Synergistic Activation of Endothelial Nitric-oxide Synthase (eNOS) by HSP90 and Akt

CALCIUM-INDEPENDENT eNOS ACTIVATION INVOLVES FORMATION OF AN HSP90-Akt-CaM-BOUND eNOS COMPLEX*

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Endothelial nitric-oxide synthase (eNOS), which generates the endogenous vasodilator, nitric oxide (NO), is highly regulated by post-translational modifications and protein interactions. We recently used purified proteins to characterize the mechanisms by which heat shock protein 90 (HSP90) increases eNOS activity at low and high Ca2+ levels (Takahashi, S. and Mendelsohn, M. E. (2003) J. Biol. Chem. 278, 9339–9344). Here we extend these studies to explore interactions between HSP90, Akt, and eNOS. In studies with purified proteins, HSP90 increased the initial rate and maximal extent of Akt-mediated eNOS phosphorylation and activation at low Ca2+ levels. Akt was not observed in the eNOS complex in the absence of HSP90, but both active and inactive Akt associated with eNOS in the presence of HSP90. Direct binding of Akt to HSP90 was observed even in the absence of eNOS. HSP90 also facilitated CaM binding to eNOS irrespective of Akt presence. Geldanamycin (GA) disrupted HSP90-eNOS binding, reduced HSP90-stimulated CaM binding, and blocked both recruitment of Akt to the eNOS complex and phosphorylation of eNOS at Ser-1179. Akt phosphorylated only CaM-bound eNOS, in an HSP90-independent manner. HSP90 and active Akt together increased eNOS activity synergistically, which was reversed by GA. In bovine aortic endothelial cells (BAECs), the effects of vascular endothelial growth factor (VEGF) and insulin on eNOS-HSP90-Akt complex formation and eNOS activation were compared. BAPTA-AM inhibited VEGF- but not insulin-induced eNOS-HSP90-Akt complex formation and eNOS phosphorylation. Insulin caused rapid, transient increase in eNOS activity correlated temporally with the formation of eNOS-HSP90-Akt complex. GA prevented insulin-induced association of HSP90, Akt and CaM with eNOS and inhibited eNOS activation in BAECs. Both platelet-derived growth factor (PDGF) and insulin induced activation of Akt in BAECs, but only insulin caused HSP90-Akt-eNOS association and eNOS phosphorylation. These results demonstrate that HSP90 and Akt synergistically activate eNOS and suggest that this synergy contributes to Ca2+-independent eNOS activation in response to insulin.

Endothelial nitric-oxide synthase (eNOS)† is a highly regulated, Ca2+/calmodulin (CaM)-dependent enzyme responsible for the physiological production of nitric oxide (NO) in the vasculature (1). eNOS is also regulated by subcellular localization, post-translational modification such as phosphorylation by Akt/protein kinase B (2–4), and interactions with several regulatory proteins, such as heat shock protein 90 (HSP90) (5–7).

Akt/protein kinase B increases eNOS activity by phosphorylation at Ser-1177 and Ser-1179 for human and bovine eNOS, respectively (2–4), while HSP90 promotes eNOS activity by direct interaction with the enzyme (5–7). Exposure of endothelial cells (ECs) to vascular endothelial growth factor (VEGF), estrogen or fluid shear stress induces both an increased association of HSP90 with eNOS and eNOS phosphorylation by Akt, leading to elevation of NO production (2–5, 8–13). It has been shown further that in endothelial cells activated by the above stimuli, inhibition of either HSP90 or Akt results in a marked reduction of NO production. Though the activation of eNOS is well characterized for HSP90 and Akt individually, only a few studies have addressed the potential interplay of these two proteins in eNOS activation. Brouet et al. (13) reported cooperative stimulation of eNOS by HSP90 and Akt in Ca2+-dependent VEGF-stimulated ECs. Their data suggested that HSP90 association is a prerequisite for subsequent Akt-mediated stimulation of eNOS. In addition, in heterologous COS cell expression studies, it was suggested that synergistic enhancement of eNOS activation is induced by HSP90 and Akt. Recently, Fontana et al. (14) also suggested that HSP90 might function as a scaffold protein for eNOS and Akt, facilitating eNOS phosphorylation and activation in VEGF-stimulated ECs. However, the synergy between HSP90 and Akt in eNOS activation cannot be explained by only the scaffolding effect of HSP90. The present study examines potential interactions between eNOS, HSP90, and Akt in vitro using purified proteins. Our results provide evidence that HSP90 and Akt synergistically activate eNOS at physiological calcium levels, and show that this occurs for Ca2+-independent activation of eNOS by insulin in endothelial cells.

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The abbreviations used are: eNOS, endothelial nitric-oxide synthase; peNOS, phospho-Ser-1179-eNOS; pAkt, phospho-Ser-473-Akt; HSP90, heat shock protein 90; CaM, calmodulin; tNAME, Nω-nitro-L-arginine methyl ester; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; BAEC, bovine aortic endothelial cells; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonic acid; GA, geldanamycin; CaM, calmodulin; EC, endothelial cells.
EXPERIMENTAL PROCEDURES

Materials—The enzymes, antibodies and reagents used in this study and their sources are as follows: active Akt/PKBa, inactive Akt1/PKBb, recombinant chicken CaM, staurosporin, vascular endothelial growth factor (VEGF), insulin, platelet-derived growth factor-BB (PDGF), geldanamycin (GA), protease inhibitor mixture set III, and BAPTA-AM (Calbiochem, La Jolla, CA), anti-eNOS antibody and anti-HSP90 antibody (BD Transduction Laboratories, Lexington, KY), anti-peNOS antibody (phospho-Ser-1177) and anti-Akt antibody, anti-pAkt antibody (phospho-Ser-473) and Phototope-HRP Western blot detection system (Cell Signaling, Beverly, MA); 2,5′-ADP Seraphose 4B, CaM Seraphose 4B, HiTrap Q, and PD10 columns (Amersham Biosciences), Bradford protein assay kit (Bio-Rad), polyvinylidene difluoride transfer membrane Immobilon-P (Millipore, Bedford, MA), t-[2,3,4-3H]arginine (45–70 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmol) (PerkinElmer Life Sciences, Boston, MA). All other chemicals were of reagent grade.

eNOS Purification—Recombinant bovine wild-type eNOS, expressed in S99 cells, was purified from the lysate by sequential chromatography using 5′-ADP Seraphose and CaM Seraphose columns according to the method of List et al. (15). The eNOS was further purified by HiTrap Q chromatography with a linear gradient of 0.1–1 mM NaCl (7). The eNOS protein was stored at −80 °C in 50 mM Tris-HCl (pH 7.5) buffer containing 1% CHAPS, 1 mM dithiothreitol, 100 mM NaCl, and 5 mM EGTA. Protein concentration was determined with bovine serum albumin as a standard. CaM-bound eNOS was prepared as we previously reported (7).

Phosphorylation of eNOS by Akt—eNOS (9.8 nM) and Akt (7.1 nM) were mixed with vehicle or HSP90 (45 nM) in the presence of CaM (300 nM) with 100 mM Ca2+ or 10 mM EGTA, and then left at room temperature for 10 min. In some experiments, HSP90 was pretreated with 1 μM GA. Phosphorylation of eNOS was initiated by addition of 50 μM ATP and 5 mM MgCl2 in kinase reaction buffer consisting of 50 mM HEPES-NaOH (pH 7.5), 1 mM dithiothreitol, 0.05% Triton X-100, and 5% glycerol. After incubation at 37 °C for the indicated time, the reaction was terminated by 10 μM staurosporine, which had no effect on eNOS activity, but completely inhibited Akt-mediated eNOS phosphorylation.

In addition to eNOS phosphorylation, Akt activity was also evaluated by using the synthetic peptide substrate, crostone (GRPRTSSFAEAG). Crostone (50 μM) was precirculated with HSP90 in the same manner as above, and then was incubated with active Akt at 37 °C for 5 min in the kinase reaction buffer containing 50 μM [γ-32P]ATP. Akt-dependent incorporation of radioactivity to crostone was measured with a liquid scintillation analyzer.

Immunoprecipitation and Immunoblotting of eNOS, HSP90, Akt, and CaM—eNOS complex was incubated with anti-eNOS antibody in the corresponding NOS reaction buffer at 4 °C for 2 h, and successively with protein G-agarose beads at 4 °C overnight, unless otherwise stated. The immunoprecipitates were subjected to SDS-PAGE and then blotted onto polyvinylidene difluoride membranes. The blots were incubated with the primary antibody at 4 °C overnight and then probed with the secondary antibody linked to peroxidase. Immunoreactive proteins were visualized on x-ray film by an enhanced chemiluminescent method. Intensity of each band was measured by densitometry and then normalized to the corresponding control. Therefore, relative intensity to the defined group as described in each figure legend was counted on a liquid scintillation analyzer. t-NAME-inhibitable activity was determined as specific eNOS activity.

eNOS Activity and Complex Formation in Endothelial Cells—Bovine aortic endothelial cells (BAECs) were allowed to grow to confluence in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B and 10% fetal bovine serum. BAECs were starved in serum-free medium over night prior to experiments. The cells were pretreated with vehicle, 100 μM BAPTA-AM for 15 min or 10 μM GA for 1 h, and then stimulated with 1 nM VEGF, 200 nM insulin, or 200 nM PDGF for 10 min, unless otherwise stated. The cells were lysed in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 10 mM sodium orthovanadate, 10 mM sodium fluoride, 10 μM β-glycerophosphate, 1% Triton X-100, 0.5% CHAPS, protease inhibitor mixture, and 5% PEG 400. The lysates were left on ice for 10 min and then were centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatants were passed through a PD10 column to remove endogenous arginine. The void fractions were collected as cell extract and subjected to eNOS activity and immunoprecipitation studies.

Statistical Analysis—Data are presented as means ± S.E. Statistical difference was evaluated by Student’s t test. P value of <0.05 was regarded as significant.

RESULTS

Synergistic Effect of HSP90 and Akt on eNOS Activation in Vitro—Recombinant bovine eNOS was purified to homogeneity by sequential chromatography as recently described (7) to a specific activity of 12–15 nmol/min/mmol protein in the presence of 10 μM Ca2+ and 300 mM CaM. The eNOS preparation did not contain detectable HSP90, Akt, or CaM by immunoblotting.

The time course of eNOS phosphorylation by Akt was examined for eNOS preincubated with active Akt, low Ca2+ (100 nM) and CaM in the presence or absence of HSP90 (Fig. 1). Phosphorylation of eNOS at Ser-1179 was examined by immunoblotting and quantitated densitometrically. eNOS phosphorylation is expressed as the percentage of peNOS at 20 min in the presence of HSP90. Data are mean ± S.E. for four determinations. * p < 0.05 from the corresponding control.
phosphorylation was detectable by 1 min and increased dramatically in the first 5–10 min, after which a plateau level was reached. Both the initial rates and the final amount of Akt-mediated eNOS phosphorylation were significantly higher in the presence of HSP90 than in the absence of HSP90. Pretreatment of HSP90 with 10 μM GA inhibited the enhancement of Akt phosphorylation of eNOS by HSP90 almost completely (data not shown).

The degree of Akt-mediated eNOS phosphorylation at Ser-1179 and the presence of potential eNOS-associated proteins were analyzed at physiological calcium levels in the absence or presence of HSP90 (Fig. 2A). When HSP90 was present in the reaction mixture, it was found associated with immunoprecipitated eNOS, and this association was not affected by the presence of inactive or active Akt. HSP90 increased CaM detected with eNOS whether or not Akt was present. As expected, eNOS at Ser-1179 was phosphorylated by active Akt, but not by inactive Akt. Active Akt was not detected with eNOS in the absence of HSP90, but both active and inactive Akt associated with eNOS in the presence of HSP90. The extent of eNOS phosphorylation by Akt was greater in the presence of HSP90 than in its absence (see Fig. 1). GA disrupted HSP90 binding to eNOS, and reduced the amount of CaM recovered with eNOS. GA also blocked the recruitment of Akt to the eNOS complex and eNOS Ser-1179 phosphorylation. As shown in Fig. 2B, when eNOS, HSP90, and Akt were incubated, and HSP90 was then immunoprecipitated, both active and inactive Akt were present in the complex with HSP90 and eNOS. In addition, Akt was found associated with HSP90 in the absence of eNOS. Further studies were done to examine proteins remaining in the postimmunoprecipitation supernatant (data not shown). In these studies, eNOS (9.8 nM), HSP90 (45 nM), and Akt (7.1 nM) were mixed as in the studies shown in Fig. 2 and immunoprecipitated with either anti-eNOS or anti-HSP90 antibody using antibody concentrations chosen so that all detectable eNOS or HSP90 were precipitated, respectively. After immunoprecipitation of eNOS, HSP90 was easily detected in the residual supernatant, but only trace Akt was detected in the supernatant. After immunoprecipitation of HSP90, ~10–15% of eNOS remained in the supernatant and Akt was not detected in the supernatant. These results indicate that virtually all Akt was bound tightly to HSP90 and incorporated into a ternary complex with eNOS. These data and those shown in Fig. 2 support that Akt is recovered in the eNOS complex only in the presence of HSP90, and that HSP90 is required for formation of the eNOS-HSP90-Akt ternary complex.

We next examined whether the presence of HSP90 bound to eNOS alters the degree of eNOS phosphorylation by Akt (Fig. 3). Control experiments for these studies were performed first using the synthetic Akt substrate, crosstide, to assess the level of Akt activity in the presence of Ca2+ or EGTA. Active Akt phosphorylated the crosstide peptide similarly in the presence of Ca2+ or EGTA in both the absence and presence of HSP90 (EGTA without HSP90, 102.2 ± 3.7%; EGTA with HSP90, 103.4 ± 3.6%; Ca2+ without HSP90, 100 ± 5.0%; Ca2+ with HSP90, 99.3 ± 1%, n = 4). To exclude HSP90-mediated increases of CaM binding to eNOS, CaM was pre-bound to eNOS and then separated from residual free (unbound) CaM (7). CaM-bound and -free eNOS were then mixed with active Akt in the presence or absence of HSP90. Akt phosphorylated only the CaM-bound form of eNOS, and CaM-bound eNOS was phosphorylated to a similar degree in the absence and presence of HSP90. Akt was unable to phosphorylate CaM-bound eNOS in...
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The presence of EGTA or staurosporine (data not shown). Akt did not phosphorylate CaM-free eNOS even with HSP90 present, indicating that HSP90 binding to eNOS is not sufficient to support eNOS phosphorylation by Akt.

Using the same conditions as the previous experiments, eNOS activity next was assayed in the presence of HSP90, active Akt, or both and in the absence or presence of EGTA (Fig. 4). In all cases, eNOS activity was negligible in the presence of EGTA. eNOS activity was easily detectable in the presence of 100 nM Ca²⁺ and HSP90 increased the activity of eNOS in the presence of inactive Akt by 3.5-fold, similar to our recent study (7). GA abolished the increase in eNOS activity observed with HSP90 and inactive Akt. Basal and HSP90-stimulated eNOS activity in the absence of any form of Akt were nearly identical to basal and HSP90-stimulated eNOS activity in the presence of inactive Akt, indicating that inactive Akt itself has virtually no effect on eNOS activity. Active Akt alone increased eNOS activity by 2-fold. The presence of both HSP90 and active Akt produced a synergistic (9-fold) increase in eNOS activity. GA also reversed the increase in eNOS activity observed in the presence of HSP90 and active Akt to the level of active Akt alone.

Role of HSP90 in Insulin- and VEGF-induced eNOS Activation in Endothelial Cells—The in vitro studies above with purified proteins support that HSP90 facilitates Akt-mediated phosphorylation of eNOS, and that HSP90 and Akt coordinately promote eNOS activity at physiologic calcium concentrations. HSP90 has been shown to serve as a scaffold for Akt recruitment and to be required for activation of eNOS in VEGF-stimulated cells (14). VEGF activates eNOS in Ca²⁺-dependent manner (13). In contrast, insulin activation of eNOS, which is also mediated by Akt, is Ca²⁺-independent (18). We therefore compared the effects of VEGF and insulin on eNOS activation and formation of an eNOS-HSP90-Akt ternary complex in intact BAECs. VEGF (1 nM) and insulin (200 nM) each caused a significant increase in eNOS activity (Fig. 5A). Consistent with previous reports (13, 18), pretreatment of the cells with BAPTA-AM completely inhibited VEGF-induced eNOS activation, but had no effect on insulin-induced eNOS activation. In response to both VEGF and insulin, HSP90, and Akt both were co-precipitated with phospho(Ser-1179)-eNOS (Fig. 5B). BAPTA-AM inhibited most eNOS-HSP90-Akt complex formation and eNOS phosphorylation induced by VEGF. However, BAPTA-AM did not decrease eNOS-HSP90-Akt complex formation and Ser-1179 phosphorylation by the Ca²⁺-independent agonist insulin. With both agonists, eNOS activation is therefore correlated directly with eNOS-HSP90-Akt complex formation and eNOS Ser-1179 phosphorylation. We next examined whether inhibition of HSP90 by GA abrogates the insulin-induced increase in eNOS activity in BAECs (Fig. 6A). Insulin caused eNOS Ser-1179 phosphorylation and an increase in both HSP90 and Akt associated with eNOS (Fig. 6B; see Fig. 5B). Akt in this complex was phosphorylated at Ser-473, its active form, as expected (pAkt, Fig. 6B). Interestingly, though insulin stimulation of eNOS was Ca²⁺-independent in these experiments, insulin also increased the binding of CaM to eNOS. Pretreatment with GA had no effect on basal eNOS
activity, but completely abolished the stimulatory effect of insulin on eNOS activity. GA also disrupted association of HSP90, CaM, and activated Akt with eNOS.

The time courses of insulin-induced eNOS activation and eNOS-HSP90-Akt complex formation are shown in Fig. 7. Insulin-stimulated eNOS activity was increased by 50% in the first 2 min, was maximal (about a 2.5-fold increase) at 5 min, and had returned nearly to baseline by 15 min. Formation of an eNOS-HSP90-Akt complex was closely correlated with the changes in eNOS activity (Fig. 7B). However, pAkt levels were maximal at 2 min, prior to development of maximal phospho(Ser-1179)-eNOS levels at 5 min. Montagnani et al. (18) reported that Akt is necessary but not sufficient for insulin activation of eNOS. BAECs next were stimulated with either PDGF or insulin, which were both known to activate Akt, and then both eNOS activity and eNOS-HSP90-Akt complex formation in BAECs were measured (Fig. 8). PDGF and insulin each induced activation of Akt, but only insulin caused activation of eNOS. Insulin caused an eNOS-HSP90-Akt complex to form, but PDGF did not. Therefore, in intact BAECs, activation of Akt alone is not sufficient to activate eNOS.

**DISCUSSION**

HSP90 and Akt both increase eNOS activity in *vivo* and *in vitro* (2–14). Recently, we reported that the HSP90 interaction with eNOS enhances NO synthesis by two mechanisms: (i) a CaM-dependent mechanism operative at low Ca<sup>2+</sup> concentrations, characterized by an increase in the affinity of eNOS for CaM and (ii) a CaM-independent mechanism apparent at high Ca<sup>2+</sup> concentrations, characterized by stimulation of eNOS reductase activity even in the absence of a change in CaM binding (7). Akt increases NO synthesis by direct phosphorylation of eNOS at Ser-1179 (2–4). Studies by McCabe et al. (19) with the phosphomimetic mutant S1179D eNOS support that Ser-1179 phosphorylation by Akt also enhances NO synthesis by both increasing the rate of eNOS electron transfer and

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**Fig. 6.** Insulin-induced NOS activation and eNOS-HSP90-Akt complex formation in BAECs in the absence or presence of geldanamycin. A, BAECs were pretreated with GA and then stimulated with vehicle or 200 nM insulin for 10 min. eNOS activity in cell extracts is expressed as a percentage of the activity measured in the absence of GA and insulin. Data are mean ± S.E. for three determinations. *, p < 0.05 from the vehicle without GA and the insulin without GA, respectively. B, eNOS was immunoprecipitated at the end of the activity assay. eNOS, peNOS, HSP90, Akt, pAkt, and CaM were evaluated by immunoblotting. Data are representative of three experiments.

**FIG. 7.** Time courses of insulin-induced eNOS activation and eNOS-HSP90-Akt complex formation in BAECs. A, BAECs were stimulated with 200 nM insulin for the indicated times. eNOS activity was determined and expressed as a percentage of basal activity. Data are mean ± S.E. for three determinations. *, p < 0.05 from basal activity. B, eNOS was immunoprecipitated at each time point and eNOS, peNOS, HSP90, Akt, and pAkt were evaluated by immunoblotting. Data are representative of three experiments.
Recent studies demonstrated that HSP90 and Akt synergistically enhance NO production by eNOS (13). In the present study, we show that HSP90 and Akt synergistically increase eNOS activity in BAECs, but only insulin causes HSP90-eNOS association, eNOS phosphorylation and enzyme activation. Following insulin, Akt phosphorylation is apparent before HSP90 and Akt are detectable in immunoprecipitates of the eNOS complex. However, eNOS phosphorylation is detectable only when HSP90 and Akt are associated with eNOS, and not with the appearance of phospho-Akt alone, supporting that activation of Akt is not simply equivalent to eNOS activation (18). Interestingly, although insulin-induced eNOS activation is Ca2+-independent, insulin stimulates CaM binding to eNOS, an effect that is blocked by GA. GA also completely abolishes the stimulatory effect of insulin on eNOS activity, suggesting that HSP90 is required for insulin-induced eNOS activation. Our results support the importance of HSP90 in Akt-mediated eNOS activation following cellular stimulation by Ca2+-independent agonists like insulin. Several other mechanisms for the effects of HSP90 on Akt-mediated eNOS activation have been identified. Sato et al. (20) reported that HSP90 binding to Akt blocks inactivation of Akt by protein phosphatase 2A-mediated dephosphorylation. HSP90 also facilitates CaM-induced eNOS release from eNOS-caveolin (6). HSP90 also can prevent eNOS uncoupling and inhibit superoxide production, increasing NO synthesis (22), suggesting that HSP90 facilitates Akt activation of eNOS in multiple ways.

Recently, the phosphorylation state of multiple serine residues on eNOS were shown to regulate basal and stimulated NO release, promote the interaction of HSP90 and Akt with eNOS, and facilitate eNOS phosphorylation on Ser-1179 (23). Those studies showed that the interactions of HSP90 and Akt with eNOS are highly regulated and suggest that an eNOS “phosphorylation code” orchestrates eNOS-protein interactions, including those with HSP90 and Akt. The HSP90 scaffold promotes eNOS activation by facilitating the association and proximity of Akt with eNOS. This function is reminiscent of a large number of scaffolding proteins that organize multienzyme complexes into signaling complexes that are capable of transmitting information between individual components in a cell (14).
zyme regulatory complexes of kinases and phosphatases to allow precise subcellular arrangement of important signal transduction pathways (24). However, it is also possible that direct binding of Akt to eNOS may occur within the eNOS complex. HSP90 binding also could induce a conformational change of eNOS, resulting in appearance of a new binding site for Akt. Alternatively, HSP90 binding may increase substrate change of eNOS, resulting in appearance of a new binding site for Akt. Thus, while it is evident that eNOS-HSP90-Akt complex is formed in response to insulin, the precise interactions between eNOS, HSP90, and Akt remain to be clarified.

In conclusion, the present studies demonstrate that HSP90 and Akt synergistically activate eNOS at physiological Ca^{2+} levels. The upstream mechanisms by which Akt is recruited and activated by many agonists are not fully clarified. The synergistic activation of eNOS by HSP90 and Akt occurs in response to the Ca^{2+}-independent agonist insulin. Our data therefore support the importance of HSP90 and Akt working together to activate eNOS when cells are stimulated by calcium-independent agonists such as insulin.

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