

AKT Participates in Endothelial Dysfunction in Hypertension

Guido Iaccarino, MD, PhD; Michele Ciccarelli, MD; Daniela Sorriento, BS; Ersilia Cipolletta, MD; Vincenzo Cerullo, BS; Gianni Luigi Iovino, MD; Alessandro Paudice, MD; Andrea Elia, MD; Gaetano Santulli, MS; Alfonso Campanile, MS; Oreste Arcucci, MD; Lucio Pastore, MD, PhD; Francesco Salvatore, MD, PhD; Gianluigi Condorelli, MD, PhD; Bruno Trimarco, MD

Background—In hypertension, reduced nitric oxide production and blunted endothelial vasorelaxation are observed. It was recently reported that AKT phosphorylates and activates endothelial nitric oxide synthase and that impaired kinase activity may be involved in endothelial dysfunction.

Methods and Results—To identify the physiological role of the kinase in normotensive Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR), we used adenoviral vectors to transfer the human AKT1 gene selectively to the common carotid endothelium. In vitro, endothelial vasorelaxations to acetylcholine, isoproterenol, and insulin were blunted in control carotids from SHR compared with WKY rats, and human AKT1 overexpression corrected these responses. Similarly, blood flow assessed in vivo by Doppler ultrasound was reduced in SHR compared with WKY carotids and normalized after AKT1 gene transfer. In primary cultured endothelial cells, we evaluated AKT phosphorylation, activity, and compartmentalization and observed a mislocalization of the kinase in SHR.

Conclusions—We conclude that AKT participates in the settings of endothelial dysfunction in SHR rats by impaired membrane localization. Our data suggest that AKT is involved in endothelium dysfunction in hypertension. (*Circulation*. 2004;109:2587-2593.)

Key Words: endothelium ■ gene therapy ■ hypertension ■ signal transduction

Nitric oxide (NO) mediates many endothelial functions, such as control of vascular tone, regulation of platelet aggregation, endothelium permeability, and neoangiogenesis.¹ NO is produced in the endothelium by the endothelial NO synthase (eNOS), which, along with inducible NOS and neuronal NOS, belongs to a family of arginine hydroxylases.² Several studies have provided evidence that eNOS is important in the regulation of vascular functions.³ In particular, mice lacking the eNOS gene show increased blood pressure levels and loss of endothelium-dependent vasorelaxation.⁴ The activity of eNOS consists of converting arginine into citrulline with stoichiometric NO production and is regulated in a calcium/calmodulin-dependent manner.³ Recently, it has been discovered that other posttranslation modifications also regulate eNOS activity.^{5,6} These modifications occur by means of phosphorylation of Ser 1177 in the eNOS sequence. Several kinases share the ability to phosphorylate this site, including protein kinase A, protein kinase C, CAMKII and the serine/threonine kinase AKT.^{7–11} This latter seems to possess the unique feature to be activated by many independent signal transduction pathways. In fact, AKT kinase is

activated primarily in response to stimulation of transmembrane receptors with intrinsic tyrosine kinase activity or indirectly coupled to tyrosine kinases or to 7-transmembrane G protein-coupled receptors.^{13–15} In the endothelium, various agonists, such as vascular endothelial growth factor, bradykinin, insulin, acetylcholine (ACh), isoproterenol (ISO), and fluid shear stress, induce AKT activation and consequent phosphorylation of eNOS on serine 1177 and NO production.^{7–9,12} Therefore, AKT seems to be an important checkpoint for eNOS activation in response to many signal transduction pathways.

Endothelial dysfunction is the impaired ability of vascular endothelium to stimulate vasodilatation and is observed in various pathological conditions, such as hypercholesterolemia, hypertension, type 2 diabetes, hyperhomocyst(e)inemia, chronic renal failure, and chronic heart failure.¹⁶ Recent observations link endothelial dysfunction to increased risk of cardiovascular accidents.¹⁷ The major cause of the endothelial dysfunction is decreased bioavailability of NO, which can be caused by reduced eNOS expression, reduced NO production, and increased NO catabolism. In hypertension, endothelial

Received August 11, 2003; de novo received December 12, 2003; accepted February 11, 2004.

From the Departments of Clinical Medicine, Cardiovascular and Immunological Sciences (G.I., M.C., D.S., E.C., G.L.I., A.P., A.E., G.S., A.C., O.A., B.T.), and Biochemistry and Medical Biotechnology (V.C., L.P., F.S.), and School of Biotechnological Sciences (L.P., F.S.), University of Naples Federico II, Italy; CEINGE-Biotecnologie Avanzate, Naples (L.P., F.S.); and San Raffaele Biomedical Science Park of Rome (G.C.), Italy.

Correspondence to Guido Iaccarino, MD, PhD, Medicina Clinica, Scienze Cardiovascolari ed Immunologiche, Federico II University, Via Pansini 5, Edificio 2, 80131 Naples, Italy. E-mail guiaccar@unina.it

© 2004 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000129768.35536.FA

dysfunction seems to be related to inadequate NO production in response to endothelial stimulation, whereas basal NO levels are increased.^{18,19} Because many endothelial signal transduction pathways converge on AKT, leading to eNOS phosphorylation and activation, this molecule can be considered an ideal target for increasing eNOS responses.

In this report, we evaluated whether endothelial AKT activation is altered in the spontaneously hypertensive rat (SHR). To this aim, we induced overexpression of the human AKT1 gene in the endothelium of SHR carotids by use of adenoviral gene transfer and evaluated the effects on endothelium-dependent responses. Furthermore, we explored possible differences in AKT signaling between normotensive and SHR endothelial cells (ECs).

Methods

Adenoviral Constructs

We used replication-defective adenoviral vectors encoding for the human AKT1 wild-type gene (ADAKT-WT) or the kinase-deficient AKT K179M mutant acting as dominant-negative (ADAKT-DN) under the control of the cytomegalovirus promoter;²⁰ as a control, we used a similar adenoviral vector without any transgene (ADempty). The ADAKT-WT and ADAKT-DN mutant constructs included an in-frame hemoagglutinin epitope (HA) 3' tag.²⁰

Animals and Surgical Procedure

Twelve-week-old normotensive rats of the Wistar-Kyoto strain (WKY, n=15) and age-matched SHR (n=18) were used in the study. Adenovirus-mediated gene transfer to the common carotids was performed as previously described.²¹ Briefly, animals were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (0.5 mg/kg), and the right carotid was exposed; through an incision on the external carotid, the vector (10^8 pfu in 100 μ L of PBS) was injected into the common carotid and incubated for 15 minutes to allow infection. Subsequently, blood flow was restored through the carotid. This maneuver achieves an \approx 2-fold overexpression of the transgene selectively at the endothelium and a 50% to 75% infection rate.²¹ The contralateral carotid was treated as described above using ADempty vector virus saline solution as control. The study was performed in accordance with institutional guidelines for animal studies.

Vascular Reactivity Using Common Carotid Rings

Four days after infection, animals were anesthetized and killed by cervical dislocation; common carotids were isolated for evaluation of vascular responses as previously described.²¹ Endothelium-dependent vasorelaxation was assessed in vessels precontracted with phenylephrine (PE, 10^{-6} mol/L) in response to the muscarinic receptor agonist ACh (10^{-9} to 10^{-6} mol/L), the β -adrenergic receptor (β AR) agonist ISO (10^{-10} to 3×10^{-8} mol/L), and insulin (10^{-9} to 10^{-6} mol/L). At the doses we used, all these agonists exert an endothelium-dependent vasorelaxation, ranging from 30% to 70%.^{21–23} Endothelium-independent vasorelaxation was tested by sodium nitroprusside (SNP, 1×10^{-9} to 5×10^{-5} mol/L). In SHR, we also explored the effects of the eNOS inhibitor *N*^G-nitro-L-arginine methyl ester (L-NAME, 10^{-5} mol/L) on both control and ADAKT-WT-treated vessels. Drugs were prepared daily, and their concentrations are reported as the final molar concentration in the organ bath. At the end of the experiment, carotids were snap-frozen in dry ice-chilled isopropyl alcohol for evaluation of AKT1 expression.

In Vivo Hemodynamics Assessment

In 3 SHR and 3 WKY rats, blood flow velocity of both carotid arteries was measured by echo Doppler ultrasounds (10 MHz, Sonos 5500, Philips) in live anesthetized animals before and 3 days after ADAKT-WT application on one carotid.

RT-PCR

Total RNA was isolated by use of RNazol (Biotech), a 1-step guanidinium-based extraction from pooled (3 per group) pulverized carotids. The RNA was treated with DNase I after extraction and used for reverse transcription (RT) into cDNA by standard methods.²⁴ cDNAs were then used as templates for the polymerase chain reaction (PCR) amplification. We designed the following primers that recognize the AKT1 sequence: forward, 5'-CCATGAA-GATCCTCAAGA-3' and the HA tag: reverse, 5'-AGCGTAAT-CTGGAACATC-3'. We also used primers for rat GAPDH sequence to normalize for cDNA production.²⁴ After amplification, samples were electrophoresed through 20% acrylamide gel in Tris 0.1 mol/L–borate 0.09 mol/L–EDTA 0.001 mol/L buffer and visualized by incubation with ethidium bromide under UV light. The presence of an \approx 900-bp PCR product confirmed viral expression.

Primary Cultured Rat Aorta Endothelial Cells

In vitro studies were performed with primary cultured ECs harvested as previously described and validated.^{21,22} Briefly, vessels were cut into rings, placed on Matrigel, incubated in DMEM supplemented with 20% FBS and EC growth supplement (10 mg/100 mL), and incubated at 37°C in 95% air–5% CO₂. After 5 to 7 days, aortic rings were removed, and the ECs remaining on Matrigel were expanded. Cells were studied between passages 3 and 7.

Western Blotting

Cells were plated on 6-well dishes and incubated overnight in absence of serum. The next day, ECs were exposed to ISO (10^{-7} mol/L, 15 minutes) or insulin (10^{-7} mol/L, 15 minutes) at 37°C and then lysed in RIPA-SDS buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% NP-40, 0.25% deoxycholate, 9.4 mg/50 mL sodium orthovanadate, 20% SDS). Alternatively, cells were homogenized in lysis buffer (12.5 mmol/L Tris, pH 6.8, 5 mmol/L EDTA, 5 mmol/L EGTA), and membrane was separated from the cytosol fraction by centrifugation as previously described.²⁵ In some experiments, total AKT was immunoprecipitated from an equal amount of whole-cell protein extracts by use of protein A/agarose beads conjugated with an antibody raised against total AKT (Cell Signaling). Clarified whole-cell or membrane proteins or immunocomplexes were resolved by PAGE and transferred to a nitrocellulose filter. Total AKT, serine 473-phosphorylated AKT (Cell Signaling), and total (Cell Signaling) and serine 1177 phospho eNOS (Upstate) were visualized by specific antibodies, followed by incubation with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and standard chemiluminescence (Renaissance, NEN).

Total AKT Activity

AKT kinase activity was analyzed by a nonradioactive immunoprecipitation-based assay (Cell Signaling Technology). Cell extracts were incubated for 2 hours with immobilized AKT monoclonal antibody. After an extensive washing, the kinase reaction was performed at 30°C for 30 minutes in the presence of 200 μ mol/L cold ATP and GSK-3 substrate. Phosphorylation of GSK-3 was measured by Western blot by using phospho-GSK-3 α/β (Ser-21/9) antibody. Autoradiographs were digitalized and quantified using Image Quant software (Molecular Dynamics).

Statistical Analysis

Data are expressed as mean \pm SEM. Because no differences were observed between the ADempty-treated and the sham-operated carotids, we pooled these data together to simplify the analysis. This group is referred to as the control group. ANOVA was used to analyze the vasoconstrictive responses to PE. Two-way ANOVA was applied to analyze dose-dependent curves. A probability value of $P < 0.05$ was considered statistically significant.

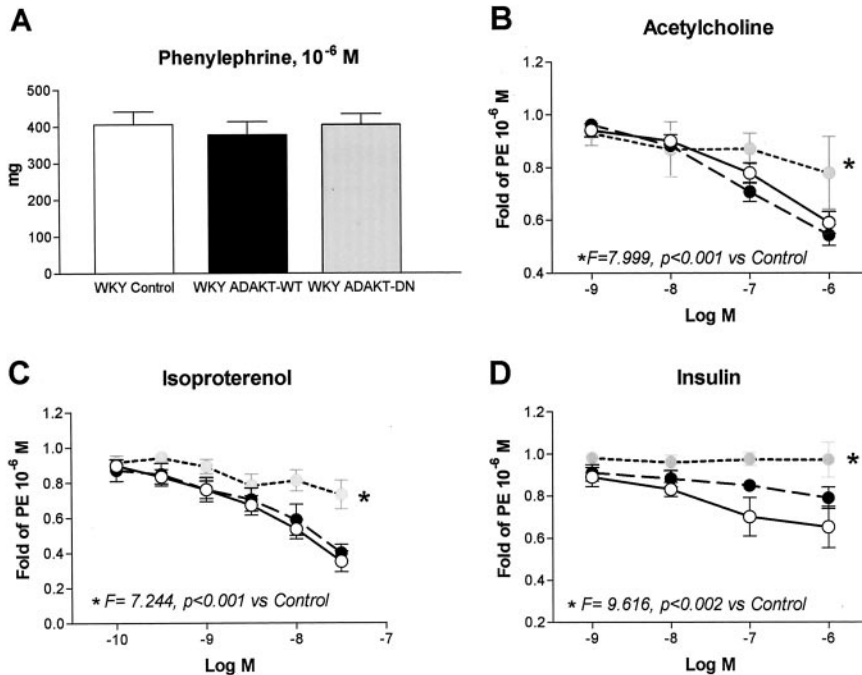


Figure 1. Vascular responses on isolated normotensive WKY carotids in vitro. **A**, Vasoconstriction to α_1 -adrenergic agonist PE was not affected by exposure to viruses. **B**, Endothelium-dependent vasorelaxation to ACh was not affected by ADAKT-WT, whereas overexpression of dominant negative mutant of AKT resulted in attenuation of response. **C**, Similarly, another endothelium-dependent response induced by β AR agonist ISO was not affected by AKT-WT overexpression, whereas it was blunted by ADAKT-DN. **D**, AKT-WT overexpression did not modify vasorelaxation to insulin. ○, Control carotids; ●, ADAKT-WT; ◐, ADAKT-DN.

Results

Adenovirus-Mediated Gene Transfer to the Endothelium

We recently demonstrated that we can selectively target common carotid endothelium using an intravascular delivery.²¹ Expression of the viral transgene was confirmed in vessels infected with the ADAKT vectors and absent in control vessels, as evaluated by the appearance of the expected 900-bp PCR fragment of the ADAKT transcripts (data not shown). GAPDH analysis confirmed the appropriate cDNA production in samples (data not shown).

Vasomotor Responses in WKY

To evaluate endothelial vasodilatation in response to independent intracellular signal transduction pathways, we studied ACh and ISO to explore G_q and the G_s protein pathways and insulin to assess the tyrosine kinase-dependent signaling. Vessels were constricted with the α_1 -adrenergic agonist PE, whose response was not affected by either ADAKT-DN or ADAKT-WT (Figure 1A). ADAKT-DN attenuated the vasodilation in response to ACh, ISO, and Ins, thereby confirming that these responses are AKT-dependent (Figure 1, B–D). Conversely, ADAKT-WT did not change the pattern of vasodilation to ISO, ACh, and Ins (Figure 1, B–D). Neither ADAKT-DN nor ADAKT-WT affected endothelium-independent response to SNP (data not shown), indicating that the gene transfer did not interfere with the intrinsic ability of the vessel to vasodilate.

Vasomotor Responses in SHR

PE vasoconstriction in control SHR carotids was not different from that observed in control WKY carotids (Figure 2A). Endothelial responses to ACh, ISO, and Ins (Figure 2, B–D) but not endothelium-independent vasorelaxation to SNP (data not shown) were significantly reduced in SHR compared with

WKY. In SHR carotids, AKT1 overexpression corrects endothelial function, because ACh, ISO, and Ins (Figure 2, B–D) endothelium-dependent vasorelaxations in ADAKT-WT-treated carotids were indistinguishable from those observed in control or ADAKT-WT WKY carotids. ADAKT-WT did not change PE (Figure 2A) and SNP responses (data not shown). The enhancement of endothelial vasorelaxation was largely dependent on NO, because the eNOS inhibitor L-NAME attenuated to a similar level the responses in ADAKT-WT and control treated carotids (Figure 3, B–D), without affecting vasoconstriction to PE (Figure 3A) or vasodilation to nitroprusside (data not shown).

In Vivo Carotid Hemodynamics

To evaluate the physiological relevance of our findings, we determined whether ADAKT-WT modifies carotid blood flow in vivo. Regarding this issue, the hemodynamics of WKY and SHR carotids were assessed by means of echocolor Doppler ultrasounds in anesthetized animals before and after ADAKT-WT gene transfer to one carotid. Before gene transfer, SHR showed reduced systolic blood flow velocity (SHR, 81.2 ± 1.33 , and WKY, 149.2 ± 3.3 cm/s, $P < 0.05$) and blood flow (SHR, 45.66 ± 4.30 , and WKY, 64.51 ± 3.96 mL/min, $P < 0.05$), whereas internal diameter was similar between rat strains (SHR, 0.70 ± 0.015 , and WKY, 0.703 ± 0.004 mm). ADAKT-WT did not exert any effect in WKY rats, whereas it ameliorated in vivo carotid hemodynamics in the SHR strain: after ADAKT-WT transduction, systolic blood velocity (132.3 ± 8.4 cm/s) and blood flow (79.26 ± 4.56 mL/min) were no longer different between SHR and WKY carotids.

AKT Assessment in WKY and SHR ECs

To study the role of AKT in endothelial responses, we evaluated AKT signaling in primary cultured endothelial cells. We assessed the responses to 2 independent signal transduction pathways, the G protein-coupled β AR and the

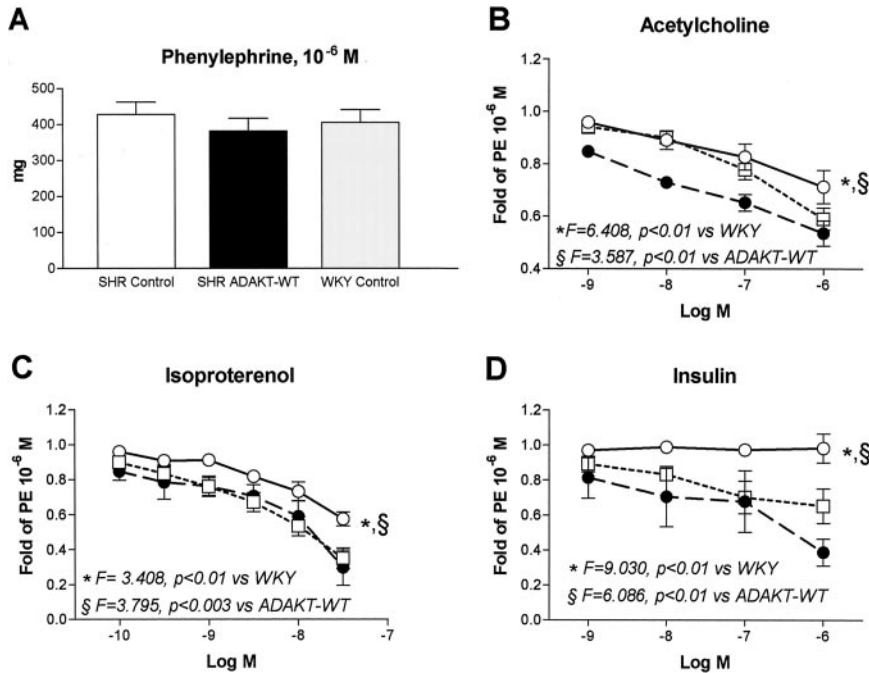


Figure 2. Vascular responses on isolated hypertensive rat carotids in vitro. To facilitate comparison with normotensive rats, responses obtained in WKY control carotids and already described in Figure 1 are duplicated in this figure. A, Vasoconstriction to PE in SHR carotids was not different from that observed in WKY, and ADAKT-WT did not modify this response. B, Endothelium-dependent vasorelaxation to ACh is impaired in hypertensive rats vs WKY, but overexpression of AKT restores this response. C, Similarly, endothelial response to ISO is attenuated in SHR control carotids vs normotensives. ADAKT-WT ameliorates response, restoring it to extent observed in WKY. D, Also in case of insulin, ADAKT-WT enhances impaired vasorelaxation in SHR. ○, SHR control; ●, SHR ADAKT-WT carotids; □, WKY control carotids.

tyrosine kinase insulin receptor. Because the activation of eNOS occurs after phosphorylation on the Ser 1177 residue by means of a series of kinases, including AKT, to pinpoint the effects of AKT on eNOS, we evaluated the phosphorylation of eNOS that immunoprecipitated with total AKT from EC whole extracts. In WKY ECs, both ISO and insulin were able to induce AKT/eNOS association and eNOS phosphorylation, but this function was impaired in SHR (Figure 4A). We then explored whether EC AKT activation in response to receptor stimulation is impaired in SHR rats. In WKY and SHR strains, both agonists were similarly able to induce AKT phosphorylation (Figure 4B). Accordingly, agonist-

stimulated AKT activity assessed in vitro by GSK3-GST fusion protein phosphorylation was similar between the 2 strains, with a slight but significant decrease in insulin-independent activation (Figure 4C). This phenomenon is compatible with the well-known insulin resistance of SHR.²⁶

Because eNOS phosphorylation by AKT occurs after translocation of the cytosolic AKT to the membranes by means of a scaffold that contains heat-shock protein 90 and eNOS itself,²⁷⁻²⁹ we explored whether AKT moved into the membranes after EC stimulation. Both agonists were able to increase the membrane content of the kinase in WKY ECs (Figure 4). Conversely, in SHR rats, AKT content seems to be

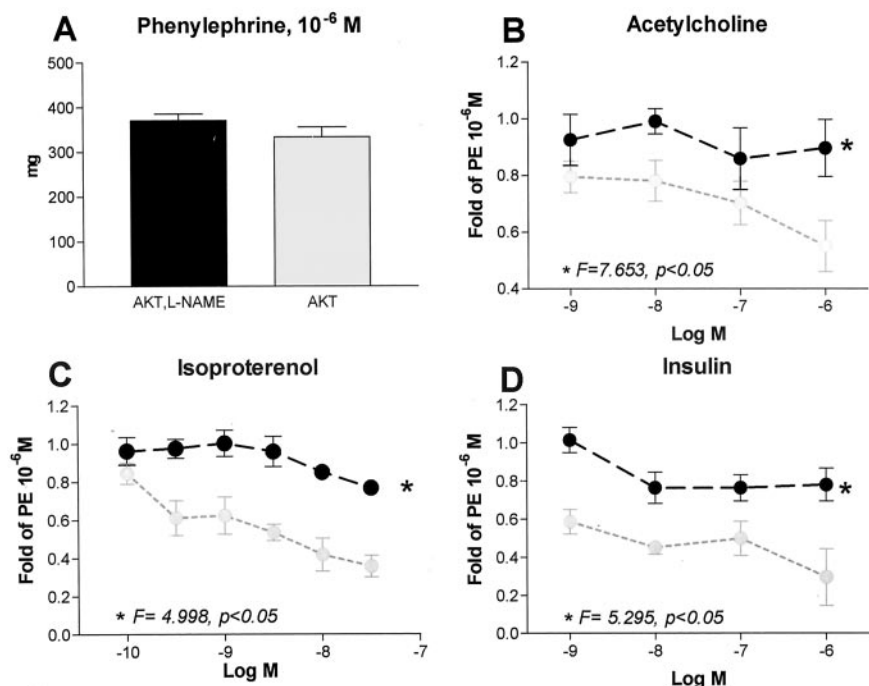


Figure 3. Effects of L-NAME on vascular responses of isolated hypertensive rat carotids in vitro. To explore role of NO in ADAKT-WT enhancement of endothelial responses, in a group of 6 SHR, we explored vasorelaxations to ISO, insulin, and ACh in ADAKT-WT-treated carotids in presence of eNOS inhibitor L-NAME (10⁻⁵ mol/L). A, L-NAME did not modify vasoconstriction to PE in ADAKT-WT and control carotids. B, Enhanced response to ACh observed in ADAKT-WT carotids was inhibited by L-NAME. C, Similarly, inhibitor prevented vasorelaxant response to ISO and D, insulin. ○, SHR ADAKT-WT; ●, SHR ADAKT-WT+L-NAME.

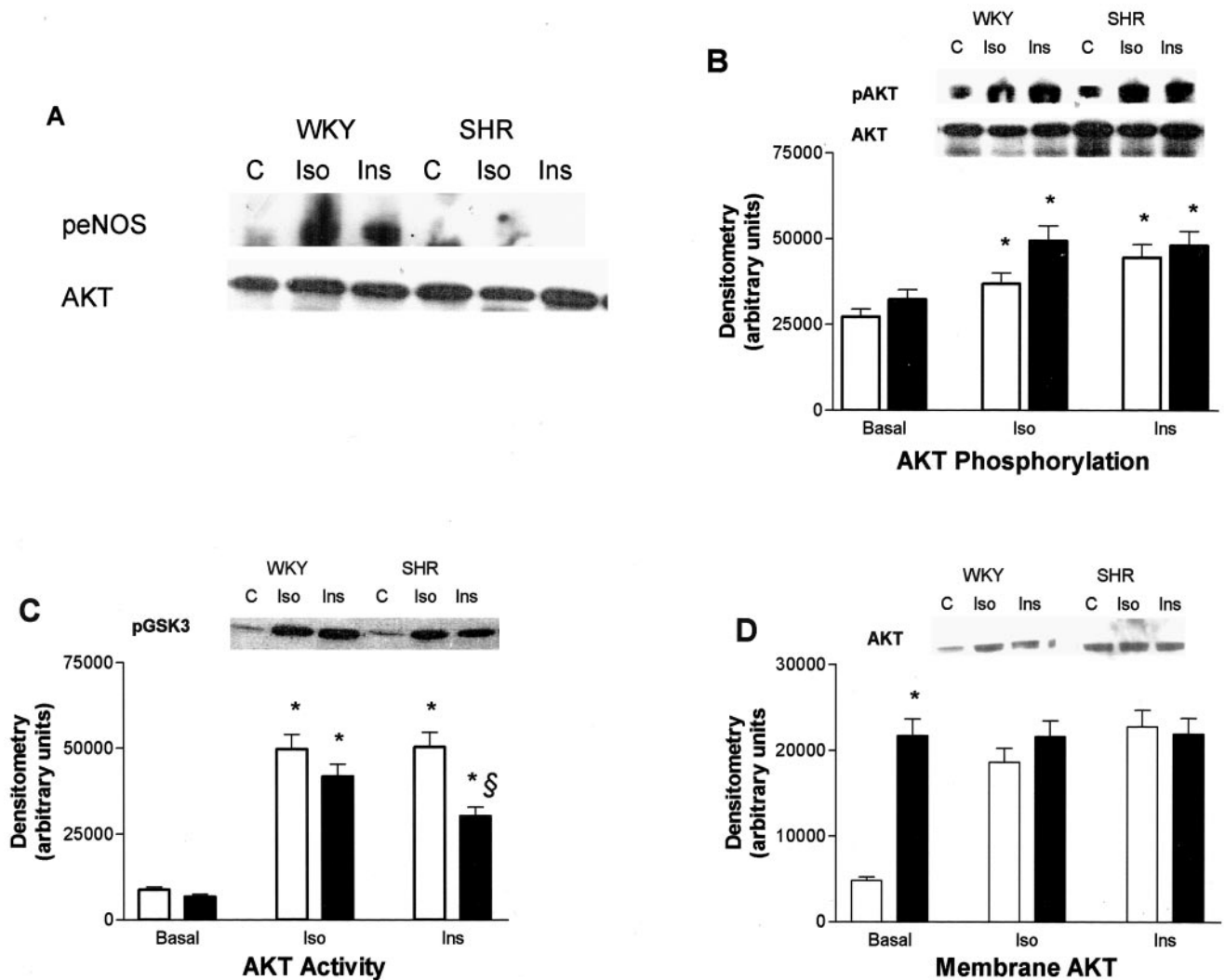


Figure 4. AKT activation and membrane localization in ECs. **A**, WKY and SHR ECs were stimulated for 15 minutes with ISO or insulin (Ins) solubilized in RIPA and immunoprecipitated with AKT antibody as indicated in Methods. Immunocomplexes were resolved by Western blot and probed for phospho-eNOS (peNOS). Equal amounts of IP are shown by total AKT. **B**, ECs were stimulated as above, solubilized, resolved by PAGE, revealed by phospho-ser 473 AKT-specific antibody (inset) and quantified by digital densitometry. Bars represent mean \pm SEM of 3 independent experiments, after normalization by total AKT. * $P < 0.05$ vs Basal. **C**, WKY and SHR ECs were stimulated as above. Cells were solubilized and immunoprecipitated with AKT antibodies. GSK3-GST fusion protein was used as substrate for AKT activity in immunoprecipitates, resolved by PAGE, visualized (inset), and quantified as described. Bars represent mean \pm SEM of determinations in 3 independent experiments. * $P < 0.05$ vs Basal. § $P < 0.05$ vs WKY. **D**, WKY and SHR EC membranes were resolved by PAGE. AKT was visualized (inset) and quantified. Bars represent mean \pm SEM of 3 independent experiments * $P < 0.05$ vs WKY. C indicates control; Iso, ISO 10^{-6} mol/L; and Ins, insulin 10^{-6} mol/L. Open bar, WKY; shaded bar, SHR.

already increased at baseline and cannot be further increased (Figure 4D).

Discussion

In hypertension, it has been proposed that impaired endothelium-dependent vasorelaxation is a result of altered signal transduction pathways leading to eNOS activation.³⁰ This hypothesis is supported by the recent observation of increased expression of negative regulators of G protein-coupled receptor signaling in the vasculature of hypertensive rats.³¹ Furthermore, we have recently demonstrated that increasing the number of β ARs at the endothelial level corrects impaired β -adrenergic vasorelaxation in carotids from SHR rats.²¹ Our study focuses on AKT because of the ability of this kinase to

activate eNOS and the central role of this kinase in multiple signal transduction pathways.¹³⁻¹⁵ Therefore, it is likely that alterations in AKT signaling may cause endothelial dysfunction in response to many agonists. Indeed, in functional studies, we showed that gene transfer of the human AKT1 to the endothelium of SHR carotids ameliorates the impaired vasorelaxation to the endothelial agents ACh, ISO and insulin. In normotensive rats, the same treatment did not achieve the same result. Similarly, although in vivo in SHR, AKT1 gene transfer to the carotid endothelium corrects impaired blood flow, in WKY, we did not observe modifications of carotid hemodynamics. Although surprising, these results testify to the complexity of the mechanisms that in normotensive animals leads to NO production. To the best of

our knowledge, this is the first time that the AKT1 wild-type gene transfer has been used in studies of endothelial vasorelaxation, because thus far, only AKT1 mutant genes have been tested⁹; therefore, our data cannot be compared with previous experiences. A parallel observation was obtained with the eNOS gene transfer to the common carotid, obtaining no changes in ACh response in normotensive nondiabetic rabbits while correcting impaired vasorelaxation in diabetic rabbits.³² There is now increasing evidence that NO participates in the regulation of vascular wall compliance.^{33,34} Our findings *in vivo* are in agreement with this notion, because the increase of wall compliance will reduce carotid resistance and correct the hemodynamics through the vessel.³⁵ In this vision, our *in vivo* data, showing that AKT1 ameliorates hemodynamics in SHR, agree with the evidence gathered on isolated carotids. Our results suggest that in hypertensive rats, there is indeed an impairment of AKT signaling that leads to endothelial dysfunction. We therefore investigated AKT expression or activity in endothelial cells isolated from WKY and SHR rats, a model that is not influenced by differences in hemodynamics. In SHR, we observed a defect in AKT-dependent phosphorylation of eNOS that cannot be ascribed to differences in expression or activity levels of AKT from WKY. To regulate eNOS, AKT has to transfer to the membranes on the formation of a scaffold including PI3K, heat-shock protein 90, and PTEN.^{27–29} Impairment of the formation of this complex has been invoked as a mechanism for reduced NO production in pulmonary hypertension³⁶; we investigated whether in SHR, AKT moves properly to the membranes after agonist stimulation. We found that in SHR, AKT is mislocalized, with an enhanced unstimulated localization to the plasma membranes. This basal increase is probably the cause of the lack of any further localization of the kinase to membranes after cellular activation by endothelial agonists. It is not clear whether higher basal AKT localization to the membranes is primary or compensatory to endothelial dysfunction, but despite the high presence of AKT on membranes, we failed to observe AKT-dependent eNOS phosphorylation. This result suggests a different localization on plasma membranes that prevents AKT from interacting with eNOS. The delivery of a transgenic kinase probably increases the amount of AKT that is available for coupling with eNOS. Our results with the eNOS inhibitor L-NAME are in agreement with this hypothesis.

An ancillary result of our study regards the involvement of AKT in the endothelium-dependent vasorelaxation in response to β AR stimulation. Indeed, although it has been very well demonstrated that endothelial β ARs couple to eNOS and induce NO vasodilatation,^{21,37} the underlying signal transduction pathway has been less investigated. Isenovic et al¹² indicated that endothelial β_2 AR can activate eNOS in a PI3K-dependent manner using a chemical inhibitor of this pathway. In this sense, our data represent the first transgenic demonstration that the endothelial β ARs activate eNOS through AKT.

In summary, our research shows for the first time that AKT is involved in endothelial dysfunction in hypertension, suggesting that mislocalization to the membrane is a possible pathogenic mechanism. Given its pathophysiological and

prognostic implication,¹⁷ correcting impaired endothelial function is an obligatory achievement of modern antihypertensive therapy. AKT represents a possible target for treating endothelial dysfunction, to be achieved with old strategies, such as pharmacological small molecules, or with new molecular tools, such as gene therapy.

References

- Schmidt HH, Walter U. NO at work. *Cell*. 1994;78:919–925.
- Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. *Cell*. 1994;78:915–918.
- Cooke JP, Dzau VJ. Nitric oxide synthase: role in the genesis of vascular disease. *Annu Rev Med*. 1997;48:489–509.
- Huang PL, Huang Z, Mashimo H, et al. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*. 1995;377:239–242.
- Butt E, Bernhardt M, Smolenski A, et al. Endothelial nitric-oxide synthase (type III) is activated and becomes calcium independent upon phosphorylation by cyclic nucleotide-dependent protein kinases. *J Biol Chem*. 2000;275:5179–5187.
- Bredt DS, Ferris CD, Snyder SH. Nitric oxide synthase regulatory sites: phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C, and calcium/calmodulin protein kinase: identification of flavin and calmodulin binding sites. *J Biol Chem*. 1992;267:10976–10981.
- Fulton D, Gratton JP, McCabe TJ, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*. 1999;399:597–601.
- Dimmeler S, Fleming I, Fisslthaler B, et al. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. 1999;399:601–605.
- Luo Z, Fujio Y, Kureishi Y, et al. Acute modulation of endothelial Akt/PKB activity alters nitric oxide-dependent vasomotor activity *in vivo*. *J Clin Invest*. 2000;106:493–499.
- Michell BJ, Chen Z, Tiganis T, et al. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J Biol Chem*. 2001;276:17625–17628.
- Schneider JC, El Kebir D, Chereau C, et al. Involvement of Ca²⁺/calmodulin-dependent protein kinase II in endothelial NO production and endothelium-dependent relaxation. *Am J Physiol*. 2003;284:H2311–H2319.
- Isenovic E, Walsh MF, Muniyappa R, et al. Phosphatidylinositol 3-kinase may mediate isoproterenol-induced vascular relaxation in part through nitric oxide production. *Metabolism*. 2002;51:380–386.
- Kohn AD, Kovacina KS, Roth RA. Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing ser/thr kinase. *EMBO J*. 1995;14:4288–4295.
- Franke TF, Yang SI, Chan TO, et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*. 1995;81:727–736.
- Burgering BM, Coffey PJ. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature*. 1995;376:599–602.
- Kurowska EM. Nitric oxide therapies in vascular diseases. *Curr Pharm Des*. 2002;8:155–166.
- Perticone F, Ceravolo R, Pujia A, et al. Prognostic significance of endothelial dysfunction in hypertensive patients. *Circulation*. 2001;104:191–196.
- Mourlon-Le Grand MC, Benessiano J, Levy BI. cGMP pathway and mechanical properties of carotid artery wall in WKY rats and SHR: role of endothelium. *Am J Physiol*. 1992;263:H61–H67.
- Qiu HY, Henrion D, Benessiano J, et al. Decreased flow-induced dilation and increased production of cGMP in spontaneously hypertensive rats. *Hypertension*. 1998;32:1098–1103.
- Skorski T, Bellacosa A, Nieborowska-Skorska M, et al. Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. *EMBO J*. 1997;16:6151–6161.
- Iaccarino G, Cipolletta E, Fiorillo A, et al. β_2 -Adrenergic receptor gene delivery to the endothelium corrects impaired adrenergic vasorelaxation in hypertension. *Circulation*. 2002;106:349–355.
- Lembo G, Iaccarino G, Vecchione C, et al. Insulin enhances endothelial α_2 -adrenergic vasorelaxation by a pertussis toxin mechanism. *Hypertension*. 1997;30:1128–1134.

23. Leosco D, Iaccarino G, Cipolletta E, et al. Exercise restores beta-adrenergic vasorelaxation in aged rat carotid arteries. *Am J Physiol.* 2003;285:H369–H374.
24. Iaccarino G, Tomhave ED, Lefkowitz RJ, et al. Reciprocal in vivo regulation of myocardial G protein-coupled receptor kinase expression by β -adrenergic receptor stimulation and blockade. *Circulation.* 1998;98:1783–1789.
25. Iaccarino G, Barbato E, Cipolletta E, et al. Cardiac β ARK1 upregulation induced by chronic salt deprivation in rats. *Hypertension.* 2001;38:255–260.
26. Klimes I, Sebokova E. Hypertension and the insulin resistance syndrome of rats: are they related? *Ann N Y Acad Sci.* 1997;827:13–34.
27. Datta K, Bellacosa A, Chan TO, et al. Akt is a direct target of the phosphatidylinositol 3-kinase: activation by growth factors, v-src and v-Ha-ras, in Sf9 and mammalian cells. *J Biol Chem.* 1996;271:30835–30839.
28. Fontana J, Fulton D, Chen Y, et al. Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ Res.* 2002;90:866–873.
29. Stambolic V, Suzuki A, de la Pompa JL, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell.* 1998;95:29–39.
30. Cardillo C, Kilcoyne CM, Quyyumi AA, et al. Selective defect in nitric oxide synthesis may explain the impaired endothelium-dependent vasodilation in patients with essential hypertension. *Circulation.* 1998;97:851–856.
31. Gros R, Tan CM, Chorazyczewski J, et al. G-protein-coupled receptor kinase expression in hypertension. *Clin Pharmacol Ther.* 1999;65:545–551.
32. Lund DD, Faraci FM, Miller FJ Jr, et al. Gene transfer of endothelial nitric oxide synthase improves relaxation of carotid arteries from diabetic rabbits. *Circulation.* 2000;101:1027–1033.
33. Fitch RM, Vergona R, Sullivan ME, et al. Nitric oxide synthase inhibition increases aortic stiffness measured by pulse wave velocity in rats. *Cardiovasc Res.* 2001;51:351–358.
34. Kinlay S, Creager MA, Fukumoto M, et al. Endothelium-derived nitric oxide regulates arterial elasticity in human arteries in vivo. *Hypertension.* 2001;38:1049–1053.
35. Cohn JN. ACE inhibition and vascular remodeling of resistance vessels: vascular compliance and cardiovascular implications. *Heart Dis.* 2000;2:S2–S6.
36. Murata T, Sato K, Hori M, et al. Decreased endothelial nitric-oxide synthase (eNOS) activity resulting from abnormal interaction between eNOS and its regulatory proteins in hypoxia-induced pulmonary hypertension. *J Biol Chem.* 2002;277:44085–44092.
37. Ferro A, Queen LR, Priest RM, et al. Activation of nitric oxide synthase by beta 2-adrenoceptors in human umbilical vein endothelium in vitro. *Br J Pharmacol.* 1999;126:1872–1880.