

Hsp90–Akt phosphorylates ASK1 and inhibits ASK1-mediated apoptosis

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Hsp90 client protein Akt has been shown to inhibit cell apoptosis in part by inhibiting proapoptotic kinase ASK1 (apoptosis signal-regulating kinase 1) activity. In the present study, we show that Hsp90 inhibits hydrogen peroxide (H₂O₂)-induced ASK1–p38 activation in endothelial cells (EC). The inhibitory effect of Hsp90 on ASK1–p38 activities is diminished when the Akt phosphorylation site on ASK1 (pSer83) is absent or when Akt is genetically deleted in cells, suggesting that Hsp90 and Akt function together to inhibit ASK1–p38 signaling. Thus, inhibition of Hsp90 by 17-allylamino-17-demethoxygeldanamycin (17-AAG) or phosphatidylinositol 3-kinase (PI3K) LY294002 induced and synergized ASK1 activation and ASK1-mediated EC apoptosis. Furthermore, we show that in resting EC Hsp90, Akt and ASK1 form a ternary complex in which both Akt and ASK1 bind to the middle domain of Hsp90, suggesting that Hsp90 may hold Akt and ASK1 in close proximity. The N-terminal domain of ASK1 containing the Akt phosphorylation site (pSer83) associates with Akt in resting state. However, Akt is released from the N-terminal domain concomitant with binding to the C-terminal domain of ASK1 in response to ASK1 activator H₂O₂, inhibitor of Hsp90 17-AAG and Akt inhibitor LY294002, leading to a more stable Hsp90–Akt–ASK1 complex. We conclude that Hsp90–Akt forms a complex with ASK1 and protect EC from stress-induced apoptosis.

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Introduction

Apoptosis signal-regulating kinase 1 (ASK1) is one of several MAP3Ks that are activated in response to proinflammatory stimuli, hydrogen peroxide (H₂O₂)

and cellular stress, leading to activation of MAP2K–JNK/p38 cascades (Ichijo *et al.*, 1997). Although ASK1 functions in cell proliferation, differentiation and survival in various cell types, its role in apoptosis has been most extensively studied (Chang *et al.*, 1998; Hatai *et al.*, 2000; Charette *et al.*, 2001; Nishitoh *et al.*, 2002). Studies in overexpression systems and from ASK1 knockout mice have shown that ASK1 is a critical mediator in TNF, ROS and stress-induced cell death (Tobiume *et al.*, 2002). ASK1 is reported to induce apoptosis by triggering a mitochondria-dependent pathway including Bid cleavage, Bax mitochondria translocation, cytochrome *c* release from mitochondria and subsequent activation of caspase 9 and caspase 3 (Hatai *et al.*, 2000). ASK1 is a 170 kDa protein that is composed of an inhibitory N-terminal domain, an internal kinase domain and a C-terminal regulatory domain. Thus, ASK1 is a central target of many cellular survival factors that bind to different domains of ASK1 to keep ASK1 in an inactive state. Redox sensor proteins thioredoxin (Trx) binds to the N-terminal domain, while glutaredoxin binds to the C-terminal domain of ASK1 to inhibit ASK1 kinase activity (Saitoh *et al.*, 1998; Liu *et al.*, 2000; Song *et al.*, 2002). Phosphoserine-binding protein 14-3-3 associates with ASK1 via the pSer967 site of ASK1 and inhibits ASK1-induced apoptosis (Zheng *et al.*, 1999; Liu *et al.*, 2001). Several proteins such as heat-shock protein hsp72 block ASK1 oligomerization and recruitment of its downstream targets (Park *et al.*, 2002). ASK1 can be phosphorylated at several sites and these phosphorylation sites regulate ASK1 activity in both positive and negative manner. Thus, protein phosphatase 5 (PP5) dephosphorylates ASK1 at the autophosphorylation site pThr845 to function as a negative feedback inhibitor of ASK1 signaling (Morita *et al.*, 2001). Conversely, several serine/threonine protein kinases such as Akt directly phosphorylates ASK1 at Ser83 to inhibit ASK1-induced apoptosis. Consistently, inhibition of Akt by phosphatidylinositol 3-kinase (PI3K) inhibitor significantly induces activation of p38 and JNK (Kim *et al.*, 2001).

The Akt (protein kinase B) serine/threonine kinase family is a critical mediators of cell survival (Dudek *et al.*, 1997; Franke *et al.*, 1997). Akt can phosphorylate a number of proapoptotic proteins including glycogen

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synthase kinase 3, BAD, caspase 9 and Forkhead transcription factors to suppress their proapoptotic activities (Cross *et al.*, 1995; Datta *et al.*, 1997; Cardone *et al.*, 1998; Kops *et al.*, 1999). Upon activation of PI3K in response to growth/survival stimuli, Akt via its PH domain targets to the cellular membrane, where it is phosphorylated by PDK1 at Ser473 and unidentified PDK2 at Thr308 (Vanhaesebroeck and Alessi, 2000). Conversely, inhibition of PI3K with inhibitors such as LY294002 blocks Akt phosphorylation leading to apoptosis. In addition, Akt is regulated by binding to a cellular chaperone protein Hsp90, which maintains stability and activity of Akt as well as other Hsp90 client proteins (such as Src, RAF-1 and RIP1) (Whitesell *et al.*, 1994; Schulte *et al.*, 1997; Lewis *et al.*, 2000; Sato *et al.*, 2000; Basso *et al.*, 2002). It has been shown that naturally occurring ansamycin antibiotics geldanamycin and its derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) bind to conserved binding pocket in the N-terminal domain of Hsp90, inhibiting ATP binding and ATP-dependent release of the client proteins undergoing refolding from Hsp90, leading to degradation of the client proteins (Schneider *et al.*, 1996; Grenert *et al.*, 1997; Prodromou *et al.*, 1997). Interestingly, many Hsp90 client proteins are involved in cell survival. Thus, prolonged treatment of 17-AAG in tumor cells induces degradation of these survival factors and AAG exerts antitumor activity in preclinical models (Neckers, 2002).

Since several Hsp90 client proteins (such as Akt and RAF-1) bind to inactive ASK1 (Chen *et al.*, 2001; Kim *et al.*, 2001), we hypothesized that Hsp90 together with its client proteins forms a complex with ASK1 and regulates ASK1 proapoptotic activity. In the present study, we show that Hsp90–Akt binds to and phosphorylates ASK1 at Ser83 to maintain ASK1 in an inactive state. Inhibition of Hsp90 or PI3K–Akt signaling, like hydrogen peroxide (H₂O₂), disrupts the Hsp90–Akt–ASK1 complex leading to activation of ASK1 signaling. Moreover, 17-AAG, LY294002 and H₂O₂ synergistically mediate ASK1–p38/JNK signaling and cell apoptosis.

Results

The inhibitory effect of Hsp90 on H₂O₂-induced activation of ASK1–p38 signaling is dependent on the Akt phosphorylation site on ASK1. Previously, it has been shown that Hsp90 client protein Akt can phosphorylate ASK1 at Ser83 (Kim *et al.*, 2001). To determine if Hsp90 has a similar effect, Hsp90 was cotransfected into bovine aortic EC (BAEC) with ASK1 followed by untreated or treatment with H₂O₂ (0.5 mM for 30 min). ASK1 activation was determined by Western blot with phospho-specific antibodies recognizing an inactive form (pSer83, the specific Akt phosphorylation site) and an active form of ASK1 (pThr845, an autophosphorylation site critical in ASK1 activation) (Tobiume *et al.*, 2002). As a control, Akt activation was also determined by Western blot with a

phospho-specific antibody. H₂O₂-induced reduction of Akt phosphorylation and ASK1 pSer83 were decreased while ASK1 pThr845 was concomitantly increased (Figure 1a). Overexpression of Hsp90 in BAEC (by threefold) increased Akt phosphorylation as well as phosphorylation of ASK1 at Ser83, while dramatically reducing H₂O₂-induced phosphorylation of ASK1 at Thr845. Similar data were obtained by measuring ASK1 activity using an *in vitro* kinase assay with GST-MKK4 as a substrate (not shown). These data suggest that Hsp90 regulates coordinated Akt activity and phosphorylation of ASK1 at Ser83.

To determine if the inhibitory effect of Hsp90 on ASK1 is Akt-dependent, Hsp90 was cotransfected into BAEC with ASK1-S83A, a mutant with a mutation at Akt phosphorylation site Ser83. As expected, ASK1-S83A did not show phosphorylation at Ser83. However, ASK1-S83A had increased basal phosphorylation at Thr845, with phosphorylation of ASK1 at Ser83 inhibits ASK1 activity. In contrast to the effect of Hsp90 on the wild type of ASK1 described in Figure 1a, expression of Hsp90 did not significantly inhibit H₂O₂-induced pThr845 of ASK1-S83A (Figure 1b). The inhibitory effect of Hsp90 on ASK1-induced p38/JNK signaling was determined by a p38/JNK-dependent reporter gene assay. A p38/JNK-reporter gene in which a c-Jun/ATF2-binding site is critical for its activation was cotransfected with vector control (VC), ASK1-WT or ASK1-S83A in the presence or absence of Hsp90, and p38/JNK-reporter gene activity was measured by luciferase assay. Hsp90 significantly inhibited ASK1-WT, but not ASK1-S83A-induced JNK/p38-reporter gene activities (Figure 1c). These data suggest that the inhibitory effect of Hsp90 on ASK1 activity is dependent on the Akt phosphorylation site (pSer83).

The inhibitory effect of Hsp90 on H₂O₂-induced activation of ASK1–p38 signaling is dependent on Akt activity

To further determine if the Hsp90 inhibitory effect on ASK1–p38 is dependent on Akt activity, we examined the effect of Hsp90 in Akt-deficient mouse embryo fibroblast (Akt^{-/-} MEF). Similar to data obtained from endothelial cells (EC), H₂O₂ induced ASK1–p38 activation by decreasing ASK1 pSer83, while increasing ASK1 pThr845 in normal MEF (Figure 2a). In Akt^{-/-} MEF, phosphorylation of ASK1 at Ser83 was diminished concomitant with a dramatic increase of phosphorylation of ASK1 at Thr845 compared to that in normal MEF. Both basal and H₂O₂-induced p38 activation were also increased in Akt^{-/-} cells in comparison to that in normal MEF (Figure 2a). We next examined effects of Hsp90 on ASK1–p38 signaling in Akt^{-/-} cells. As MEF cells show much less transfection efficiency compared to BAEC with the Lipofactamine method, MEF cells were infected with adenoviral Hsp90 (Ad-Hsp90, HA-tagged) or Ad-LacZ followed by untreated or treatment with H₂O₂ (0.5 mM for 30 min). Adenoviral infection of Ad-LacZ was determined by staining for β -galactosidase and infection was over 90% at 2 days postinfection.

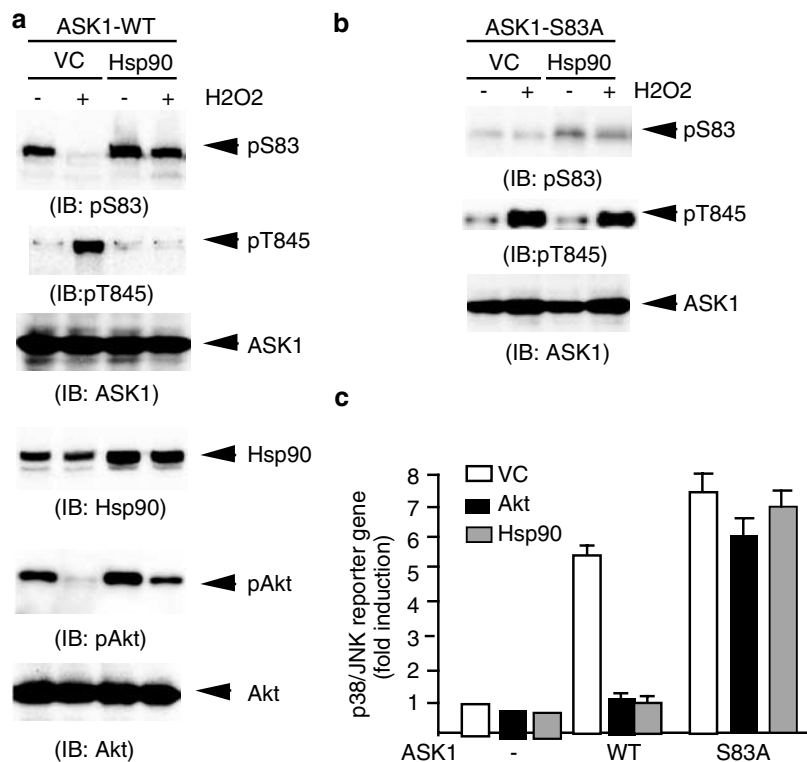


Figure 1 The inhibitory effect of Hsp90 on H₂O₂-induced activation of ASK1-p38/JNK signaling is dependent on the Akt phosphorylation site on ASK1. (a) Hsp90 expression blocked H₂O₂-induced ASK1 activation (reduction of pSer83 and increase of pThr845). BAEC were transfected with ASK1 in the presence or absence of Hsp90, followed by untreated or treatment with H₂O₂ (0.5 mM for 30 min). Phosphorylation of ASK1 (pSer83 and pThr845) and that of Akt were determined by Western blot with phospho-specific antibodies. (b) Hsp90 failed to block ASK1-S83A activity. BAEC were transfected with ASK1 in the presence or absence of Hsp90 and ASK1 phosphorylation was determined as in (a). (c) Hsp90 failed to inhibit ASK1-S83A-induced p38/JNK reporter gene activity. A p38/JNK reporter gene was cotransfected into BAEC with ASK1-WT, ASK1-S83A or ASK1-T845A in the presence or absence of Hsp90 or Akt. ASK1-induced p38/JNK reporter gene activity was determined by luciferase assay. P38/JNK reporter gene activities (relative luciferase activities) are presented from the mean of duplicate samples by taking vector control as 1. Similar results were obtained from two additional experiments

Expression of infected Hsp90 was determined by Western blot with anti-HA. As observed in BAEC, expression of Hsp90 (compared to LacZ) diminished H₂O₂-induced activation of ASK1-p38 in normal MEF cells (Figure 2b, WT MEF). However, Akt^{-/-} MEF cells showed augmented responses in H₂O₂-induced activation of ASK1-p38. Moreover, expression of Hsp90 on ASK1-p38 failed to inhibit H₂O₂-induced ASK1-p38 activation in Akt^{-/-} MEF cells (Figure 2b). These data demonstrated that Akt protein is critical for the inhibitory effect of Hsp90 on ASK1-p38 signaling.

Inhibition of Hsp90 or Akt induces and enhances H₂O₂-induced activation of ASK1-p38 signaling and EC apoptosis

The results above prompted us to reason that inhibition of Hsp90 or Akt would induce ASK1-p38 activation and cell apoptosis. To this end, BAEC were treated with Hsp90 inhibitor 17-AAG (1 μM) and PI3K inhibitor LY294002 (10 μM, to inhibit Akt activation) for various times (0, 30, 6 h and 16 h). ASK1 activation by H₂O₂ (0.5 mM) was used as a control. Activation of ASK1 (pThr845 and pSer83) and p38 was determined by

phospho-specific antibodies. Results showed that 17-AAG or LY294002, like H₂O₂, induced activation of ASK1 (increase in pThr845 and decrease in pSer83) as well as activation of p38 (Figure 3a). 17-AAG significantly induced degradation of Hsp90 client protein Akt at 16 h. Prolonged treatments (for 16 h) with 17-AAG, LY294002 or H₂O₂ induced significant cell death (not shown). To determine if 17-AAG and LY294002 enhance H₂O₂-induced ASK1-p38 signaling, BAEC were treated with a suboptimal dose of H₂O₂ (0.2 mM) in the absence or presence of 17-AAG (1 μM), LY294002 (10 μM) or 17-AAG + LY294002 for 6 h. Results showed that a combination of 17-AAG and LY294002 enhance H₂O₂-induced activation of ASK1 and p38 (Figure 3b).

We have recently shown that the ASK1-mediated mitochondria-dependent pathway is characterized by Bax mitochondrial translocation, cytochrome *c* release and nuclei condensation (Zhang R *et al.*, 2004). We examined if 17-AAG, LY294002 and H₂O₂ synergistically induced EC apoptosis. Bax translocation was determined by indirect immunofluorescence microscopy with anti-Bax, which specifically recognizes mitochondrial conformation of Bax. Cytochrome *c* release was

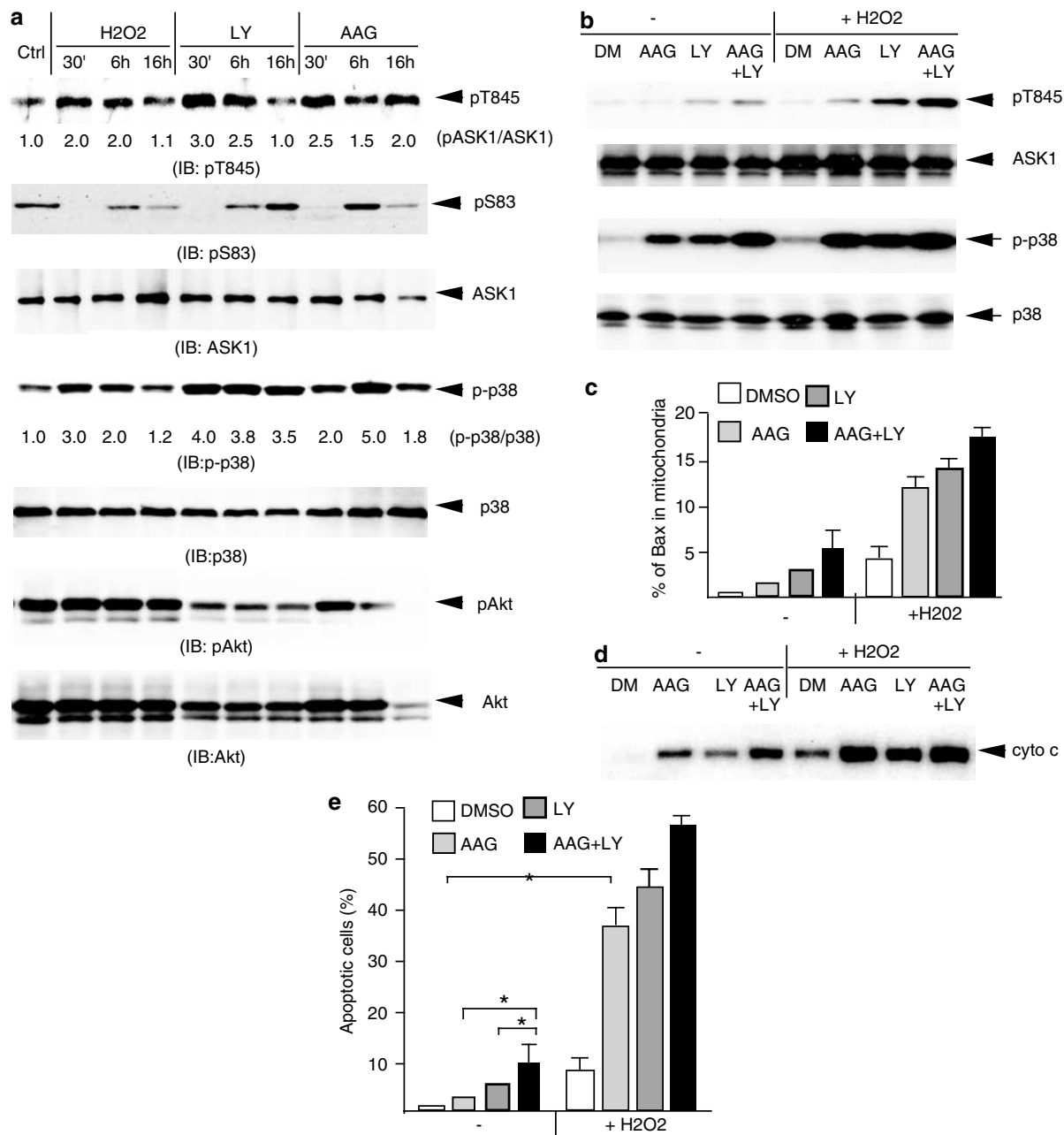


Figure 3 Inhibition of Hsp90 or Akt induces and enhances H₂O₂-induced activation of ASK1-p38 signaling and EC apoptosis. **(a)** 17-AAG and LY294002 induce ASK1-p38 activation. BAEC were treated with H₂O₂ (0.5 mM), 17-AAG (1 μ M) or LY294002 (10 μ M) for various times as indicated (30 min, 6 and 16 h). Phosphorylation of ASK1, p38 and Akt was determined by phospho-specific antibodies. Total ASK1, p38 and Akt were also determined. Relative activation (pASK1/ASK1 and p-p38/p38) is shown, with Ctrl as 1.0. **(b-e)** 17-AAG and LY294002 enhance H₂O₂-induced ASK1-p38 activation. BAEC were treated with DMSO (DM), 17-AAG (1 μ M), LY294002 (10 μ M) or 17-AAG + LY294002 in the presence or absence of H₂O₂ (0.2 mM) for 6 h. Phosphorylation of ASK1 (pT845) and p38 was determined by phospho-specific antibodies **(b)**. Bax translocation was determined by indirect immunofluorescence microscope using a double staining with anti-Bax (followed by FITC-conjugated anti-rabbit 2nd antibody). The percentage of Bax-staining cells is presented. Data are presented as mean of duplicates from two independent experiments **(c)**. Cytosolic fractions from EC were isolated and cytochrome *c* was detected by Western blot with anti-cytochrome *c*. Relative levels of cytochrome *c* are shown (setting mock-treated as 1.0) **(d)**. Apoptotic cells (with nuclear condensation) were determined by DAPI staining. The apoptosis rate is shown. Data are presented as mean of duplicates from two independent experiments. **P* < 0.05 **(e)**

and a C-terminal dimerization domain (Neckers, 2002) (Figure 5a). Flag-tagged Hsp90 β and its truncate (Hsp90-N, M or C) was transfected into BAEC, and association of Hsp90 proteins with endogenous ASK1 and Akt was determined by immunoprecipitation with

anti-ASK1 and anti-Akt, respectively. Hsp90-F and Hsp90-M but not Hsp90-N and Hsp90-C interacted with ASK1, suggesting that the middle domain of Hsp90 is critical for association with ASK1 (Figure 5b). Consistent with the previous reports (Sato *et al.*, 2000;

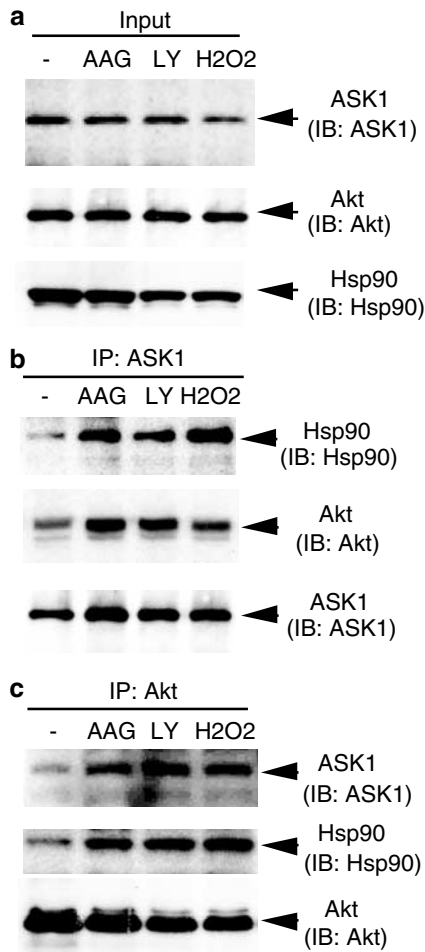


Figure 4 Akt and Hsp90 form a complex with ASK1. BAEC were treated with 17-AAG (1 μ M), LY294002 (10 μ M) and H₂O₂ (0.5 mM) for 30 min. Hsp90–Akt–ASK1 association was determined by immunoprecipitation assay with anti-ASK1 (b) or anti-Akt (c). Hsp90, Akt and ASK1 proteins in the input (a) and immunoprecipitates were determined by Western blot with the respective antibodies

Basso *et al.*, 2002), Akt also binds to the middle domain of Hsp90 (Figure 5c). These data suggest that Hsp90 may serve as a scaffold to hold Akt–ASK1 in close proximity.

Hsp90 activity is required to maintain Akt in association with the N-terminal domain of ASK1

To determine the critical domains in ASK1 for interactions with Akt and Hsp90, Flag-tagged ASK1 truncates containing the N-terminal domain (ASK1-N), the kinase domain (ASK1-K) and the N-terminal deletion (ASK1- Δ N) were generated (Figure 6a). Flag-tagged ASK1 truncates were transfected into BAEC, and association of Akt with ASK1 domains was determined by immunoprecipitation with anti-Akt followed by Western blot with anti-Flag. Results show that Akt associates with ASK1-N, but not with ASK1-K or ASK1- Δ N in untreated cells, suggesting that Akt binds to the N-terminal domain of ASK1 in resting EC

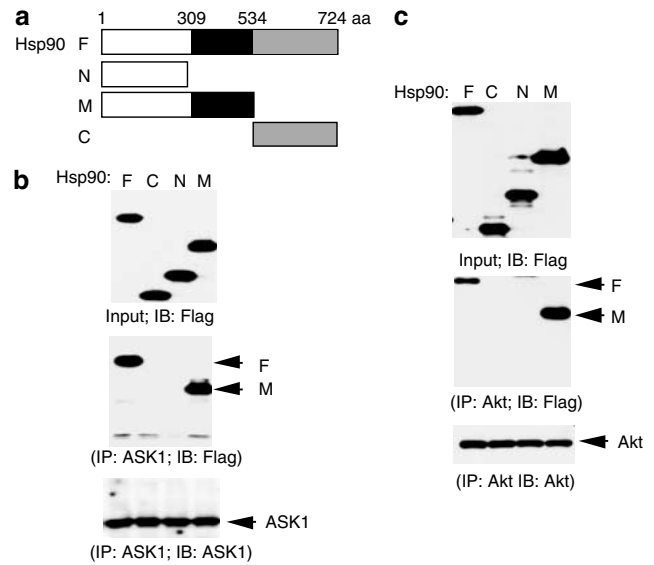


Figure 5 Hsp90 via its middle domain binds to both ASK1 and Akt. (a) Schematic diagram of Hsp90 domains and expression constructs. (b, c) Association of Hsp90 with ASK1 and Akt. Hsp90 expression constructs were transfected into BAEC. Association of Hsp90 proteins with endogenous ASK1 and Akt was determined by immunoprecipitation with anti-ASK1 (b) and anti-Akt (c) followed by Western blot with anti-Flag. ASK1 and Akt protein in the immunoprecipitates were determined by Western blot with the respective antibodies

(Figure 6b). To determine if Hsp90–Akt–ASK1 complex is regulated by activities of Hsp90 and Akt, BAEC were treated with LY294002 (10 μ M) or 17-AAG (1 μ M). Treatment of EC with LY294002, 17-AAG or H₂O₂ (0.5 mM) for 30 min completely abolished the association of Akt with ASK1-N. However, these treatments enhanced binding of Akt to the C-terminal domain of ASK1 (Figure 6b). Association and regulation of Hsp90 with ASK1 domains were also examined by immunoprecipitation assay with anti-Hsp90. Different from Akt, Hsp90 appears to bind to ASK1-N and ASK1- Δ N (but not ASK1-K). Treatments with 17-AAG, LY294002 or H₂O₂ did not significantly disrupt (and slightly increased) association of Hsp90 with ASK1 domains (Figure 6c). These data suggest that activities of Hsp90 and Akt are required to maintain Akt in association with the N-terminal domain of ASK1 where the Akt phosphorylation site (ser83) is located.

Discussion

Based on our data, we propose the following model (Figure 7): Hsp90 and Akt form a complex with ASK1 in resting cells to retain ASK1 in an inactive state. Hsp90 via its middle domain binds to both Akt and ASK1 and allows Akt in close proximity to the N-terminal domain of ASK1 to phosphorylate ASK1 at Ser83. In response to apoptotic stimuli such as H₂O₂, Hsp90–Akt–ASK1 forms a more stable complex in which Akt shifts to the C-terminal domain of ASK1,

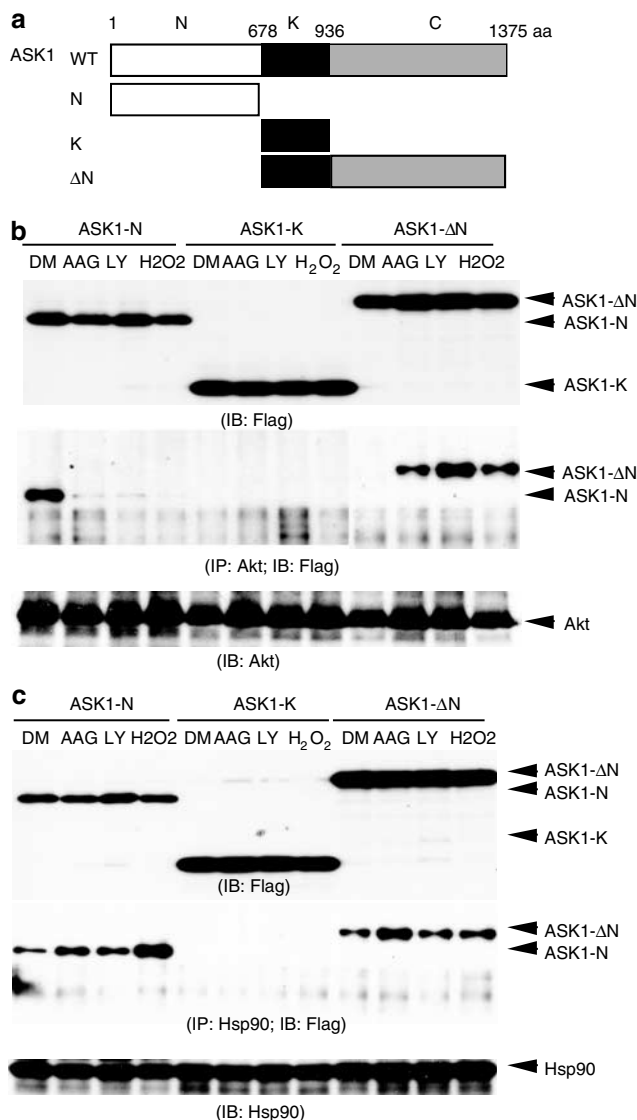


Figure 6 Hsp90 activity is required to maintain Akt in association with the N-terminal domain of ASK1. (a) Schematic diagram of ASK1 domains and expression constructs. (b, c) Flag-tagged ASK1-N, -K or -ΔN was transfected into BAEC, and association of endogenous Akt (b) or Hsp90 (c) with ASK1 domain was determined by immunoprecipitation with anti-Akt and anti-Hsp90, respectively. ASK1 proteins were determined by Western blot with anti-Flag. Akt and Hsp90 in the immunoprecipitates were determined by Western blot with the respective antibodies

leading to reduction of pSer83 and activation of ASK1. Inhibition of Hsp90 activity (by 17-AAG) or PI3K–Akt activity (by LY294002) induces a similar conformational change in Akt–Hsp90–ASK1 complex and enhances H₂O₂-induced ASK1–p38/JNK signaling and EC apoptosis.

Similar to other cellular inhibitors (e.g., Trx, Glutaredoxin and 14-3-3), Hsp90 and Akt bind to ASK1 and contribute to retaining ASK1 in an inactive state. Different from other ASK1 inhibitors, Hsp90–Akt are not dissociated from ASK1 in response to stress stimuli, but rather undergo a conformational change leading to a more stabilized complex. The mechanism for stabiliza-

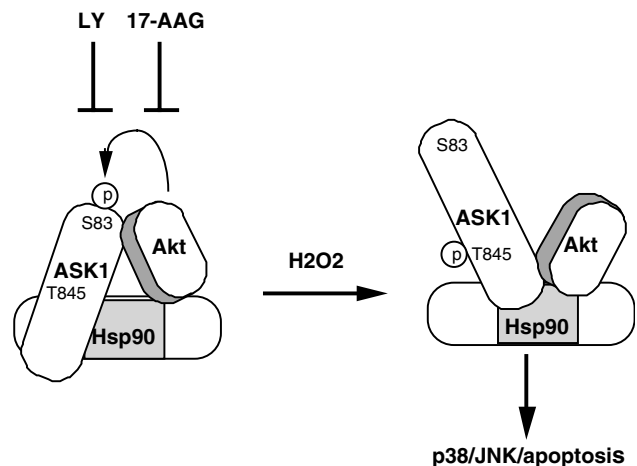


Figure 7 A model for regulation of ASK1-mediated apoptosis by Hsp90–Akt. See text for details

tion of the Hsp90–Akt–ASK1 complex is not known. It is likely that the C-terminal domain of ASK1 forms a more stable complex with Hsp90–Akt compared to its N-terminal domain. Alternatively, dissociation of other ASK1 inhibitors such as Trx and 14-3-3 from ASK1 may stabilize Hsp90–Akt on ASK1. The exact mechanism by which Hsp90–Akt inhibits ASK1 is not clear. However, we provide evidence to support the fact that Hsp90–Akt inhibits ASK1 activity by phosphorylating ASK1 at Ser83. First, overexpression of Akt or Hsp90 increases phosphorylation of ASK1 on Ser83 concomitant with decrease of ASK1 pThr845. Second, ASK1 pSer83 is strongly blunted in Akt-deficient cells. Third, 17-AAG, LY294002 or H₂O₂ reduces pSer83 with increase of pThr845 of ASK1 and activation of p38/JNK. Moreover, ASK1-S83A-induced p38/JNK is resistant to Hsp90–Akt inhibition. More importantly, the inhibitory effect of Hsp90 on ASK1–p38 is diminished in Akt-deficient cells, suggesting that Akt and Hsp90 are functionally dependent to target on Ser83 of ASK1.

ASK1 is a target of many survival factors, suggesting that ASK1 is a central player in regulating cell death. ASK1 is proposed to mediate multiple cell death pathways (intrinsic and extrinsic, caspase-dependent and independent) induced by various proapoptotic stimuli including death receptors, DNA-damaging agents, oxidants and cellular stresses such as growth factor deprivation and endoplasmic reticulum (ER) stresses caused by protein aggregation (Nishitoh *et al.*, 2002). It is conceivable that the survival proteins cooperate to protect insults from different stimuli and to secure ASK1 in an inactive state. The mechanism for ASK1 activation in response to apoptotic stimuli has not been fully elucidated. Recent studies have demonstrated that ASK1 activation involves several steps including association with upstream activators such as TRAF2 and AIP1 (Zhang R *et al.*, 2003; Zhang *et al.*, 2004a), release of cellular inhibitors such as thioredoxin, glutaredoxin, glutathione S-transferase Mu, heat-shock proteins and 14-3-3 (Saitoh *et al.*, 1998; Zheng *et al.*,

1999; Liu *et al.*, 2000, 2001; Song *et al.*, 2002), ASK1 oligomerization and autophosphorylation at Thr-845 (Tobieme *et al.*, 2002), and scaffold protein-mediated association of ASK1 with downstream MKK and JNK (Matsuura *et al.*, 2002). These steps likely occur sequentially although it has not been formally approved. Thus, different ASK1 inhibitors may block different steps in ASK1 activation. For example, heat-shock protein hsp72 blocks ASK1 oligomerization and recruitment of its downstream targets (Park *et al.*, 2002). However, PP5 dephosphorylates ASK1 at the autophosphorylation site pThr845 to function as a negative feedback inhibitor of ASK1 signaling (Morita *et al.*, 2001). Alternatively, different survival factors inhibit ASK1 in different cellular compartments. As we demonstrated recently, mitochondria Trx (Trx2) specifically inhibits mitochondria-located ASK1-induced apoptosis (Zhang R *et al.*, 2004).

Recently, it has been shown that prolonged treatment of cells with 17-AAG (up to 16 h) induces degradation of Hsp90 client proteins including RIP, IKK α/β and RAF-1, but not of other components such as TRADD, TRAF2 and FADD in TNF signaling. The degradation of RIP, IKK α/β and other unidentified proteins by 17-AAG leads to inhibition of TNF-induced NF- κ B and JNK pathways and induction of cell apoptosis (Vanden Berghe *et al.*, 2003; Zhang *et al.*, 2004b). Our data show that 17-AAG treatment for a short time (up to 6 h) did not induce degradation of Hsp90 client proteins (Akt, RAF-1 and RIP1), but induces an ASK1-mediated apoptotic signaling. Our study provides an alternative apoptotic pathway induced by 17-AAG.

The function of Hsp90–Akt in vascular EC has been previously addressed in our group (Garcia-Cardena *et al.*, 1998; Fontana *et al.*, 2002). In response to stimulation of VEGF, Hsp90 serves as a molecular scaffold to recruit endothelial nitric oxide synthase (eNOS) and Akt to a close proximity, thereby facilitating eNOS phosphorylation at Ser1179 and enzyme activation. eNOS and its product NO are known to promote EC survival and angiogenesis. Our present work suggests that ASK1 is another substrate of the Hsp90–Akt complex. In contrast to eNOS, proapoptotic kinase ASK1 is phosphorylated and is thereby inactivated by Hsp90–Akt. Akt has been shown to phosphorylate and inactivate a number of proapoptotic factors such as glycogen synthase kinase 3, BAD, caspase 9 and Forkhead transcription factors in EC and tumor cells. It is conceivable that the Hsp90–Akt complex functions as a general survival mechanism in both vascular and tumor cells. Thus, inhibition of Hsp90–Akt pathway or activation of ASK1 apoptotic signaling may provide novel antitumor and antiangiogenic strategies.

Materials and methods

Constructs

Mammalian expression plasmids for Hsp90, Akt and ASK1 were described previously (Fulton *et al.*, 1999; Fontana *et al.*,

2002; Zhang R *et al.*, 2003, 2004). ASK1 mutants (S83A, T845A) were constructed by site-directed mutagenesis using Quickchange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol, and mutations at the desired sites were confirmed by DNA sequencing.

Antibodies

A rabbit polyclonal antibody against phospho-specific antibodies against phospho-ASK1 (pSer83 and pThr845), phospho-p38 and phospho-Akt (pSer473) were from Cell Signaling. We obtained anti-ASK1 (H300), anti-Hsp90, anti-Akt, anti-Myc, anti-RIP1, anti-Bax (N20) and anti-caspase 8 from Santa Cruz Biotechnology. Anti-cytochrome *c* and anti- β -tubulin were purchased from BD Pharmingen. Anti-HA was from Roche and anti-Flag was from Sigma.

Cells, cytokines and inhibitors

Human umbilical vein endothelial cells (HUVEC) and BAEC were purchased from Clonetics Corp. (San Diego, CA, USA). HUVEC were cultured in modified M199 culture medium, containing 20% v/v heat-inactivated bovine fetal calf serum (FCS), 100 μ g/ml heparin sodium salt, 30 μ g/ml EC growth supplement, 2 mM L-glutamine, 60 U/ml penicillin and 0.5 μ g/ml streptomycin at 37°C, in 5% CO₂ on gelatin-coated tissue culture plastic as described previously (Zhang R *et al.*, 2003, 2004). Cells were used at passages 2–4. Akt-deficient embryonic fibroblasts (MEFs) were isolated from Akt^{-/-} mice, which we have described previously (Fulton *et al.*, 1999). 17-AAG and LY294002 were purchased from Calbiochem.

Cell transfection and reporter gene assay

Transfection of HUVEC was performed by the DEAE-dextran method as described previously (Min and Pober, 1997) and transfection of BAEC was performed by Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen Corp., San Diego, CA, USA). For reporter gene assay, cells were cultured at 90% confluence in six-well plates and were transfected with a total of 2.5 μ g plasmid constructs including the p38/JNK reporter gene (1 μ g), renilla gene (0.5 μ g) and various transgenes (1 μ g) as indicated. Cells were harvested at 36–48 h post-transfection and cell lysates (10 μ l) were measured for firefly luciferase activity followed by firefly renilla activity twice in duplicates with Promega reagents (Promega Corp., Madison, WI, USA) using a Berthold luminometer (EG&G Wallac, Gaithersburg, MD, USA). All data were normalized as relative luciferase light unit/renilla unit.

Preparation of subcellular fractions, immunoprecipitation and immunoblotting

Subcellular fractions from EC, immunoprecipitation assay and immunoblotting were prepared as described (Zhang R *et al.*, 2003, 2004). After measuring the protein concentrations (Bio-Rad reagents), proteins were detected by Western blot with the indicated antibodies.

ASK1 and JNK kinase assays

ASK1 and JNK assays were performed as described previously (Zhang R *et al.*, 2003, 2004) using GST-MKK4 and GST-c-Jun (1–80) fusion protein as a substrate, respectively.

GST pull-down assay

GST-hsp90 fusion protein preparation and GST pull-down assay were performed as described previously (Fulton *et al.*, 1999; Fontana *et al.*, 2002; Zhang R *et al.*, 2003, 2004).

Adenoviral expression and preparation

Replication-deficient adenovirus expressing HA-tagged Hsp90, Akt or β -galactosidase under the control of the cytomegalovirus (CMV) promoter was generated using pAdTrack-CMV vector and the AdEasy System (Fulton *et al.*, 1999; Fontana *et al.*, 2002). The viruses were amplified in HEK293 cells, purified using CsCl, and titered using the cytopathic effect (CPE). Infection of 50 MOI of virus resulted in close to 100% of the cells expressing the gene of interest with no signs of toxicity.

Quantitation of apoptosis

Bax mitochondrial translocation, cytochrome *c* release and nuclei fragmentation were performed as described previously (Zhang R *et al.*, 2003, 2004).

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Statistical analysis

Data are presented as means (\pm s.d.). For Western blot, kinase activity, apoptosis and reporter gene assays, experiments were performed at least twice with duplicates. Analysis of densitometry was performed using NIH Image 1.60. Results were then normalized for comparison among different experimental groups by arbitrarily setting the value of control cells to 1.0. Statistical analyses were performed with StatView 4.0 package (ABACUS Concepts). Differences were analysed by unpaired two-tailed Student's *t*-test. Values of $P < 0.05$ were taken as significant.

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