, it is interesting to note that statins, a widely used class of drugs for cholesterol lowering, also exert 'pleiotropic' effects that include improved NO bioavailability via both rhoA inhibition (Laufs and Liao, 1998) and stimulation of the PI3K/Akt pathway (Mukai et al., 2003). Furthermore, it is conceivable that the beneficial clinical effects of the rho-kinase inhibitor fasudil in the treatment of cerebral vasospasm, a severe complication characterized by endothelial dysfunction following subarachnoid hemorrhage (Sobey and Faraci, 1998), are in part related to improved endothelial function as well as its direct effects on vascular contractility.

Selectivity of pharmacological inhibition of PI3K

It is important to recognize that eight distinct isoforms of PI3K have been identified. These are grouped into three main classes based on their protein structure, substrate specificity and regulation (Foster et al., 2003; Wymann et al., 2003). Whilst wortmannin is well established as a selective PI3K inhibitor at the concentration used here (0.1 μmol/L), it inactivates all eight PI3K isoforms as does LY294002 (Wymann et al., 2003), and there are currently no isoform-selective PI3K inhibitors

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commercially available. Thus, it is conceivable that the different roles of endothelial versus vascular smooth muscle PI3K in regulation of vascular tone are mediated by different isoforms of PI3K. To answer this question, future studies will require the development of isoform-selective PI3K inhibitors and/or the use of gene knockout technology (Katso et al., 2001).

In summary, endothelial and vascular smooth muscle PI3K appear to have opposing roles in regulating vascular tone, with endothelial PI3K modulating GPCR-dependent vasoconstriction via the effects of NO. Moreover, this study has provided the first functional evidence for negative regulation of endothelial PI3K by endothelial rho-kinase. In angiotensin II-induced hypertension, endothelial dysfunction and enhanced vascular contraction are associated with impaired PI3K function in endothelium, conceivably due to enhanced rho-kinase activity. Thus, inhibition of excessive rho-kinase activity may serve to improve endothelial NO production in vascular disease.

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Footnotes

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Legends for Figures

Figure 1 Effects of PI3K inhibition

Left column: Concentration-response curves to phenylephrine in the presence of vehicle (VEH) or wortmannin (WORT, 0.1 μmol/L) in endothelium-intact (EI, *n*=16; **A**), endothelium-denuded (EX, *n*=6; **C**) and L-NAME (100 μmol/L)-treated aortic rings (*n*=6; **E**). **Right column:** Concentration-response curves to KCl in the presence of VEH or WORT in EI (*n*=14; **B**), EX (*n*=6; **D**) and L-NAME-treated aortic rings (*n*=6; **F**). All values are mean±SE. **P*<0.05 vs VEH maximum, Student's paired *t* test.

Figure 2 Effects of rho-kinase inhibition alone or combined with PI3K inhibition

Left column: Concentration-response curves to phenylephrine in the presence of Y-27632 (1 μmol/L) alone, and in combination with wortmannin (WORT, 0.1 μmol/L) in endothelium-intact (EI, *n*=8; **A**), endothelium-denuded (EX, *n*=6; **C**) and L-NAME (100 μmol/L)-treated aortic rings (*n*=6; **E**). **Right column:** Concentration-response curves to KCl in the presence of Y-27632 alone, and in combination with WORT in EI (*n*=6; **B**), EX (*n*=7; **D**) and L-NAME-treated aortic rings (*n*=6; **F**). All values are mean±SE. **P*<0.05 vs VEH maximum, †*P*<0.05 vs Y-27632 maximum, one-way ANOVA (repeated measures) and Newman-Keuls post-hoc test.

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Figure 3 Effects of angiotensin II treatment

(A) Mean arterial pressure (MAP) of rats at Days 0 and 14 of angiotensin II (0.7 mg/kg per d s.c.) treatment (*n*=5); **(B)** relaxation responses to acetylcholine (ACh, 10 μmol/L) of aortic rings from normotensive control (NBP, *n*=33) and hypertensive rats (HBP, *n*=5); **(C)** concentration-response curves to phenylephrine in aortic rings from NBP (*n*=16) and HBP rats (*n*=5); **(D)** concentration-response curves to phenylephrine in the presence of vehicle (VEH) or wortmannin (WORT, 0.1 μmol/L) in aortic rings from HBP rats (*n*=5). All values are mean±SE. *P<0.05 vs Day 0, Student's paired *t* test; †P<0.05 vs NBP, Student's unpaired *t* test.

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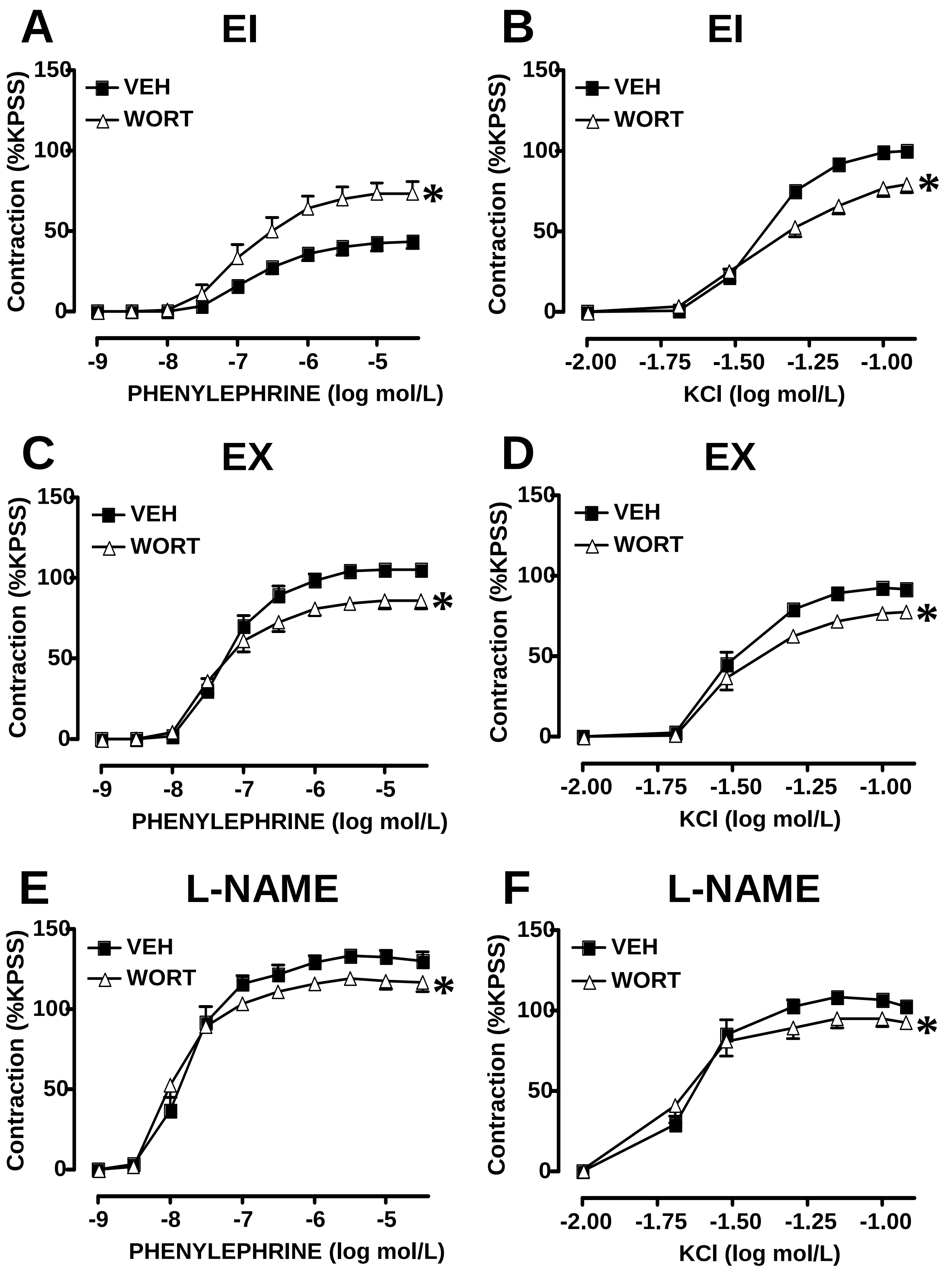

Figure 1

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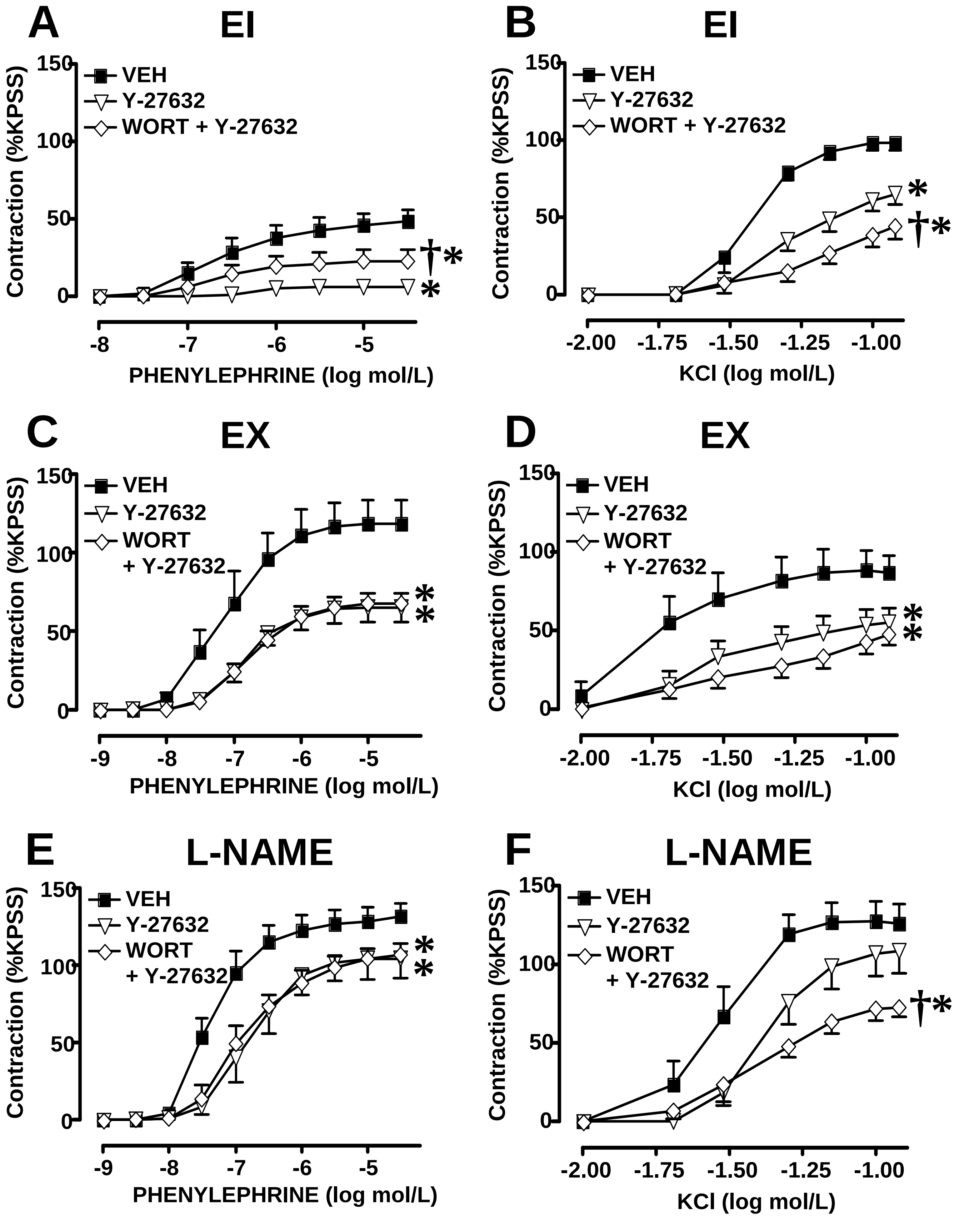

Figure 2

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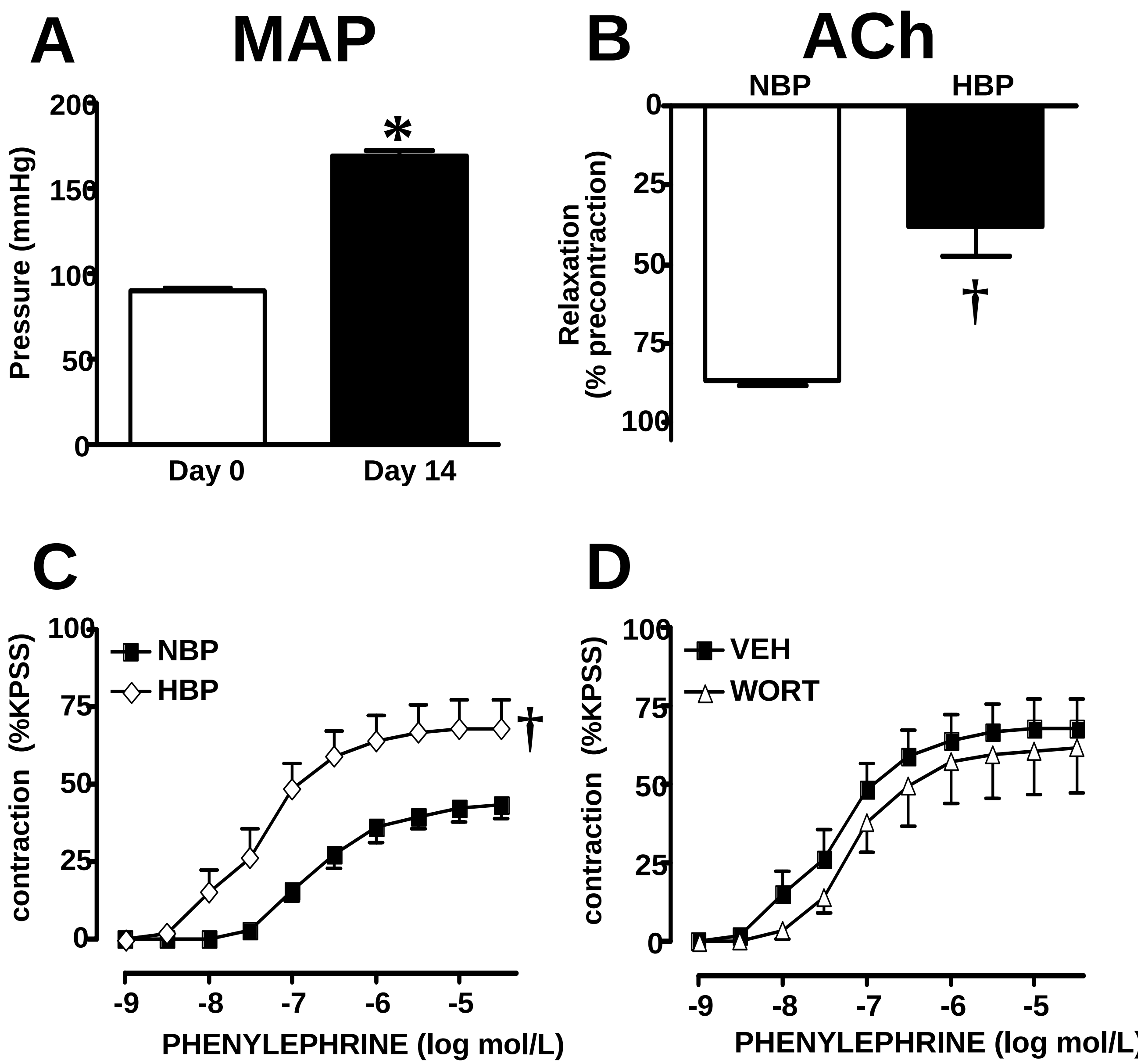

Figure 3