Adrenomedullin Induces Endothelium-Dependent Vasorelaxation via the Phosphatidylinositol 3-Kinase/Akt–Dependent Pathway in Rat Aorta

Hiroaki Nishimatsu, Etsu Suzuki, Daisuke Nagata, Nobuo Moriyama, Hiroshi Satonaka, Kenneth Walsh, Masataka Sata, Kenji Kangawa, Hisayuki Matsu, Atsuo Goto, Tadaichi Kitamura, Yasunobu Hirata

Abstract—To study the mechanisms by which adrenomedullin (AM) induces endothelium-dependent vasorelaxation, we examined whether AM-induced endothelium-dependent vasodilation was mediated by the phosphatidylinositol 3-kinase (PI3K)/Akt-dependent pathway in rat aorta, because it was recently reported that PI3K/Akt was implicated in the activation of endothelial NO synthase. AM-induced vasorelaxation in thoracic aorta with intact endothelium was inhibited by pretreatment with PI3K inhibitors to the same level as that in endothelium-denuded aorta. AM elicited Akt phosphorylation in a time- and dose-dependent manner. AM-induced Akt phosphorylation was inhibited by pretreatment with a calmodulin-dependent protein kinase inhibitor as well as with PI3K inhibitors. When an adenovirus construct expressing a dominant-negative Akt mutant (Ad/dnAkt) was injected into abdominal aortas so that the mutant was expressed predominantly in the endothelium layer, AM-induced vasodilation was diminished to the same level as that in endothelium-denuded aortas. Finally, AM-induced cGMP production, which was used as an indicator for NO production, was suppressed by PI3K inhibition or by Ad/dnAkt infection into the endothelium. These results suggested that AM induced Akt activation in the endothelium via the Ca2+/calmodulin-dependent pathway and that this was implicated in the production of NO, which in turn induced endothelium-dependent vasodilation in rat aorta. (Circ Res. 2001;89:63-70.)

Key Words: adrenomedullin ■ phosphatidylinositol 3-kinase ■ Akt ■ nitric oxide ■ gene transfer

Vascular endothelial cells (ECs) are critically implicated in the modulation of vascular tone by producing a variety of vasoactive substances. Among them, endothelin has a vasoconstrictive activity, whereas NO and prostacyclin possess a vasorelaxant activity. The modulation of vascular tone by ECs is known to be perturbed in pathophysiological states such as hypertension, diabetes mellitus, and hyperlipidemia.1

A novel peptide, which increases the level of cAMP in platelets, was recently isolated from human adrenal medulla and was dubbed adrenomedullin (AM).2 AM is a 52-amino acid peptide with a potent vasorelaxant activity and natriuretic activity.3–5 Although AM was originally isolated from human pheochromocytoma tissue, it is now well known that AM is also produced by vascular ECs, vascular smooth muscle cells, and macrophages,6–8 suggesting its role as a local mediator in normal and/or diseased vessels. Although little is known of the intracellular signaling pathways that are activated by AM, it is reported that AM increases intracellular cAMP and [Ca2+]i.9 AM also activates extracellular signal–regulated kinase (ERK), which appears to be involved in AM-induced cell proliferation.10–12 although AM seems to inhibit cell proliferation in some cells,13 depending on cell types. It was originally postulated that AM-induced vasorelaxation might be mediated by its effect on intracellular cAMP. However, we have recently shown that AM induced vasorelaxation, at least partly, in an endothelium-dependent manner and that endothelium-dependent vasorelaxation was mediated via the NO/cGMP-dependent pathway in rats.14 Furthermore, it has been reported that transgenic mice that were designed to express an excess amount of AM in vascular ECs showed lower blood pressure than wild-type mice and that the difference in blood pressure was abolished by administration of Nω-monomethyl-L-arginine to those mice,15 suggesting the involvement of an NO/cGMP-dependent pathway for the AM-induced vasorelaxation, although the molecular mechanisms by which AM activated the NO/cGMP-dependent pathway remained to be elucidated.
It is well established that Ca\(^{2+}\)/calmodulin (CaM) is a major activator of endothelial NO synthase (eNOS). When [Ca\(^{2+}\)]\(_i\) is increased, CaM binds to the CaM binding domain of eNOS, which in turn changes the conformation of eNOS by an “allosteric” effect and activates eNOS.\(^{16–18}\) It is therefore possible that AM activates eNOS via the Ca\(^{2+}\)/CaM-dependent pathway. However, it has been reported that eNOS is activated by direct phosphorylation of serine 1179 (Ser1179) by Akt, a downstream target of phosphatidylinositol 3-kinase (PI3K). When eNOS is phosphorylated on Ser1179, eNOS is activated under a very low concentration of Ca\(^{2+}\), which suggests that the affinity of eNOS to CaM increases by Ser1179 phosphorylation.\(^{19,20}\) Thus, it seemed to be of interest to examine whether AM induced activation of eNOS via the PI3K/Akt-dependent pathway.

In the present study, we examined the mechanisms by which AM induced endothelium-dependent vasorelaxation. We show that AM induces endothelium-dependent vasorelaxation and cGMP production via the PI3K/Akt-dependent pathway. We also show that AM induces phosphorylation of Akt and that AM-induced Akt phosphorylation depends on the Ca\(^{2+}\)/CaM-mediated pathway.

### Materials and Methods

#### Reagents
Phosphospecific anti-ERK1/2 antibody and phosphospecific anti-Akt antibody that recognize catalytically active ERK1/2 and Akt, respectively, were obtained from New England BioLabs. Anti-Akt and -ERK1 antibodies were obtained from Santa Cruz Biotechnologies. N\(^2\)-nitro-arginine methyl ester (L-NAME), forskolin, sodium nitroprusside (SNP), KN-93, A23187, wortmannin, and LY294002 (LY) (Jackson ImmunoResearch Laboratories) and with To-PRO-3 iodide for nuclear staining at a dilution of 1:500 (Molecular Probes). Antibodies that recognize catalytically active ERK1/2 and Akt were used at a dilution of 1:500. Antibodies that recognize catalytically active ERK1/2 and Akt were used at a dilution of 1:500. Antibodies that recognize catalytically active ERK1/2 and Akt were used at a dilution of 1:500. Antibodies that recognize catalytically active ERK1/2 and Akt were used at a dilution of 1:500.

#### Ex Vivo Experiments
Effects of AM on the tension of rat aortic rings were examined as previously described.\(^{14}\) In brief, thoracic aortas were excised from 12-week-old male Wistar rats, and aortic rings with or without endothelium were mounted in organ chambers filled with Krebs-Ringer bicarbonate solution at 37°C with constant bubbling of 95% oxygen/5% carbon dioxide. Isometric tension was recorded with a force transducer. The endothelium was denuded by gentle rubbing with a twist of cotton, and this was confirmed by the lack of acetylcholine-induced vasodilation. The aortic rings were precontracted with L-norepinephrine, and the effects of AM on vasodilation were studied. In some experiments, several reagents such as L-NAME, wortmannin, LY, and PD were added to the chambers before precontraction with L-norepinephrine to examine their effects on AM-induced vasorelaxation. To prepare protein extracts, rat aortas were placed in tubes containing oxygenated Krebs-Ringer bicarbonate solution at 37°C and incubated with AM or with or without pretreatment with several reagents such as KN-93, wortmannin, L.Y, and PD.

#### Preparation of Protein Extracts
Rat aortas were homogenized on ice in a Triton X-100 homogenization buffer (in mmol/L, HEPES [pH 7.5] 50, NaCl 150, EDTA 1, and DTT 1, as well as 2% Triton X-100 and 10% glycerol) containing 1 mmol/L PMSF, 2 μg/mL leupeptin, and 2 μg/mL aprotinin. After centrifugation for 20 minutes at 4°C, the cleared supernatant was used for Western blot analysis. Protein concentration was measured according to Bradford’s method (Bio-Rad).

#### Western Blot Analysis
Western blot analysis was performed as previously described.\(^{21}\) Antibodies that recognize catalytically active ERK1/2 and Akt were used at a dilution of 1:500.

#### Immunohistological Analysis of Adenovirus-Infected Abdominal Aorta
Rats were anesthetized with pentobarbital 48 hours after the injection of Ad/GFP into the abdominal aorta. Abdominal aortas were fixed by perfusion with 4% paraformaldehyde in PBS for 5 minutes. They were then excised and embedded in OCT (Sankyo-Miles Inc). Sections of 10 μm each were prepared using a cryostat at −28°C and then air dried and immersed in PBS. The sections were treated with 5% normal donkey serum (Jackson ImmunoResearch Laboratories), followed by overnight incubation at 4°C with anti-GFP antibody at a dilution of 1:200 (3E6 monoclonal antibody, Qbiogene). After washing them with PBS, the sections were incubated for 1 hour with 3.75 μg/mL of Cy-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories) and with To-PRO-3 iodide for nuclear staining at a dilution of 1:500 (Molecular Probes). Thereafter, the sections were washed with PBS, mounted with antifade substance, and observed under a confocal laser scanning microscope (MRC-1024, Japan Bio-Rad Laboratories).

#### Measurement of cGMP
Rat aortas were homogenized in 4% trichloroacetic acid (pH 4.0) on ice. After centrifugation, the supernatant was extracted 4 times with water-saturated ether and then evaporated. The pellets were redisolved in a buffer included in the ELISA kit. ELISA was performed according to the manufacturer’s recommendation (Amersham).

#### Statistical Analyses
Values are mean±SEM. The statistical analyses were performed using ANOVA followed by the Student-Neumann-Keul test. Differences with a P value of <0.05 were considered statistically significant.

### Results

#### AM-Induced Endothelium-Dependent Vasorelaxation Is Inhibited by Pretreatment With PI3K Inhibitors
We first examined the effects of PI3K inhibition on AM-induced endothelium-dependent vasorelaxation using rat tho-
AM ranging from $10^{-9}$ to $10^{-7}$ mol/L induced vasorelaxation in aortic rings with endothelium (E/1 aortic rings) precontracted with norepinephrine in a dose- dependent manner, whereas AM-induced vasorelaxation was diminished in aortic rings when endothelium was denuded (E/2 aortic rings). In fact, AM-induced vasorelaxation was negligible in E/2 aortic rings when $10^{-9}$ mol/L AM was administered, whereas $10^{-7}$ mol/L AM did induce vasodilation in E/1 aortic rings. Although AM, at concentrations of $10^{-8}$ and $10^{-7}$ mol/L, elicited slight vasorelaxation in E/2 aortic rings, the magnitude was 14.4% and 32.6%, respectively, compared with its effects on E/1 aortic rings at each concentration. Forskolin and SNP induced a potent vasodilation in E/2 aortic rings (data not shown). Thus, the reduction in AM-induced vasorelaxation observed in E/1 aortic rings did not seem to be due to potential injuries to smooth muscle layers when endothelium was denuded. Pretreatment of E/1 aortic rings with 20 μmol/L LY or 100 nmol/L wortmannin reduced AM-induced vasorelaxation almost to the same levels as AM-induced vasorelaxation in E/2 aortic rings. In contrast, pretreatment of E/1 aortic rings with 25 μmol/L PD, an inhibitor of mitogen-activated protein kinase kinase (MEK) 1/2, did not inhibit AM-induced vasodilation. AM-induced vasodilation in E/2 aortic rings was abolished by pretreatment with L-NAME. Furthermore, pretreatment of E/1 aortic rings with LY or wortmannin did not suppress forskolin- or SNP-induced vasodilation (data not shown). These results indicated that AM elicited endothelium-dependent vasodilation, at least in part, via the NO-dependent pathway in rat aorta and that AM-induced endothelium-dependent vasodilation was suppressed by PI3K inhibition but not by MEK inhibition. The results also suggested that the suppression of AM-induced endothelium-dependent vasodilation by PI3K inhibitors did not seem to be a result of the cytotoxic effects of LY and wortmannin on vascular smooth muscle cells, because vasodilation induced by cAMP and NO donor was intact.

**AM Induces Akt Phosphorylation in Rat Aorta**

We next examined whether AM induced phosphorylation of Akt in rat aorta. AM induced Akt phosphorylation in a time-dependent manner (Figure 2A). The phosphorylation of Akt peaked ~15 minutes after stimulation with $10^{-7}$ mol/L AM. Plotted amplitudes for Akt phosphorylation in rat aorta with or without endothelium. #P<0.05 vs E(-) (n=4).
AM also induced Akt phosphorylation in a dose-dependent fashion (Figure 2B). AM, even at a low concentration of $10^{-11}$ mol/L, phosphorylated Akt and $10^{-7}$ mol/L AM increased Akt phosphorylation by 5.3-fold ($n=4$, $P<0.01$). We also examined the extent of Akt phosphorylation in rat aorta in the presence and absence of endothelium (Figure 2C). The extent of Akt phosphorylation in denuded aorta was 69.5% ($n=3$, $P<0.05$) compared with that in intact aorta. This finding indicated that $\approx 30\%$ of total Akt phosphorylation occurred in a monolayer of endothelium in rat aorta, suggesting that enormous amounts of Akt were phosphorylated by AM in vascular endothelium.

Previous reports showed that AM induced activation of ERK. We, therefore, examined whether AM induced ERK phosphorylation in rat aorta (Figure 3). AM induced ERK phosphorylation in a dose-dependent manner in our ex vivo system. AM at $10^{-7}$ mol/L increased ERK phosphorylation by 2.4-fold ($n=3$, $P<0.01$) 15 minutes after stimulation, although the ERK-dependent pathway did not seem to be involved in AM-induced endothelium-dependent vasorelaxation.

We next examined whether LY and wortmannin indeed inhibited AM-induced Akt phosphorylation in our system. As shown in Figure 4, AM-induced Akt phosphorylation was significantly inhibited by pretreatment of aortas with LY or wortmannin, whereas pretreatment with PD did not significantly inhibit AM-induced Akt phosphorylation. In fact, AM-induced Akt phosphorylation was suppressed to a level lower than the control (nonstimulated) level by pretreatment with LY or wortmannin, suggesting a basal level of phosphorylation of Akt in the vessels.

**AM-Induced Akt Phosphorylation Is Mediated by the Ca$^{2+}$/CaM-Dependent Pathway**

Previous reports showed that PI3K activation was mediated by the Ca$^{2+}$/CaM-dependent pathway in some cells. It was also reported that Akt was activated by the Ca$^{2+}$/CaM-dependent pathway in some cells. We, therefore, wondered whether AM-induced Akt phosphorylation was mediated by the Ca$^{2+}$/CaM-dependent pathway. When the Ca$^{2+}$ concentration of Krebs-Ringer bicarbonate solution was reduced, AM-induced Akt phosphorylation decreased significantly in a dose-dependent manner (Figure 5A). We also examined the effects of the CaM-dependent protein kinase inhibitor KN93 on AM-induced Akt phosphorylation. AM-induced Akt phosphorylation was inhibited by pretreatment with KN93 in a dose-dependent fashion (Figure 5B). Furthermore, A23187, a calcium ionophore, increased Akt phosphorylation in a dose-dependent fashion, and A23187-induced Akt phosphorylation was abolished by pretreatment with PI3K inhibitors, suggesting that Akt phosphorylation induced by Ca$^{2+}$/CaM was mediated by the PI3K-dependent pathway in rat aorta (see online Figure 1 in data supplement available at http://www.circresaha.org).

**AM-Induced Endothelium-Dependent Vasodilation Is Diminished in Rat Aortas Expressing a Dominant-Negative Akt Mutant in the Endothelium**

To examine more specifically the role of Akt expressed in the endothelium on AM-induced endothelium-dependent vasodi-
in aortic rings prepared from Ad/dnAkt-infected aortas in a dose-dependent manner, AM-induced vasodilation was significantly reduced compared with that in Ad/GFP-infected aortas. In fact, the AM-induced vasodilation observed in Ad/dnAkt-infected aorta was reduced almost to the same extent as that observed in endothelium-denuded aorta, suggesting that Akt expressed in endothelium played a major role in AM-induced endothelium-dependent vasodilation.

**AM-Induced cGMP Production Is Suppressed by PI3K/Akt Inhibition**

We originally tried to examine whether AM induced eNOS phosphorylation and whether AM-induced eNOS phosphorylation was mediated by the PI3K/Akt-dependent pathway. We immunoprecipitated eNOS in protein extracts prepared from rat aortas and examined its phosphorylation by immunoblotting with antiphosphoserine antibody. However, we did not detect a significant amount of eNOS phosphorylation in aortas stimulated with AM (data not shown), probably because the amount of phosphorylated eNOS was under detectable levels. We therefore decided to use cGMP production in aortas as an indicator for NO production in endothelium. We examined whether AM induced cGMP production in rat aortas and whether AM-induced cGMP production was mediated by the PI3K/Akt-dependent pathway. AM significantly increased cGMP production in rat thoracic aortas in a dose-dependent manner (Figure 7A). AM at $10^{-7}$ mol/L increased the production of cGMP by 2.2-fold (n=3, P<0.05). AM-induced increase of cGMP production was significantly inhibited to basal levels by pretreatment with LY. AM-induced increase of cGMP production was also significantly inhibited to basal levels by chelating extracellular Ca$^{2+}$ with EGTA. Therefore, the suppression of Akt phosphorylation by PI3K inhibition or by inhibition of the Ca$^{2+}$/CaM-dependent pathway correlated with suppression of cGMP production by inhibition of the PI3K- or Ca$^{2+}$/CaM-dependent pathway in rat aortas. Furthermore, pretreatment with L-NAME also significantly inhibited AM-induced cGMP production to basal levels, suggesting that cGMP production could be an indicator for NO production in endothelium. We measured cGMP production in abdominal aorta infected with Ad/GFP or Ad/dnAkt (Figure 7B). Infection of the abdominal aorta with those adenovirus constructs did not remarkably change the basal production of cGMP. In contrast, AM-induced increase of cGMP production was significantly inhibited in Ad/dnAkt-infected aortas compared with that in Ad/GFP-infected and noninfected aortas, indicating that Akt expressed in the endothelium was involved in AM-induced NO/cGMP production.


**Discussion**

Although it was reported that AM had a potent vasorelaxant activity, the precise mechanisms were not clear. Because AM stimulates cAMP production, it was originally thought that cAMP was a sole second messenger for AM-induced vasodilation. On the other hand, we and others reported that AM elicited endothelium-dependent vasodilation via, at least
partly, the NO/cGMP-mediated pathway. Thus, it was possible that AM activated eNOS by stimulating the direct binding of CaM to eNOS and a subsequent conformational change of eNOS because AM reportedly has the capacity to increase \([\text{Ca}^{2+}]_{i}\) in endothelium. Here, we have shown that AM-induced endothelium-dependent vasodilation and AM-induced increase of cGMP production, which we used as an indicator for NO production, depended on the PI3K/Akt-mediated pathway. To our knowledge, this is the first report that shows that AM induces Akt phosphorylation. Although we detected Akt phosphorylation in whole vessels, several data presented in this paper indicated that Akt phosphorylation occurring in vascular endothelium played a major role in AM-induced endothelium-dependent vasodilation. First, pretreatment with LY or wortmannin of rat aortas with intact endothelium suppressed AM-induced vasodilation to the same level as that in endothelium-denuded aortas. LY and wortmannin did not seem to cause remarkable cytotoxic effects on vascular smooth muscle cell layers, because forskolin and SNP potently relaxed aortic rings pretreated with LY. Furthermore, pretreatment with PD did not inhibit AM-induced vasodilation in aortas with intact endothelium. Second, Akt phosphorylation occurring in a single layer of vascular endothelium was estimated to be \(\sim 30\%\) of the total phosphorylation of Akt occurring in the whole aorta, suggesting that a huge amount of Akt phosphorylation occurred in vascular endothelium. Third, cGMP production in rat aorta was significantly inhibited by pretreatment with LY, and this inhibition correlated with the suppression by LY of AM-induced endothelium-dependent vasorelaxation. Fourth, a dnAkt mutant that expressed predominantly in endothelium after infection with Ad/dnAkt inhibited AM-induced vasodilation to the same level as that observed in endothelium-denuded aortas. Finally, AM-induced increase of cGMP production was significantly suppressed in aortas that expressed dnAkt in the endothelium. Recently, two studies have shown that Akt directly phosphorylates eNOS on Ser1179 and that phosphorylated eNOS is active at a low \([\text{Ca}^{2+}]_{i}\) concentration. Thus, eNOS phosphorylation on Ser1179 appears to be critical for facilitating CaM binding to eNOS. Our results indicated that AM-induced vasodilation was almost entirely endothelium dependent at a low concentration of AM (10^{-9} \text{ mol/L}), at which concentration AM could stimulate Akt phosphorylation and cGMP production. In contrast, at high concentrations (10^{-8} \text{ mol/L} and 10^{-7} \text{ mol/L}), AM-induced vasodilation was mediated, partly, by an endothelium-independent mechanism; this was probably a direct effect of AM on vascular smooth muscle cells leading to an increase of intracellular cAMP and vasodilation. Thus, although endothelium dependence of AM-induced vasodilation reportedly relies on the species and vessels used, it appears that, under physiological conditions, AM-induced vasorelaxation is largely endothelium dependent and mediated by Akt activation, at least, in rat aorta.

Although it was reported that \([\text{Ca}^{2+}]_{i}\) was dispensable for shear stress-induced Akt phosphorylation, our results

**Figure 6.** AM-induced vasodilation is suppressed in rat aorta expressing a dominant-negative Akt mutant in endothelium. A, Adenovirus particles injected into rat abdominal aorta are predominantly expressed in the endothelial layer. Ad/GFP (10^9 PFU) was injected into rat abdominal aortas, and aortas were fixed 48 hours after infection. Noninfected rat abdominal aortas were used as negative control. GFP-positive cells were stained with Cy (red fluorescence). Nuclei in aortas were stained with To-PRO-3 iodide (blue fluorescence). Areas showing green fluorescence represent autofluorescence in vessels. Note that nuclei in the endothelium that were double-stained with Cy and To-PRO-3 iodide show pink fluorescence. Arrowheads indicate GFP-positive nuclei in the endothelium. Bars = 10 \mu m. B, Ad/dnAkt (○) or Ad/GFP (●) was infected in the endothelium of rat abdominal aorta, and AM-induced vasodilation was examined 48 hours after adenovirus infection. Noninfected abdominal aortas with (□) or without (●) endothelium were used as the control. #P < 0.05 vs Ad/GFP infection at each concentration of AM stimulation (n = 4).
clearly showed that the Ca\textsuperscript{2+}/CaM-dependent pathway was necessary for AM-induced Akt phosphorylation. Our results also indicated that Ca\textsuperscript{2+}/CaM-induced Akt phosphorylation was mediated by the PI3K-dependent pathway (see online Figure 1 in data supplement available at http://www.circresaha.org). Thus, Ca\textsuperscript{2+}/CaM appeared to be necessary not only for direct activation of eNOS by binding to the CaM binding domain in eNOS but also for activation of eNOS via its phosphorylation by Akt. The involvement of Ca\textsuperscript{2+}/CaM in the activation of PI3K/Akt has been suggested in several reports. CaM-dependent protein kinase directly phosphorylated Akt on threonine 308 and activated Akt, which was required for antiapoptotic effects induced by the Ca\textsuperscript{2+}/CaM-dependent pathway.\textsuperscript{24} Insulin-induced activation of PI3K was inhibited in hepatocytes by pretreatment with EGTA or the Ca\textsuperscript{2+} channel inhibitors gadolinium and nickel.\textsuperscript{25} Furthermore, the p110 subunits of PI3K contained a \(\cal C\)2A binding site, which was involved in PI3K activation and required for its association with the cognate receptors for AM. Therefore, CaM appears to be necessary for AM receptors available at present. Thus, future studies are required to elucidate how AM receptors transmit signals to the Ca\textsuperscript{2+}/CaM- and PI3K-dependent pathways.

Accumulated evidence has indicated that PI3K/Akt is implicated in a variety of biological processes including glycogen synthesis, antiapoptotic actions, modulation of cell proliferation and eNOS activation.\textsuperscript{19,20,32–34} Furthermore, it has been reported that PI3K/Akt is implicated in stimulation of angiogenesis.\textsuperscript{35} Thus, the PI3K/Akt-dependent pathway seems to play pivotal roles in vascular endothelium such as maintenance of vascular tone, protection against apoptotic cell death, repair of endothelium, and modulation of angiogenesis. Because AM has an activity to activate Akt and induce NO production, modulation of AM activity in the endothelium may be a useful strategy to inhibit progression of atherosclerosis.

Acknowledgments

This study was supported in part by Grants-in-Aid 09281206 and 10218202 from the Ministry of Education, Culture and Science of Japan (to Y.H.). We thank Reiko Sato, Etsuko Taira, and Marie Morita for technical assistance.

References


Adrenomedullin Induces Endothelium-Dependent Vasorelaxation via the Phosphatidylinositol 3-Kinase/Akt–Dependent Pathway in Rat Aorta
Hiroaki Nishimatsu, Etsu Suzuki, Daisuke Nagata, Nobuo Moriyama, Hiroshi Satonaka, Kenneth Walsh, Masataka Sata, Kenji Kangawa, Hisayuki Matsuo, Atsuo Goto, Tadaichi Kitamura and Yasunobu Hirata

Circ Res. 2001;89:63-70; originally published online June 21, 2001; doi: 10.1161/hh1301.092498

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/89/1/63

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2001/06/13/hh1301.092498.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Results

To study more specifically the role of the Ca\(^{2+}\)/CaM-dependent pathway in Akt phosphorylation, we examined the effect of the Ca\(^{2+}\) ionophore A23187 on Akt phosphorylation in rat aorta. A23187 stimulated Akt phosphorylation in a dose-dependent manner (Online Figure 1). The effect of A23187 reached a maximal level when 10\(^{-8}\) mol/L A23187 was used. A23187, at concentrations of 10\(^{-8}\) mol/L and 10\(^{-6}\) mol/L, increased Akt phosphorylation by 2.2-fold and 2.1-fold, respectively, compared with control aorta. Akt phosphorylation induced by 10\(^{-8}\) mol/L or 10\(^{-6}\) mol/L A23187 was suppressed to a level lower than the control (nonstimulated) level by pretreatment with 20 \(\mu\)mol/L LY or 100 nmol/L WT, suggesting that the Ca\(^{2+}\)/CaM-dependent Akt phosphorylation was mediated by the activation of PI3K.

FIGURE LEGEND

Online Figure 1  
Effects of A23187 on Akt phosphorylation. Rat aortas were incubated with increasing doses of A23187 for 15 min in the presence or absence of 20 \(\mu\)mol/L LY294002 (LY) or 100 nmol/L wortmannin (WT). One hundred \(\mu\)g of each protein extract was immunoblotted with a phospho-specific anti-Akt antibody (*pAkt), which recognizes catalytically active Akt, or anti-Akt antibody (Total Akt) which recognizes total Akt1/2, regardless of whether Akt is phosphorylated or not. The relative intensity of phosphorylated Akt is also indicated. Shown is a representative result of two independent experiments in which the same results were obtained.
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>LY</th>
<th>WT</th>
<th>LY</th>
</tr>
</thead>
<tbody>
<tr>
<td>A23187 (M)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10^{-10}</td>
<td>10^{-8}</td>
<td>10^{-6}</td>
</tr>
<tr>
<td>Relative Intensity</td>
<td>1.0</td>
<td>1.4</td>
<td>2.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Total Akt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*pAkt*