Inhibition of Phosphoinositide 3-Kinase Potentiates Relaxation of Porcine Coronary Arteries Induced by Nitroglycerin by Decreasing Phosphodiesterase Type 5 Activity

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Background: Vessel tension can be modulated by phosphoinositide 3-kinase (PI3K) acting on \( \alpha \)-type calcium channel, rho kinase and phosphodiesterase (PDE) type 3 in smooth muscle cells. Inhibition of PI3K could increase the relaxation of porcine coronary arteries to nitroglycerin independent of this pathway, and the aim of the present study was therefore to determine the underlying mechanisms.

Methods and Results: Isolated porcine coronary arteries were dissected from the heart and cut into rings in ice-cold modified Krebs-Ringer bicarbonate buffer. The response of these vessels was studied by using the organ chamber technique; the content of cyclic guanosine monophosphate (cGMP) was determined by using enzyme-linked immunosorbent assay kit; and PI3K and Akt activity were determined by measuring the phosphorylation level of their downstream signaling molecule on Western blot. Inhibition of PI3K with 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002) potentiated the relaxation of porcine coronary arteries to nitroglycerin and nitric oxide (NO), but not to 8-bromo-guanosine 3’5’-cyclic monophosphate, isoproterenol or (R)-(+)trans-4-(1-Aminoethyl)-N-(4-Pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate (Y27632). Increased relaxation induced by LY294002 was eliminated by Akt1/2 kinase inhibitor (Akt-I: 1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazo(4,5-g)quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate) or zaprinast, but was not affected by 1H-(1,2,4)oxadiazolo(4,3-\( \alpha \))quinoxalin-1-one, nifedipine or milrinone. Inhibition of Akt caused similar effects as LY294002. Incubation with LY294002 or Akt-I decreased the activity of PI3K and Akt but augmented the elevation of cGMP caused by NO. Enhanced cGMP elevation by LY294002 or Akt-I was also eliminated by zaprinast.

Conclusions: PI3K–Akt signaling may affect vascular tone through a stimulatory effect on PDE type 5. (Circ J 2012; 76: 230–237)

Key Words: Nitric oxide; Smooth muscle; Vasodilatation

Phosphoinositide 3-kinase (PI3K) participates in various cellular functions such as cell survival, metabolism, angiogenesis, adhesion and proliferation in vascular system.1–3 Some studies have found that PI3K signaling is also a key regulator of vascular tone.4 In the endothelium, activation of the PI3K/Akt pathway activates endothelial nitric oxide synthase (eNOS) by phosphorylating eNOS at ser-1177, and also enhances nitric oxide (NO) release.5 Olmesartan and azel-nidipine improve severely impaired vasodilatation in diabetic ApoE(–/–) mice by activation of the Akt/eNOS pathway.6 Studies also suggest that PI3K affects the constriction of vascular smooth muscle via actions on \( \alpha \)-type calcium channel,7–11 rho kinase12–14 and phosphodiesterase type 3 (PDE3).15,16 Nitroglycerin is widely used in the treatment of angina pectoris, hypertension and acute heart failure. It causes vasodilatation after being converted to NO or NO-related intermediate, followed by elevation of cyclic guanosine monophosphate (cGMP) via soluble guanylyl cyclase (sGC) and activation of...
cGMP-dependent protein kinase (PKG). Phosphodiesterase type 5 (PDE5) is essential to cGMP hydrolysis in vascular smooth muscle cells and plays an important role in the vasodilation caused by nitroglycerin and NO. Activity of PDE5 is enhanced by phosphorylation of the enzyme at serine 92 by PKG and cAMP-dependent protein kinase (PKA). Previous studies have shown that PDE3 activity is modulated by PI3K, prompting us to test whether PI3K may also regulate the activity of PDE5. The present study demonstrates that inhibition of PI3K increases the relaxation of porcine coronary arteries induced specifically by nitrate, but not by 8-bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP), isoproterenol or Y27632, a specific inhibitor of rho kinase. Inhibition of PI3K also enhanced the cGMP elevation of porcine coronary arteries induced by NO. The effect was eliminated by zaprinast, a specific PDE5 inhibitor, but not by nifedipine, milrinone or 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), the specific inhibitor of sGC. Hence, the present results suggest that PI3K may stimulate the activity of PDE5 and thereby increase the degradation of cGMP and attenuate the relaxation mediated by nitrovasodilators.

**Methods**

**Porcine Coronary Artery Preparation**

Fresh porcine hearts were collected from a local slaughterhouse. The porcine coronary left circumflex arteries and left anterior descending arteries were carefully dissected and cut into rings (length: 5 mm) in ice-cold modified Krebs-Ringer bicarbonate buffer (composition [in mmol/L]: NaCl, 118.3; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25.0; glucose, 11.1). Vessel Tension

Rings of coronary arteries were repeatedly rinsed and suspended in organ chambers filled with 10 ml of the modified Krebs-Ringer bicarbonate solution maintained at 37±0.1°C and aerated with 95% O₂–5% CO₂ (pH=7.4). Two stirrups passed through the lumen suspended on each ring. One stirrup was anchored to the bottom of the organ chamber, the other

![Figure 1](image-url)
one was connected to a strain gauge, and the isometric force was measured with a force transducer (MLT0202/D; ADInstruments, Castle Hill, NSW, Australia) and recorded with an ML785 PowerLab/8sp Recording and Analysis System (ADInstruments). 3,28

At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension. This was achieved by step-wise stretching until the active contraction of the vessel ring to 100 nmol/L KCl reached a plateau. The optimal resting tension of porcine coronary arteries was approximately 2.5 g. After the vessels were brought to optimal resting tension, 1 h of equilibration was allowed. Effects of nitroglycerin, 2,2’-((hydroxy)nitrosohydrazono) bis(ethanamine) (DETA NONOate), 8-Br-cGMP, (R)-(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate (Y27632) and isoproterenol were determined in vessels pre-constricted with (9,11)-dideoxy-(11β,9α)-epoxymetha-9,12-dienoic acid (U46619). The concentration–response curves for these dilators were constructed in a cumulative fashion.

In some experiments 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002), Akt1/2 kinase inhibitor (Akt-I: 1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinolin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazo[2-1]aziridinyl)-2-one trifluoroacetate salt hydrate; nifedipine (10−5 mol/L), milrinone (10−4 mol/L) and zaprinast (10−6 mol/L, 3×10−6 mol/L, 3×10−5 mol/L) were added 30 min before vessels were constricted with U46619 and remained in contact with the tissue throughout the experiment. All experiments were carried out in a parallel fashion under control conditions or with different treatments. To eliminate the possible involvement of endogenous prostanoids and endothelium-derived NO, indomethacin (10−5 mol/L) and nitro-l-arginine (10−4 mol/L) were included in the present study throughout the experiments. 29

Western Blot
After endothelium was denuded, isolated porcine coronary arteries were incubated in Krebs-Ringer bicarbonate buffer maintained at 37±0.5°C and aerated with 95% O2–5% CO2 (pH 7.4) for 3 h. Tissue samples were ground to a fine powder under liquid nitrogen. Tissue lysates were prepared from isolated porcine coronary arteries after treatment as aforementioned. Lysates each containing 20 μg of protein were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis, and electrotransferred to polyvinylidene fluoride. Non-specific binding of antibody was blocked by washing with TBS buffer containing 10% milk for 1 h. The blot was then subjected to 2 brief washes with TBS plus 0.5% Tween-20, incubated with the first antibody of Akt (Cell Signaling Technology, MA, USA; 1:1,000 dilution), Akt-p (S473; Cell Signaling Technology; 1:1,000 dilution), FoxO1 (Cell Signaling Technology; 1:1,000 dilution), FoxO1-p (S256; Cell Signaling Technology; 1:1,000 dilution), FoxO1 (Cell Signaling Technology; 1:1,000 dilution), FoxO1-p (S256; Cell Signaling Technology; 1:1,000 dilution), overnight at 4°C and the secondary antibody for 1 h at room temperature. The blot was developed using the chemiluminescent detection method (Amershams ECLTM). Proteins of Akt, Akt-p, FoxO1 or FoxO1-p in blots were quantified on densitometry using a Gel Doc 2000 densitometer (Bio-Rad, CA, USA) and normalized to scanning signals of actin (Calbiochem, CA, USA).

Enzyme-Linked Immunosorbutent Assay (ELISA) of cGMP
Rings of porcine coronary arteries were incubated in control solution containing indomethacin and nitro-l-arginine (10−4 mol/L; 37°C, 95% O2–5% CO2, pH 7.4) in the presence of solvent, LY294002, or Akt-I. In some experiments zaprinast (3×10−5 mol/L), a specific inhibitor of PDE5, was also present. After 30-min equilibration, DETA NONOate (10−4 mol/L) was added. Ten min later vessel rings were snap-frozen with liquid nitrogen. Tissue samples were ground to a fine powder under liquid nitrogen in a mortar. After the liquid nitrogen had evaporated, the frozen tissue was weighed and homogenized in 10 volumes of 0.1 mol/L HCl. Tissues were sonicated 3 times for...
3 s, and centrifuged (13,000 g for 10 min) at room temperature. The supernatant was extracted and the content of cGMP was determined using a monoclonal anti-cGMP antibody-based direct cGMP ELISA kit (catalog: 80103; NewEast Biosciences, Malvern, PA, USA). The protein content was determined based on the Bradford dye-binding procedure.

**Statistical Analysis**

Data are given as mean±SEM. Means of 2 groups were compared with 1-way ANOVA with Student-Newman-Keuls test for post-hoc testing of multiple comparisons. Statistical significance was accepted for P<0.05 (2 tailed). In all experiments, n represents the number of animals.

**Reagents**

The following drugs were used (unless otherwise specified, all were obtained from Sigma, St Louis, MO, USA): 8-Br-cGMP, DETA NONOate, Akt-I, LY294002, milrinone, nitroglycerin (NTG; Beijing Yimin Pharmaceutical, Beijing, China), ODQ, nifedipine, isoproterenol, U46619, Y27632 and zaprinast.

Akt-I, LY294002, milrinone, nitroglycerin and zaprinast were dissolved in DMSO (final concentration: <0.2%). Preliminary experiments showed that DMSO at the concentration used had no effect on contraction to U46619 and relaxation induced by the NO donor and cGMP analogs. The other drugs were prepared using distilled water.

**Results**

**Vessel Tension**

Rings of porcine coronary arteries were constricted with U46619 (3×10⁻⁷ mol/L) prior to testing their relaxant responses. There was no significant difference in tension of vessels evoked by U46619 between the control and LY294002 (10⁻⁵ mol/L) groups (16.2±1.6 g vs. 16.1±2.4 g, n=6–7 for each group, P>0.05).

Inhibition of PI3K with LY294002 at 10⁻⁵ mol/L but not at 10⁻⁶ mol/L or 3×10⁻⁶ mol/L significantly enhanced the relaxation of porcine coronary arteries to nitroglycerin (Figure 1A). Inhibition of PI3K with LY294002 (10⁻⁵ mol/L) also increased the relaxation of porcine coronary arteries to DETA NONOate (Figure 1B) but not to 8-Br-cGMP (Figure 1C).

Relaxation of the artery to nitroglycerin in the presence of ODQ (10⁻⁷ mol/L, a specific inhibitor of sGC) was less than that of the control. The augmentation of relaxation to nitroglycerin caused by inhibition of PI3K was not affected by ODQ (Figure 2A). The relaxation to nitroglycerin in the presence of...
zaprinast (3×10⁻⁵ mol/L or 3×10⁻⁶ mol/L, a specific inhibitor of PDE5) was greater than that in the absence of zaprinast. The augmentation of relaxation to nitroglycerin caused by inhibition of PI3K was fully eliminated by zaprinast at 3×10⁻⁵ mol/L (Figure 2B).

The augmentation of relaxation to nitroglycerin caused by LY294002 (10⁻⁵ mol/L) was not affected by nifedipine (10⁻⁵ mol/L, a specific inhibitor of L-type calcium channel; Figure 3A). Inhibition of PI3K with LY294002 (10⁻⁵ mol/L) had no effect on relaxation of the arteries induced by Y27632 (10⁻⁵ mol/L, a specific inhibitor of rho kinase; Figure 3B) or isoproterenol (Figure 3C). The enhanced relaxation to nitroglycerin caused by inhibition of PI3K was not affected by milrinone (10⁻⁴ mol/L, a specific inhibitor of PDE3; Figure 3D).

Inhibition of Akt with Akt-I (10⁻⁵ mol/L, a specific inhibitor of Akt1/2 kinase) significantly potentiated the relaxation of the artery induced by nitroglycerin. Akt-I plus LY294002 caused a similar effect on the relaxation of porcine coronary arteries to nitroglycerin as compared with LY294002 alone (Figure 4A). Inhibition of Akt also enhanced relaxation induced by DETA NONOate (Figure 4B), but not that induced by 8-Br-cGMP (Figure 4C). Relaxation induced by nitroglycerin was potentiated by zaprinast (3×10⁻⁵ mol/L; a specific inhibitor of PDE5). The augmentation of relaxation to nitroglycerin caused by inhibition of Akt was eliminated by zaprinast (Figure 4D).

Western Blot
Incubation of porcine coronary arteries with LY294002 for 30 min reduced the protein level of phosphorylated Akt significantly. The protein level of total Akt in the different groups was not significantly changed (Figure 5A). Incubation with LY294002 or Akt-I for 30 min decreased the protein level of phosphorylated FoxO1 significantly. The protein level of total FoxO1 in the different groups was not significantly affected (Figure 5B).

cGMP Assay
DETA NONOate (10⁻⁴ mol/L; 10 min) significantly increased the cGMP content of porcine coronary artery. Inhibition of PI3K with LY294002 potentiated the elevation of cGMP of the artery caused by DETA NONOate. The elevation of cGMP induced by DETA NONOate in the presence of zaprinast (3×10⁻⁵ mol/L, a specific inhibitor of PDE5) was more than that in those not treated with zaprinast. The augmentation of

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**Figure 4.** (A) Increased relaxation of nitroglycerin induced by 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002; 10⁻⁵ mol/L) was prevented by 1,3-dihydro-1-[(4-(6-phenyl-1H-imidazo(4,5-g)quinolin-7-yl)phenyl)methyl]-4-piperidinyl]-2H-benzimidazol-2-one trifluoroacetate salt hydrate (Akt-I; 10⁻⁵ mol/L) in porcine coronary arteries; (B) inhibition of Akt enhanced relaxation induced by 2,2'-(hydroxynitrosohydrazono) bis(ethanamine) (DETA NONOate) but (C) not that caused by 8-bromo-guanosine 3'5'-cyclic monophosphate (8-Br-cGMP). (D) Increased relaxation of nitroglycerin induced by Akt-I (10⁻⁵ mol/L) was prevented by zaprinast (3×10⁻⁵ mol/L). Data are given as mean±SEM; n=4–6 for each group. *Significant difference between control group and LY294002 group (P<0.05).
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Figure 5. (A) Akt-p (S473) and total Akt protein of porcine coronary arteries following 0.5-h incubation with solvent or 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002; 10−5 mol/L). (B) FoxO1-p (S256) and total FoxO1 protein of porcine coronary arteries following 0.5-h incubation with solvent, LY294002 (10−5 mol/L) or Akt-I (10−6 mol/L). (Lower panels) Densitometric scanning of (A) Akt-p (S473) protein normalized to total Akt or (B) FoxO1-p (S256) protein normalized to total FoxO1. Data given as mean±SEM; n=6 for each group. *Significantly different from the solvent group (P<0.05).

Figure 6. Elevation of the intracellular content of cyclic guanosine monophosphate (cGMP) of porcine coronary arteries caused by 2,2’-(hydroxynitrosohydrazono) bis(ethanamine) (DETA NONOate) (10−4 mol/L) in the (A) absence of solvent, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002; 10−5 mol/L), zaprinast (10−4 mol/L) and LY294002 plus zaprinast or (B) in the presence of solvent, 1,3-dihydro-1-[(4-((6-phenyl-1H-imidazo(4,5-g)quinolin-7-yl)phenyl)methyl)-4-piperidinyl]-2H-benimidazol-2-one trifluoracetate salt hydrate (Akt-I; 10−5 mol/L), zaprinast (10−4 mol/L) and Akt-I plus zaprinast. Data are given as mean±SEM; n=6 for each group. *Significantly different from the respective controls (P<0.05); †significantly different from the solvent group (P<0.05).

elevation of cGMP caused by inhibition of PI3K, however, was eliminated by zaprinast (Figure 6A).

Inhibition of Akt also potentiated the elevation of cGMP of the arteries caused by DETA NONOate. Elevation of cGMP induced by DETA NONOate was augmented by zaprinast but the augmentation of elevation of cGMP caused by inhibition of Akt was eliminated by zaprinast (Figure 6B).

Discussion

Previous studies have demonstrated that nitroglycerin is mainly converted into NO or NO-related intermediates by mitocon-
glycerin. LY294002 plus Akt inhibitor, however, had no additional effect. Akt, a serine/threonine protein kinase, is a key downstream signaling molecule of PI3K and plays a critical role in multiple processes involved. While inhibition of PI3K significantly potentiates the relaxation of porcine coronary arteries caused by nitroglycerin and NO, but not by 8-Br-cGMP (Figure 1). Hence, it is likely that inhibition of PI3K augments the elevation of cGMP but not the activation of PKG by cGMP.

It is well known that cGMP is a second messenger and plays a key role in regulation of vessel tension. cGMP is synthesized by guanylyl cyclase (GC), which converts guanosine-5'-triphosphate (GTP) to cGMP. GC exists in both membrane-bound (pGC; particulate guanylyl cyclase) and cytosolic forms (sGC). The pGC is activated by peptide hormones such as the atrial natriuretic factor, whereas sGC is typically activated by NO to stimulate cGMP synthesis. Cyclic nucleotide phosphodiesterase (PDE) degrades cGMP by hydrolyzing cGMP into 5'-GMP. Although there are 11 different PDE gene families expressed in mammalian tissues, the major PDE present in arterial smooth muscle are PDE1A, 1B and 1C, PDE3A and 3B, and PDE5. It is generally recognized that PDE5 is the most active cGMP-hydrolyzing PDE, while PDE1 and PDE3 can hydrolyze both cAMP and cGMP. In the present study, we found that the increased relaxation of porcine coronary arteries caused by inhibition of PI3K was not affected by ODQ, a specific inhibitor of sGC (Figure 2A), but was prevented by zaprinast (3×10⁻⁷ mol/L), a specific inhibitor of PDE5 (Figure 2B). Inhibition of PI3K by LY294002 potentiated the elevation of cGMP induced by DETANONOate significantly in porcine coronary arteries, and this was also blocked by zaprinast (Figure 2A). These results suggest that inhibition of PI3K may affect vasodilatation by inhibiting the degradation of cGMP through PDE5. PI3K inhibitor had no effect on the relaxation induced by 8-Br-cGMP (Figure 1C). This may be because 8-Br-cGMP is more resistant to PDE than cGMP.

PI3K may modulate vasoconstriction by acting on l-type calcium channels. The present study showed that the increased relaxation caused by LY294002 could not be prevented by nifedipine (Figure 3A). It has been reported that PI3K is involved in the activation of rho kinase induced by endothelin-1 in rabbit basilar artery. The present study showed that inhibition of PI3K had no effect on the relaxation of porcine coronary arteries to Y27632, the specific inhibitor of rho kinase (Figure 3B). Recent studies also showed that activation of PI3K could activate Akt and then increase the activity of PKG, which catalyzes and activates PDE5 instead of PDE3.

Activation of PI3K increases phosphorylation of Akt at serine 473, and active Akt can phosphorylate FoxO1 on serine 256. Thus, the levels of phosphorylation of Akt (S473) and FoxO1 (S256) represent the activity of PI3K and Akt. The present study showed that incubation of vessel with LY294002 or Akt-1 significantly decreased the activity of PI3K and Akt (Figure 3B), which provides evidence for the effectiveness of these inhibitors at the concentrations used. In conclusion, the present study has demonstrated that inhibition of PI3K specifically potentiates the vasodilatation induced by nitroglycerin and/or NO by decreasing the activity of PDE5. It may represent a novel mechanism by which PI3K modulates vascular tone. The present results also suggest that the modulation of PDE5 by PI3K is mediated by Akt. Recent studies have shown that both expression and activity of PI3K are upregulated in hypertension and diabetes. This new mechanism may be involved in the development of hypertension or diabetic arterial disease.

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