Hsp90 Ensures the Transition from the Early Ca\textsuperscript{2+}-dependent to the Late Phosphorylation-dependent Activation of the Endothelial Nitric-oxide Synthase in Vascular Endothelial Growth Factor-exposed Endothelial Cells*

Received for publication, February 13, 2001, and in revised form, June 24, 2001
Published, JBC Papers in Press, June 25, 2001, DOI 10.1074/jbc.M101371200

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Vascular endothelial growth factor (VEGF) exerts its angiogenic effects partly through the activation of endothelial nitric-oxide synthase (eNOS). Association with heat shock protein 90 (hsp90) and phosphorylation by Akt were recently shown to separately activate eNOS upon VEGF stimulation in endothelial cells. Here, we examined the interplay between these different mechanisms in VEGF-exposed endothelial cells. We documented that hsp90 binding to eNOS is, in fact, the crucial event triggering the transition from the Ca\textsuperscript{2+}-dependent activation of eNOS to the phosphorylation-mediated potentiation of its activity by VEGF. Accordingly, we showed that early VEGF stimulation first leads to the Ca\textsuperscript{2+}/calmodulin disruption of the caveolin-eNOS complex and promotes the association between eNOS and hsp90. eNOS-bound hsp90 can then recruit VEGF-activated (phosphorylated) Akt to the complex, which in turn can phosphorylate eNOS. Further experiments in transfected COS cells expressing either wild-type or S1177A mutant eNOS led us to identify the serine 1177 as the critical residue for the hsp90-dependent Akt-mediated activation of eNOS. Finally, we documented that although the VEGF-induced phosphorylation of eNOS leads to a sustained production of NO independently of a maintained increase in [Ca\textsuperscript{2+}], this late stage of eNOS activation is strictly conditional on the initial VEGF-induced Ca\textsuperscript{2+}-dependent stimulation of the enzyme. These data establish the critical temporal sequence of events leading to the sustained activation of eNOS by VEGF and suggest new ways of regulating the production of NO in response to this cytokine through the ubiquitous chaperone protein, hsp90.

Nitric oxide (NO)\textsuperscript{1} contributes to the cardiovascular homeo-

\* This work was supported in part by grants from the Fonds de la Recherche Scientifique Médicale, the Belgian Federation against Cancer, the J. Maisin and Bekales Foundations, and the Fonds de la Recherche Scientifique (FNRS)-Televie.

‡ Recipient of a grant from Fonds National de la Recherche Scientifique (FNRS)-Televie.

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\textsuperscript{1} The abbreviations used are: NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; CaM, calmodulin; hsp90, 90-kDa heat shock protein; HUVEC, human umbilical vein endothelial cells; EC, endothelial cells; [Ca\textsuperscript{2+}], intracellular [Ca\textsuperscript{2+}]; VEGF, vascular endothelial growth factor; PKB, phosphatidylinositot 3-kinase; BAFTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetracetic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
VEGFs ability to activate eNOS in target tissues, as recently verified in animal models (24, 25), the recent insights in the post-translational regulation of eNOS posed novel fundamental questions that need to be addressed. For instance, is the Ca\(^{2+}\)/CaM-mediated caveolin/eNOS dissociation a prerequisite for eNOS phosphorylation in response to VEGF? Does eNOS/hsp90 association precede eNOS phosphorylation or do these two phenomena occur independently? Is Akt part of the eNOS-hsp90 multicomplex? Is the phosphorylation of eNOS truly a calcium-independent process? To answer these questions, we used a model of human EC in culture as well as the heterologous COS cell expression system to dissect the specific protein-protein interactions, their inter-dependence, and temporal sequence involved in the post-translational regulation of eNOS by VEGF.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Freshly isolated human umbilical vein EC (HUVEC) were cultured to confluence in 100-mm dishes in a 2:1 mixture of M199 endothelial cell basal medium containing 10% serum; control experiments were also performed with myocytes isolated from neonatal mice as previously detailed (26) and plated at 5 × 10\(^{4}\) cells/ml. Serum-starved and myocytes were exposed for the indicated periods of time to VEGF (50 ng/ml) and insulin (12.5 milliunits/ml), respectively, in the presence or absence of various pharmacological modulators (30–60 min preincubation with 1 µg/ml geldanamycin (Invitrogen Life Technologies)).

**Transfection**—COS-7 cells were transfected with the different plasmid cDNAs using LipofectAMINE (Life Technologies Inc.) according to the manufacturer’s protocol; eNOS and Akt constructs were a gift from Dr. S. Dimmeler and the hsp90 cDNA was provided by Dr. W. C. Sessa. The expression of appropriate proteins was confirmed by Western blotting and an irrelevant plasmid encoding β-galactosidase was used as a control to maintain identical amounts of DNA in each transfection.

**Immunoprecipitation, Immunoblotting, and Kinase Assay**—EC were homogenized in a buffer containing phosphatase and protease inhibitors (11), and processed for immunoblotting or immunoprecipitation in an octyl glucoside-containing buffer as described previously (4). Caveolin, eNOS, and hsp90 antibodies were from BD Transduction Labs, Akt and phospho-Akt antibodies from BD PharMingen, CaM antibody from Santa Cruz, and phospho-eNOS antibody from NEB Cell Signaling Technology. Akt activity was measured using a non-radioactive immunoprecipitation kinase assay kit (NEB Cell Signaling Technology).

**Measurement of Changes in [Ca\(^{2+}\)]\(_{i}\)**—EC plated on coverslips were incubated with 2 µM Fura-2/AM at 37°C for 30 min and then extensively washed and placed in a temperature-controlled perfusion chamber for 15 min. Cells were observed with a Zeiss Axiovert 100 microscope in the epifluorescence mode using a long pass filter cut-off for excitation and a 510 nm dichroic filter at 405 nm. Alternating wavelength excitation of 340 and 380 nm was provided by a motorized filter wheel. The images pairs monitored by the IonOptix Mycam camera were processed by the IonWizard software (IonOptix, Milton, MA) and are presented as the fluorescence ratio (340/380 nm), a direct index to the [Ca\(^{2+}\)]\(_{i}\). In some experiments, Fura-2-loaded cells were preincubated with the intracellular calcium chelator BAPTA/AM (20 µM). After rinsing coverslips, EC were left to recover for 5 min before VEGF stimulation.

**Nitrite Detection**—Quantitative analysis of nitrite (NO\(_{2}\)) was used as an index of NO production. Briefly, aliquots of the medium bathing intact EC were collected at the time intervals corresponding to either 0–2 or 5–30 min of VEGF exposure. For the latter period, the medium bathing the VEGF-stimulated cells for the first 5 min was discarded and replaced by fresh VEGF-containing medium for the next 25 min. In COS cells experiments, NO\(_{2}\) accumulation in the interval time of 44 to 48 h after transfection was measured. Acidic iodide was used to convert NO\(_{2}\) to NO that was electrochemically measured with an NO-selective microsensor (WPI), as recommended by the manufacturer; adequate controls using either vehicle or NO inhibitors were routinely performed in parallel. Data are normalized for the amount of protein in the dish, and are presented for convenience as mean ± S.E. Statistical analyses were made using Student’s t test or one-way ANOVA where appropriate.

**RESULTS**

**Endothelial Cell Exposure to VEGF Promotes Caveolin-eNOS Complex Disruption and hsp90/eNOS Interaction**—Following exposure to VEGF (50 ng/ml) for increasing periods of time (0, 0.5, 2, 5, and 30 min), EC were collected, lysed, and cell extracts used for immunoprecipitation as follows. First, extracts were immunoprecipitated with caveolin antibodies and the immunoprecipitation immunoblotted with eNOS antibodies (Fig. 1A, top). In a parallel series of experiments, we examined the time course of both Akt and eNOS phosphorylation in our experimental conditions, we used the PI3K inhibitor LY294002 known to prevent phosphorylation of Akt and eNOS phosphorylation in EC. HUVECs were exposed to VEGF for the indicated periods of time. A, in some experiments, lysates were immunoprecipitated with caveolin or eNOS antibodies. Caveolin immunoprecipitation was analyzed by immunoblotting with eNOS antibodies (top) and eNOS immunoprecipitation by immunoblotting with hsp90 antibodies (bottom). B, lysates were also directly immunoblotted with antibodies directed against phospho-Ser\(^{117}\)-eNOS (top) or phospho-Ser\(^{117}\)-Akt (bottom). Note that the PI3K inhibitor LY294002 blocked both eNOS and Akt phosphorylation upon 30 min VEGF stimulation (right panels). C, densitometric analysis of three independent experiments (as shown in B) demonstrates the lag between the time courses of VEGF-induced Akt and eNOS phosphorylation (*, p < 0.01 versus phospho-Akt time control).

**Akt Phosphorylation Precedes eNOS Phosphorylation in EC Exposed to VEGF**—In a parallel series of experiments, we examined the time course of both Akt and eNOS phosphorylation in VEGF-stimulated EC using antibodies directed against phosphorylated Ser\(^{117}\) and Ser\(^{117}\) in Akt and eNOS, respectively. We observed that maximal eNOS phosphorylation occurred after at least 2 min of VEGF exposure (Fig. 1B, top) whereas the stimulatory effect of VEGF on Akt phosphorylation was already observed at 30 s in our experimental conditions (Fig. 1B, bottom); densitometric analysis of these observations is presented in Fig. 1C. To further assess if the VEGF/Akt signaling cascade accounted for the observed eNOS phosphorylation in our experimental conditions, we used the PI3K inhibitor LY294002 known to prevent phosphorylation and activation of the downstream effector Akt (16, 21). In Fig.
Hsp90 Binding Is a Prerequisite for eNOS Phosphorylation

In an attempt to appreciate the proportion of eNOS involved in the geldanamycin-sensitive recruitment of Akt, we further developed the reverse co-immunoprecipitation assay: hsp90 and Akt antibodies were used to immunoprecipitate eNOS (which was detected by immunoblotting) and the supernatants of these immunoprecipitations were checked for residual eNOS presence; for this purpose, the immunoprecipitated supernatants were submitted to a second immunoprecipitation with eNOS antibodies. Since both hsp90/eNOS (Fig. 1A) and Akt/eNOS co-immunoprecipitation (Fig. 2A) appeared maximal after 5 min VEGF exposure, we chose to perform the immunoprecipitation from extracts of EC pre-exposed for 5 min to VEGF in the presence or absence of geldanamycin. Fig. 2B (first and second lanes) shows that approximately the same amount of eNOS that was immunoprecipitated by hsp90 antibodies was found in the supernatant of this immunoprecipitation. A similar pattern was found for the Akt/eNOS immunoprecipitation (Fig. 2B, fifth and sixth lanes), indicating that around half of the total eNOS pool was engaged in hsp90 and Akt interaction in the conditions of our assay. The geldanamycin pretreatment did not lead to any detectable amount of eNOS immunoprecipitated either by hsp90 or Akt antibodies (Fig. 2B, third and seventh lanes); all the pool of eNOS was found in the supernatants of these immunoprecipitations, as detected by immunoblotting after a second immunoprecipitation with eNOS antibodies (Fig. 2B, fourth and eighth lanes).

eNOS Phosphorylation Requires the Hsp90-mediated Recruitment of Activated Akt—To further test our hypothesis that hsp90/eNOS interaction is necessary for Akt phosphorylation of eNOS, we also examined Akt and eNOS phosphorylation patterns in the presence of geldanamycin. We observed that although Akt phosphorylation was detectable as soon as 30 s after VEGF addition despite the presence of geldanamycin (Fig. 2C, bottom), no phospho-eNOS signal was detectable even upon prolonged VEGF exposures (Fig. 2C, top) in striking contrast with the control condition (Fig. 2C, right). We also verified that geldanamycin treatment had no significant effect on the time course of caveolin/eNOS dissociation (not shown).

To exclude a direct effect of geldanamycin on Akt, we measured Akt activity in EC exposed for 30 min to VEGF in the presence or absence of geldanamycin. After selective immunoprecipitation of Akt from the corresponding lysates, immunoprecipitates were incubated with purified GSK-3 and Akt-induced phosphorylation of GSK-3 was evaluated by immunoblotting using specific anti-phospho-GSK-3 antibody. Fig. 2D shows that the 30-min VEGF-stimulated Akt activity was not altered by the pretreatment of cells with geldanamycin, whereas not surprisingly, the PI3K inhibitor LY294002 abrogated the Akt activation in VEGF-exposed EC. We then sought to determine whether the inhibitory effect of geldanamycin on eNOS phosphorylation could be reproduced in another cell type and upon another agonist stimulation. Accordingly, we exposed neonatal mouse cardiac myocytes to insulin (a known activator of Akt in myocytes (27)) for 5 min with or without geldanamycin and examined the phosphorylation status of Akt and eNOS.

Fig. 2. Recruitment of Akt in the hsp90-eNOS complex promotes eNOS phosphorylation in EC exposed to VEGF and in insulin-stimulated cardiac myocytes. A. HUVECs were exposed to VEGF for the indicated periods of time, and then rapidly collected. Corresponding lysates were immunoprecipitation with eNOS antibodies, resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with Akt antibodies. Akt was detectable in the immunoprecipitation as a function of time exposure in cells treated with VEGF (top) but not in cells exposed to VEGF and geldanamycin (bottom). B, lysates from 5-min VEGF-treated EC (preincubated or not with geldanamycin) were submitted to the reverse immunoprecipitation with either hsp90 or Akt antibodies, and analyzed by immunoblotting for eNOS expression. The supernatants (Spnt) of these immunoprecipitations were submitted to another immunoprecipitation with eNOS antibodies before immunoblotting analysis to estimate their residual eNOS content; the proportion of detected eNOS (versus total eNOS pool) is indicated for each couple of lanes. C, lysates from VEGF/geldanamycin-treated EC were also directly analyzed by immunoblotting with antibodies directed against phospho-Ser1177-eNOS (top) or phospho-Ser1177-Akt (bottom). As a positive control for phosphorylation, lysates from EC exposed for 30 min to VEGF in the absence of geldanamycin were analyzed in parallel (see sixth lane). D, the Akt activity was measured as the extent of phospho-GSK-3 detected by immunoblotting after incubation of a GSK-3 fusion protein and Akt immunoprecipitates from the lysates of EC exposed for 30 min to VEGF in the absence (veh.) or presence of geldanamycin (gelda) or LY294002 (LY) versus unstimulated control cells (CTL). E, neonatal mouse cardiac myocytes were exposed to insulin for 5 min in the absence (veh.) or presence of geldanamycin (gelda). Corresponding lysates as well as lysates from control, unstimulated cells (CTL) were analyzed by immunoblotting with antibodies directed against phospho-Akt, phospho-eNOS, and phospho-GSK-3. These experiments were repeated two to four times with similar results.

1B (right), we show that a 1-h preincubation with LY294002 (10 μM) completely prevented both Akt and eNOS phosphorylation upon 30 min VEGF stimulation.

eNOS Recruits Akt through a Mutual Hsp90 Interaction—We next sought to determine whether eNOS and Akt interact together in eNOS/Akt co-immunoprecipitation experiments. Interestingly, as illustrated in Fig. 2A (top), we consistently observed the co-immunoprecipitation of Akt with eNOS with a time course in agreement with the observed pattern of eNOS phosphorylation (see Fig. 1B) and hsp90/eNOS interaction (see Fig. 1A); using anti-phospho-Akt antibodies, we verified that the eNOS-bound Akt was the activated (phosphorylated) form of the kinase (not shown). Based on this correlation in the kinetics, we then examined whether these different events were directly related. EC were pre-exposed for 30 min, before VEGF stimulation, to geldanamycin (1 μg/ml), an agent known to inhibit hsp90 function (11), and corresponding lysates were submitted to immunoprecipitation with eNOS antibodies. In these conditions, hsp90 was no longer detectable in eNOS immunoprecipitation (not shown) and neither was Akt (Fig. 2A, bottom).
Fig. 2E shows that while the pretreatment with geldanamycin (1 µg/ml) did not alter the insulin-induced Akt activation, it completely abolished eNOS phosphorylation. We also took advantage of the native expression of GSK-3 in myocytes to determine whether geldanamycin could influence the phosphorylation of this other Akt substrate. Fig. 2E (lower panel) shows that the insulin-induced increase in GSK-3 phosphorylation was not altered by geldanamycin. Of note, the stimulatory effect of insulin on Akt, eNOS, and GSK-3 phosphorylation was blocked by preincubation of myocytes with 50 µM LY294002 (not shown).

Hsp90 Facilitates Akt-dependent NO Production through eNOS Phosphorylation on Ser1177.—Since our finding of hsp90-dependent phosphorylation of Ser1177 eNOS could be confounded by the phosphorylation or dephosphorylation of eNOS on other sites as recently reported by others (18–20, 28), we carried out transfection experiments to evaluate the effect of geldanamycin treatment and hsp90 overexpression in COS cells expressing either wild-type eNOS or S1177A eNOS (construct in which the serine 1177 is mutated in alanine). We first validated that when COS cells were co-transfected with active Akt (T308D/S473D), a significant increase in nitrite production was observed in WT-eNOS-expressing cells (+64 ± 4%) whereas no change in eNOS activity was detected with the non-phosphorylatable S1177A eNOS cells (Fig. 3, see bars 3 and 4). Interestingly, when COS cells were pretreated for 30 min with geldanamycin, around 70% of the Akt-dependent increase in nitrite production in WT-eNOS cells was blocked (Fig. 3, bar 5). In another series of experiments, the cellular pool of hsp90 was increased by 2-fold (see Fig. 3, lower panel) by co-transflecting hsp90 cDNA either with WT- or S1177A-eNOS constructs. As shown in Fig. 3 (bars 7 and 8), the consecutive increase in nitrite production (+49 ± 4 and +40 ± 3%, respectively) was similar in both conditions. By contrast, when active Akt (T308D/S473D) was also present in the system, a synergistic increase of 152 ± 8% over basal level of nitrite production was observed in cells expressing WT-eNOS (Fig. 3, bar 9), whereas no further change (over the hsp90-alone condition) was observed in S1177A eNOS cells (Fig. 3, compare bars 8 and 10); preincubation with geldanamycin blocked 70–95% of the recombinant hsp90- and hsp90/Akt-mediated increase in NO production (not shown). The lower panel of Fig. 3 shows the immunoblots for eNOS (WT and S1177A), Myc-tagged Akt and hsp90 (recombinant + endogenous).

Late eNOS Phosphorylation Is Dependent on the Early VEGF-evoked Increase in [Ca2+]i.—We next studied the calcium dependence of the Akt/hsp90 recruitment and eNOS phosphorylation process by preincubating EC with the intracellular calcium chelator BAPTA before exposure to VEGF. Fig. 4A shows the pattern of changes in [Ca2+]i, in EC exposed to VEGF in the absence and presence of 20 µM BAPTA-AM. VEGF exposure led to a slightly delayed calcium transient peaking after 1 min and slowly returning to basal levels in agreement with the reported phospholipase C-γ-dependent calcium release from internal stores (29). In the presence of BAPTA, the VEGF-evoked calcium transient was completely abrogated, indicating that under these experimental conditions, any further change in eNOS activity may be considered as independent of changes in measurable [Ca2+]i.

In conditions where BAPTA blocked the VEGF-induced increase in [Ca2+]i, we observed a complete inhibition of caveolin/eNOS dissociation as reflected by the consistent amount of eNOS co-immunoprecipitated with caveolin over time in response to VEGF exposure (Fig. 4B, top). Calcium clamping by BAPTA also prevented the VEGF-induced Akt/eNOS co-immunoprecipitation (Fig. 4B, middle) and eNOS phosphorylation (Fig. 4B, bottom). Of note, the hsp90/eNOS co-immunoprecipitation was also completely prevented by the use of BAPTA (not shown). Importantly, when using the 30-min VEGF exposure in the absence of BAPTA as a control condition, we consistently observed the dissociation of eNOS from caveolin, the eNOS/Akt interaction as well as eNOS phosphorylation (Fig. 4B, right).

Hsp90 Promotes the Phosphorylation-dependent Component of eNOS Activation.—We next measured the activity of eNOS following VEGF stimulation in the presence and absence of...
addition in order to better distinguish between direct Ca\textsuperscript{2+}/CaM-dependent and phosphorylation-mediated activation of eNOS.

As shown in Fig. 5B (where the nitrite production is normalized per min), geldanamycin pretreatment significantly inhibited the late VEGF-induced eNOS activation in agreement with the observed inhibition of interaction with hsp90 and Akt, whereas no significant effect on the early VEGF-induced eNOS activation was observed. Importantly, similar results were obtained using the PI3K inhibitor LY294002 confirming that the effect of Akt phosphorylation on eNOS activation was limited to the late activation of the enzyme. Of note, the basal level of [Ca\textsuperscript{2+}], and the profile of the VEGF-induced calcium transient were not altered by pretreatments with geldanamycin or LY294002 (not shown).

We next determined the calcium dependence of the early and late activation of eNOS by measuring the VEGF-induced NO\textsubscript{2} production in the presence of the intracellular calcium chelator BAPTA. As shown in Fig. 5B, preincubation with 20 \mu M BAPTA-AM, in conditions similar to those used in Fig. 4A, was sufficient to block not only the early (0–2 min) but also the late eNOS activation (5–30 min). Importantly, when BAPTA was added after the early phase of enzyme activation (at t = 5 min), we did not observe any significant reduction in the level of eNOS activation for the 5–30-min period (versus vehicle condition; not shown). Finally, we also used the calcium ionophore A23187 to examine whether a temporary increase in [Ca\textsuperscript{2+}], similar to the initial Ca\textsuperscript{2+} rise evoked by VEGF was sufficient to produce both the early and late activation of eNOS. Accordingly, cells were incubated in the presence of 1 \mu M A23187 for 2 min and then extensively washed and placed in Ca\textsuperscript{2+}-free medium containing 1 mM EGTA; in these conditions, [Ca\textsuperscript{2+}]i increased upon A23187 exposure and returned to its basal level with a time course similar to that observed with VEGF (not shown). Fig. 5B shows that while the A23187 pulse led to an increase in early NO\textsubscript{2} production (0–2 min) amounting to ~3-fold the level obtained with VEGF, the long-term A23187-stimulated NO\textsubscript{2} production was significantly lower than in VEGF-exposed cells.

In order to further dissect the calcium dependence of the Akt-mediated phosphorylation of eNOS, we examined the eNOS/CaM association after 2 and 30 min VEGF exposure, i.e., at the maximum of the calcium transient and after the return of [Ca\textsuperscript{2+}]i to its basal level, respectively. Fig. 5C (top) reveals that, in our experimental conditions, CaM could be detected in the eNOS immune complex 2 min after VEGF stimulation and that this association persisted for at least 30 min. Interestingly, while pretreatment with BAPTA completely abrogated the CaM/eNOS interaction (Fig. 3B, top), the Akt phosphorylation was not altered by calcium chelation (Fig. 3B, bottom), thereby excluding an effect of calcium chelation on Akt activity to account for the blockade of late eNOS activation (see Fig. 5C). Finally, we also observed that the temporary increase in [Ca\textsuperscript{2+}]i, induced by a short exposure to A23187 (see above) led to the early association between eNOS and CaM but not to the prolonged Akt activation, in agreement with the low level of NO production in the 5–30-min interval of exposure to VEGF (see last column in Fig. 5B).

**DISCUSSION**

The recent discovery of the regulatory phosphorylation of eNOS has considerably challenged the original assumption that NO production by this Ca\textsuperscript{2+}/CaM-dependent enzyme primarily reflected changes in intracellular [Ca\textsuperscript{2+}]. Even though several pharmacological studies had recently suggested the existence of a “Ca\textsuperscript{2+}-independent” activation of eNOS (mainly induced by fluid shear-stress) (30–32), the molecular basis for
such a mechanism remained elusive. Independent investigators have now reported that eNOS phosphorylation on serine 1177 (human sequence) by Akt (13, 14), AMP-activated (18), and cyclic nucleotide-dependent protein kinases (15) considerably enhanced the apparent calcium sensitivity of the enzyme, potentiating its activity at submaximal [Ca$^{2+}$]$_i$. In this study, we now document that although eNOS phosphorylation (and sustained activation) by Akt persists well after the initial calcium transient induced by VEGF, both regulatory processes of NO production are intimately linked in endothelial cells. Moreover, we show that hsp90 binding to eNOS is required for this transition from the early Ca$^{2+}$-dependent activation of eNOS to the late enzyme phosphorylation by Akt observed upon VEGF exposure.

Our demonstration that in EC, the hsp90/eNOS interaction triggered by VEGF stimulation is a key event allowing eNOS phosphorylation by Akt and prolonged NO release is based on the following evidence. (i) Time course studies revealed that, chronologically, EC exposure to VEGF first led to eNOS dissociation from caveolin, a hallmark of the Ca$^{2+}$/CaM-mediated activation of eNOS, and then to hsp90/eNOS interaction. The late occurrence of eNOS phosphorylation perfectly matched the time course of the hsp90/eNOS interaction. Furthermore, while we consistently observed the Akt phosphorylation in the first 30 s of EC exposure to VEGF, we could only detect Akt association to eNOS after 2 min of VEGF stimulation, e.g. concomitantly to hsp90/eNOS interaction. (ii) In transfected COS cells, hsp90 overexpression synergistically increased the extent of eNOS activation by Akt. Furthermore, these data clearly identified serine 1177 as the critical residue within the eNOS sequence accounting for the hsp90-dependent Akt-mediated activation of the enzyme. (iii) The use of the pharmacological hsp90 inhibitor, geldanamycin, allowed us to further demonstrate the obligatory and specific role of hsp90 in the eNOS phosphorylation process. Indeed, geldanamycin completely blocked the Akt recruitment and consecutive eNOS phosphorylation and activation whereas it did not interfere with Akt activation, Akt binding to hsp90 (33), nor phosphorylation of another Akt substrate (see Fig. 2E).

Our data also shed some light on the Ca$^{2+}$ dependence of both early and late eNOS activation. The increase in [Ca$^{2+}$], upon VEGF stimulation has been documented to result from c-Src-dependent activation of phospholipase C-$\gamma$ (29) whereas the Akt activation is known to occur following VEGF-induced activation of PI3K (17). Our data indicate that the concomitant occurrence of both signaling cascades leads to the potentiation of NO release in VEGF-exposed EC. Thus, the early VEGF-induced calcium transient is obligatory to promote the efficient binding of CaM to the enzyme (see Fig. 5C) and the short-term burst of NO release independently of eNOS phosphorylation by Akt (see Fig. 5B). Importantly, our data also revealed that the CaM/eNOS association, that persists even though [Ca$^{2+}$], has returned to baseline (see Figs. 4A and 5C) is critical for the late phase of enzyme activation. Indeed, the calcium chelator BAPTA, by preventing the VEGF-induced CaM/eNOS association, blocked not only the early phase of eNOS activation but also the late enzyme phosphorylation and prolonged NO production. However, although the VEGF-induced [Ca$^{2+}$], increase appears to be necessary for both aspects of eNOS activation, the only calcium transient is not sufficient to promote the prolonged NO release since we did not observe long-term eNOS activation by mimicking a temporary increase in [Ca$^{2+}$], by a sequential protocol of cell exposure to the calcium ionophore A23187 and Ca$^{2+}$-free medium. These data still do not exclude that in the case of G-protein-coupled receptors that mobilize [Ca$^{2+}$], in EC to a much larger extent than VEGF, the initial rise in calcium could directly lead to Akt phosphorylation through the activation of P13K (34–36) or the CaM-dependent protein kinase kinase (37). Of note, other kinases than Akt, such as the CaM-dependent protein kinase II (28) or the mitogen-activated protein kinase (19), have been recently reported to promote the phosphorylation-dependent activation of eNOS following G-protein-coupled receptor stimulation. Also, deinhibition of eNOS through dephosphorylation at Thr$^{1177}$, instead of as described in Ref. 38, or as a prerequisite to as described in Ref. 28, activation of the enzyme by Ser$^{1177}$ phosphorylation, was shown to contribute to the burst of NO production in EC upon stimulation of the G-protein-coupled bradykinin receptor.

A recent study by Pritchard and colleagues (39) reveals that altering the interaction of hsp90 with eNOS can uncouple the enzyme activity resulting in an increased superoxide anion production. Although, according to these authors, geldanamycin appears to exert its effects by preventing the ability of eNOS-bound hsp90 to change eNOS conformation and not by blocking the interaction between both proteins (our study, see Fig. 2B), these data emphasize the role of hsp90 as a major control point to integrate opposing properties of eNOS (coupled versus uncoupled enzyme activity) or to bridge early and late (independent) signaling cascades leading to the acute and prolonged NO release. While the ATP/ADP state of hsp90 seems to determine its modulatory effect on eNOS-derived NO production (39), the molecular details of this protein-protein interaction remain unclear at this time. Interestingly, the time course of the association of hsp90 with eNOS paralleled the changes in detergent solubility of eNOS induced by stimuli which mostly elicit an apparent calcium-independent activation (30, 32). Moreover, a very recent study by Sato and colleagues (33) documented that hsp90 potentiates Akt kinase activity by preventing PP2A-mediated dephosphorylation through a direct protein-protein interaction. Together, these findings suggest that VEGF stimulation promotes the formation of a stabilized multiprotein complex containing eNOS, hsp90, and Akt. It should be noted that when CHAPS or a Triton/molybdate mixture were used instead of octyl glucoside to lyse the cells and perform the immunoprecipitation, we consistently observed a low but significant amount of hsp90 associated to eNOS in the absence of any VEGF stimulation (not shown). Moreover, experiments in COS cells revealed that hsp90 also stimulates eNOS activation independently of Akt phosphorylation on serine 1177 (see Fig. 3). Together with the recent demonstration by Gratton et al. (12) that hsp90 facilitates eNOS/caveolin dissociation, it can therefore be postulated that a multicomplex including eNOS, caveolin, and hsp90 does exist and that local changes in calcium induces, in fact, the re-organization of the different partners within the complex and the recruitment of other players such as Akt.

Although, chronologically, the disruption of the caveolin/eNOS interaction precedes the hsp90/Akt recruitment, and [Ca$^{2+}$], chelation blocks both phenomena, we cannot conclude that only the pool of eNOS initially bound to caveolin participates in the phosphorylation-mediated activation. The extent of eNOS associated to caveolin seems to vary according to the activating EC type and the state of cell confluence. However, we and others have reported that an increase in the abundance of caveolin (native protein (40) or peptido-mimetics (28, 41)) resulted in a marked decline in agonist-stimulated NO generation. Altogether, these observations suggest that caveolae could be a critical pool of activable eNOS in native cells. Further studies need to be performed to elucidate whether Akt selec-

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A. Brouet and O. Feron, unpublished observations.
tively phosphorylates caveolar or intracellular pools of eNOS.

In conclusion, we found that the binding of hsp90 is necessary for the recruitment of Akt to Ca\(^{2+}\)/CaM-activated eNOS, thereby leading to the enzyme phosphorylation and the long-lasting NO release even in conditions of low [Ca\(^{2+}\)]. Our findings also revealed that the binding of CaM to eNOS, as evoked by the initial Ca\(^{2+}\) transient, is obligatory to promote the long-term NO release at submaximal levels of [Ca\(^{2+}\)]. Thus, the so-called “calcium-independent” activation of eNOS appears misleading and we think that the term “phosphorylation-dependent eNOS activation” should be preferred at least in the context of VEGF stimulation. More generally, the identification of hsp90 as a key player in the eNOS phosphorylation pathway in EC (but also in myocytes, see Fig. 2E) opens new perspectives in the pharmacological regulation of NO-mediated pathways such as vasorelaxation and angiogenesis but also in understanding of the pathophysiology of diseases associated with alterations in hsp90 abundance such as hypoxia (42).

REFERENCES

1. Ignarro, L. J., Cirino, G., Casini, A., and Napoli, C. (1999) J. Cardiovasc. Pharmacol. 34, 879–886
2. Balligand, J. L., Feron, O., and Kelly, R. A. (2000) Nitric Oxide: Biology and Pathobiology (Ignarro, L. J., ed) Academic Press, San Diego, CA
3. Feron, O. (1999) Curr. Opin. Clin. Nutr. Metab. Care 2, 201–206
4. Feron, O., Saldana, F., Michel, J. B., and Michel, T. (1998) J. Biol. Chem. 273, 3125–3128
5. Prabhakar, P., Thatte, H. S., Cho, M. R., Golan, D. E., and Michel, T. (1998) J. Biol. Chem. 273, 27383–27388
6. Feng, Y., Venema, W. J., Venema, R. C., Tsai, N., and Caldwell, R. B. (1999) Biochem. Biophys. Res. Commun. 256, 192–197
7. Sowa, G., Liu, J., Papapetropoulos, A., Rea-Haffer, M., Hughes, T. E., and Sessa, W. C. (1999) J. Biol. Chem. 274, 22524–22531
8. Nuszkowski, A., Grabher, R., Marsche, G., Unhehau, A., Malle, E., and Heller, R. (2001) J. Biol. Chem. 276, 14212–14221
9. Michel, J. B., Feron, O., Sacks, D., and Michel, T. (1997) J. Biol. Chem. 272, 15583–15586
10. Rizzo, V., McIntosh, D. P., Oh, P., and Schnitzer, J. E. (1998) J. Biol. Chem. 273, 34724–34729
11. Garcia-Cardenas, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., and Sessa, W. C. (1998) Nature 392, 821–824
12. Gratton, J. P., Fontana, J., O'Connor, D. S., Garcia-Cardenas, G., McCabe, T. J., and Sessa, W. C. (2000) J. Biol. Chem. 275, 22268–22272
13. Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) Nature 399, 597–601
14. Dintzler, S., Fleming, I., Fisslthaler, B., Herrmann, C., Busse, R., and Zeiher, A. M. (1999) Nature 399, 601–605
15. Butt, E., Bernardt, M., Smolen斯基, A., Kotsonis, P., Frohlich, L. G., Sickmann, A., Meyer, H. E., Lohmann, S. M., and Schmidt, H. H. (2000) J. Biol. Chem. 275, 5179–5187
16. Gallis, R., Corthals, G. L., Goodlett, D. R., Ueba, H., Kim, F., Presnell, S. R., Figeys, D., Harrison, D. G., Berk, B. C., Abersold, R., and Corson, M. A. (1999) J. Biol. Chem. 274, 30101–30108
17. Michell, B. J., Griffiths, J. E., Mitchellhill, K. I., Rodriguez-Crespo, I., Tiganis, T., Bozinovski, S., de Montellano, P. R., Kemp, B. E., and Pearson, R. B. (1999) Curr. Biol. 9, 845–848
18. Chen, Z. P., Mitchellhill, K. I., Michell, B. J., Stapleton, D., Rodriguez-Crespo, I., Witters, L. A., Power, D. A., Ortiz de Montellano, P. R., and Kemp, B. E. (1999) FEBS Lett. 443, 285–289
19. Bernier, S. G., Haldar, S., and Michel, T. (2000) J. Biol. Chem. 275, 30707–30715
20. Michell, B. J., Chen, Z., Tiganis, T., Stapleton, D., Katsia, F., Power, D. A., Sim, A. T., and Kemp, B. E. (2001) J. Biol. Chem. 276, 17625–17628
21. Dimmeler, S., Dernbach, E., and Zeiher, A. M. (2000) FEBS Lett. 477, 258–262
22. McCabe, T. J., Fulton, D., Roman, L. J., and Sessa, W. C. (2000) J. Biol. Chem. 275, 6125–6128
23. Carmeliet, P. (2000) Nat. Med. 6, 389–395
24. Murohara, T., Horowitz, J. R., Silver, M., Tsurumi, Y., Chen, D., Sullivan, A., and Isner, J. M. (1998) Circulation 97, 99–107
25. Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J. F., Fishman, M. C., Huang, P. L., and Isner, J. M. (1998) J. Clin. Invest. 101, 2567–2578
26. Feron, O., Deysy, C., Opel, D. J., Arstall, M. A., Kelly, R. A., and Michel, T. (1998) J. Biol. Chem. 273, 30249–30254
27. Pham, F. H., Sugden, P. H., and Clerk, A. (2000) Circ. Res. 86, 1252–1258
28. Fleming, I., Fisslthaler, B., Dimmeler, S., Kemp, B. E., and Busse, R. (2001) Circ. Res. 88, E63–E75
29. He, H., Venema, V. J., Gu, X., Venema, R. C., Marrero, M. B., and Caldwell, R. B. (1999) J. Biol. Chem. 274, 25130–25135
30. Fleming, I., and Busse, R. (1999) Cardiovasc. Res. 43, 532–541
31. Fisslthaler, B., Dimmeler, S., Herrmann, C., Busse, R., and Fleming, I. (2000) Acta Physiol. Scand. 168, 81–88
32. Fleming, I., Baurersachs, J., Fisslthaler, B., and Busse, R. (1998) Circ. Res. 82, 696–695
33. Sato, S., Fujita, N., and Tsuuro, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10832–10837
34. Murga, C., Pakhuhara, S., and Gutkind, J. S. (2000) J. Biol. Chem. 275, 12069–12073
35. Igarashi, J., Bernier, S. G., and Michel, T. (2001) J. Biol. Chem. 276, 12420–12426
36. Morales-Ruiz, M., Lee, M. J., Zollner, S., Gratton, J. P., Scotland, R., Shiojima, I., Walsh, K., Hla, T., and Sessa, W. C. (2001) J. Biol. Chem. 276, 19672–19677
37. Yano, S., Tokumitsu, H., and Soderling, T. R. (1998) Nature 396, 584–587
38. Harris, M. B., Ju, H., Venema, V. J., Liang, H., Zou, R., Michell, B. J., Chen, Z. P., Kemp, B. E., and Venema, R. C. (2001) J. Biol. Chem. 276, 16587–16591
39. Prichard, R. A., Jr., Ackerman, A. W., Gross, E. R., Stepp, D. W., Shi, Y., Fontana, J. T., Baker, J. E., and Sessa, W. C. (2001) J. Biol. Chem. 275, 17621–17624
40. Feron, O., Deysy, C., Monzillo, S., Desager, A. J., and Balligand, J. L. (1999) J. Clin. Invest. 103, 897–905
41. Bucci, M., Gratton, J. P., Rudik, R. D., Avevedo, L., Roviozzo, F., Cirino, G., and Sessa, W. C. (2000) Nutr. Mol. 6, 1362–1367
42. Su, Y. and Block, E. R. (2000) Am. J. Physiol. Lung Cell Mol. Physiol. 278, L1204–L1212
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J. Biol. Chem. 2001, 276:32663-32669.
doi: 10.1074/jbc.M101371200 originally published online June 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101371200

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