Heat Shock Cognate Protein 70 Is Essential for Akt Signaling in Endothelial Function

Masayuki Shiota, Hiromi Kusakabe, Yasuakazu Izumi, Yuko Hikita, Takafumi Nakao, Yoshihiko Funae, Katsuyuki Miura, Hiroshi Iwao

Objective—Heat shock protein 70s (Hsp70s) are molecular chaperones that protect cells from damage in response to various stress stimuli. However, the functions and mechanisms in endothelial cells (ECs) have not been examined. Herein, we investigate the role of Hsp70s, including heat shock cognate protein 70 (Hsc70), which is constitutively expressed in unstressed healthy cells and tissues. However, its expression is rapidly induced by a variety of physical and chemical stresses; therefore, it is often called the major stress-inducible Hsp70. It is suggested that Hsp72 and Hsc70 expression is essential for certain cells to respond to some cytotoxic factors. There are several chaperone mechanisms based on inducible Hsp72 and constitutive Hsc70. Although both bind to misfolded proteins, newly synthesized polypeptides (ie, Hsp72 and Hsc70) function in the cytosol, suggesting that they display specificity for their client proteins or, alternatively, serve particular chaperone-independent functions.

Methods and Results—The Hsp70 inhibitor, KNK437, significantly decreased vascular endothelial growth factor (VEGF)–induced cell migration and tube formation in vitro. KNK437 inhibited the phosphorylation of VEGF-induced Akt and endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cells. In a mouse hind limb model of vascular insufficiency, intramuscular inhibition of Hsp70s attenuated collateral and capillary vessel formation. Silencing the Hsc70 gene by short interfering RNA abolished VEGF-induced Akt phosphorylation and VEGF-stimulated human umbilical vein endothelial cell migration and tube formation. As the molecular mechanisms, Hsc70 knockout reduced the expression of phosphatidylinositol 3-kinase.

Conclusion—Collectively, Hsc70 plays a significant role in ECs via the phosphatidylinositol 3-kinase/Akt pathway. Hsc70 may provide the basis for the development of new therapeutic strategies for angiogenesis. (Arterioscler Thromb Vasc Biol. 2010;30:491-497.)

Key Words: heat shock cognate protein 70 ■ endothelial cells ■ angiogenesis ■ Akt ■ VEGF
ous stress conditions; however, there is little research on Hsc70 in the cardiovascular system.

In the present study, we investigated whether Hsp70s modulate the angiogenic process. Recently, it has been reported that RNAi-mediated knockdown of human Hsc70 A12B, a member of the Hsp70 family, disrupted normal zebra fish blood vessel development and inhibited in vitro angiogenesis and migration in human umbilical vein endothelial cells (HUVECs). Additional Hsp70 family members could modulate these important functions as well. However, the role and mechanism of Hsp70s, including Hsc70, in endothelial cells (ECs) were not examined. We examined the ability of Hsp70s to induce EC migration, the formation of capillary-like structures, and endothelial nitric oxide synthase (eNOS) activation. We also tested whether Hsp70s are required for angiogenesis in a hind limb ischemia model. Our observations indicate that Hsp70s are essential for Akt activation and the maintenance of endothelial function. Furthermore, the knockdown of Hsc70 revealed the reduction of phosphatidylinositol 3-kinase (PI3K) p110 protein in HUVECs. Hsc70 may play a critical role in the regulation of EC function through the phosphorylation of Akt.

Methods
Additional methods and details are presented in the Data Supplement available online at http://atvb.ahajournals.org.

Materials and Cell Culture
Vascular endothelial growth factor (VEGF) 165 and N-formyl-3,4-methyleneoxy-benzylidine-γ-butyrolactam (a KNK437, Hsp70 inhibitor) were obtained from Calbiochem (La Jolla, Calif). Endothelial cell growth medium (EGM2) and endothelial cell basal medium (EBM2) culture media and HUVECs (passage ≤5) were obtained from Lonza (Walkersville, Md). HUVECs were grown at 37°C in a humidified atmosphere of 5% carbon dioxide in EGM2 medium.

The supplemental data also provide information regarding immunoblotting, migration assay, in vitro angiogenesis, and real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis.

In Vivo Models of Angiogenesis
All procedures were in accordance with institutional guidelines for animal research. Male C57BL/6 mice (aged 8 weeks) were purchased from Japan Clea (Tokyo, Japan). Unilateral hind limb ischemia was induced by resecting the left femoral arteries and veins, which were measured on day 5, as described previously.13 Immediately after the left femoral artery and vein were resected, we used an ischemic hind limb model of in vivo angiogenesis. In this study, we investigated the effects of a Hsp inhibitor, N-formyl-3,4-methyleneoxy-benzylidine-γ-butyrolactam (KNK437), on ECs. KNK437 was reported to inhibit the acquisition of thermotolerance and the inhibition of various Hsps, including Hsp110, Hsp72, and Hsp40, in human colon cancer cells.14 To evaluate the function of Hsp70s in ECs, we first studied the effects of an HSP inhibitor, KNK437, on angiogenesis. Angiogenesis incorporates cell survival, migration, intercellular connections with other ECs, and lumen formation. In vitro angiogenesis was assessed by tube formation assay using Matrigel. KNK437, 100 μmol/L, significantly inhibited VEGF, 10 ng/mL, induced tube formation (Figure 1A). Because angiogenesis involves cell migration, we investigated the role of Hsp70s in VEGF-promoted migration of HUVECs using a modified Boyden chamber method. KNK437 treatment suppressed VEGF-induced cell migration (Figure 1B).

Results
Blockade of Hsp70 Suppresses VEGF-Induced Migration and Tube Formation
In this study, we investigated the effects of a Hsp inhibitor, N-formyl-3,4-methyleneoxy-benzylidine-γ-butyrolactam (KNK437), on ECs. KNK437 was reported to inhibit the acquisition of thermotolerance and the inhibition of various Hsps, including Hsp110, Hsp72, and Hsp40, in human colon cancer cells. To evaluate the function of Hsp70s in ECs, we first studied the effects of an HSP inhibitor, KNK437, on angiogenesis. Angiogenesis incorporates cell survival, migration, intercellular connections with other ECs, and lumen formation. In vitro angiogenesis was assessed by tube formation assay using Matrigel. KNK437, 100 μmol/L, significantly inhibited VEGF, 10 ng/mL, induced tube formation (Figure 1A). Because angiogenesis involves cell migration, we investigated the role of Hsp70s in VEGF-promoted migration of HUVECs using a modified Boyden chamber method. KNK437 treatment suppressed VEGF-induced cell migration (Figure 1B).

Supplemental Figure I). Taken together, these results suggest that blockade of Hsp70s suppressed angiogenesis, indicating that endogenous Hsp70s are important for angiogenic activity of ECs.

DBF Analysis and Tissue Capillary Density
To further substantiate a role for Hsp70s in angiogenic activity, we used an ischemic hind limb model of in vivo angiogenesis. Immediately after the left femoral artery and vein were resected, the ratio of ischemic (left) to nonischemic (right) hind limb LDBF (the LDBF ratio) decreased from 1.01±0.02 to 0.06±0.02 in
vehicle mice and from 1.01±0.02 to 0.07±0.04 in KNK437-treated mice, indicating a similar level of ischemia. Figure 2A shows representative LDBF images of hind limb blood flow on day 5. A serial LDBF examination showed that hind limb blood flow recovered in vehicle mice after the induction of ischemia, but treatment with KNK437 blocked this recovery; the LDBF ratio in KNK437-treated mice was low at 5 days after the induction of ischemia (55% decrease, \( p<0.05 \); Figure 2A). To investigate microcirculation, we measured capillary density in histological sections harvested from the ischemic tissues by anti-CD31 antibody. KNK437 significantly reduced capillary density on postoperative day 5 compared with vehicle mice (Figure 2B).

**Effects of KNK437 on Akt Phosphorylation**

Akt phosphorylation is one of the key steps in transducing angiogenic signals, including those initiated by VEGF, an important regulator of various cellular processes (ie, metabolism, cell survival, migration, and nitric oxide release).\(^1\)\(^6\)\(^1\)\(^7\) VEGF-stimulated activation of the PI3K/Akt pathway plays an essential role in the regulation of eNOS activity. To determine the role of Hsp70 in VEGF-provoked Akt phosphorylation, HUVECs were pretreated with KNK437, 100 \( \mu \)mol/L, or vehicle for 1 hour, and then stimulated with or without VEGF for 30 minutes. KNK437 inhibited VEGF-induced Akt-Ser473 phosphorylation, whereas total Akt protein expression barely changed (Figure 3A). These results indicate that Hsp70s mediate VEGF-induced Akt phosphorylation in HUVECs.

Next, to determine whether Hsp70s are directly involved in Akt activity in vivo, we examined the effect of KNK437 on the phosphorylation of Akt in ischemic mouse tissues. Hind limb ischemia induced Akt phosphorylation, which peaked at 60 minutes (data not shown). Pretreatment of KNK437 completely blocked this peaked Akt phosphorylation (Figure 3B). Treatment with KNK437 did not affect Akt activity in nonischemic muscles. These data demonstrate that pharmacological inhibition of the function of Hsp70s attenuated the activity of Akt, leading to suppression of angiogenesis.

**Interference With Hsc70 Induces Endothelial Dysfunction**

KNK437 was reported to broadly affect Hsp family members.\(^1\)\(^4\) The induction of heat-inducible Hsp70s was inhibited at the messenger RNA (mRNA) level by treatment with KNK437, although the precise mechanism of KNK437-induced suppression of Hsp70 transcription has not been elucidated. Therefore, we analyzed mRNA expression of the Hsp family in KNK437-treated HUVECs by quantitative RT-PCR under non–heat-stressed conditions. KNK437 reduced the expression of Hsps to less than the basal level under nonstressed conditions. Treatment with KNK437 for 6 hours (the time point of migration and in vitro angiogenesis assay) significantly reduced the expression of Hsc70, Hsp72, Hsp40, and Hsp90α, but not of Hsp90β or Hsp110. A 1.5-hour treatment (time point of immunoblotting) significantly reduced the expression of Hsp72, Hsp40, and Hsc70, but not of Hsp90α (Supplemental Figure II). Taken together, the data indicate that KNK437 suppressed Hsp72, Hsp40, and Hsc70 under a nonstress condition. Because KNK437 treatment decreased mRNA expression of Hsc70, a stress-noninducible Hsp, we next focused on the role of Hsc70 in endothelial function.

To determine whether Hsc70 is also required to regulate EC function, we used siRNA to block Hsc70 transcription. Three siRNAs (siRNA1, siRNA2, and siRNA3) and a negative control were tested against endogenous Hsc70 in HUVECs. These siRNA constructs were specific for Hsc70 and did not cross-react with Hsp72. All constructs resulted in moderate knockdown of Hsc70 expression by reducing mRNA expression (Supplemental Figure III). Because Hsc70-reduced cells detached from the substratum after 48 hours of transfection, we investigated cell number (Figure 4A). Hsc70-knockdown cells reduced their number to 60% compared with that of the control cells. Nevertheless, there were no significant differences in the level of cleaved caspase 3 (Supplemental Figure IV). Next, to investigate the specificity of Hsc70 knockdown, we determined the level of expression of Hsp family genes, which is affected by KNK437 treatment in quantitative RT-PCR (Supplemental Fig-
Although Hsc70 siRNA significantly inhibited Hsc70 mRNA expression in HUVECs, it did not inhibit expression of the other Hsps. Rather, it induced their expression (Supplemental Figure V). Therefore, it is believed that the influence of siRNA is specific for Hsc70 but not Hsp72. HUVECs were then transfected with Hsc70 siRNA (siRNA1) or the negative control, and angiogenesis was evaluated by tube formation assay after 48 hours. Hsc70 knockdown significantly blocked the normal formation of tubelike structures (Figure 4B). A similar result was obtained with the siRNA2 construct. siRNA1 treatment significantly inhibited VEGF-induced migration (Figure 4C). Thus, silencing of Hsc70 blocked endothelial migration and tube formation, indicating that endogenous Hsc70 is essential for angiogenic activity in ECs.

Hsc70 Is Essential for Akt Phosphorylation in ECs

Furthermore, we tested the effects of Hsc70 siRNA on VEGF-induced Akt phosphorylation. At 48 hours after transfection, cells were starved and treated with VEGF for 30 minutes. HUVECs transfected with siRNA showed diminished phosphorylation of Akt (Ser473) in the presence of VEGF (Figure 5 and Supplemental Figure VI), but total levels of Akt1 protein did not change. These results are in good agreement with the data shown Figure 3A.

Depletion of Hsc70 Reduces PI3K Expression

Finally, to clarify how Hsc70 affected Akt activation, we evaluated the activation of molecules that play a role in its activation. As shown in Supplemental Figure VII, the depletion of Hsc70 prevented VEGF-induced phosphorylation of threonine 308 and Ser473 on Akt. However, knockdown of Hsc70 did not affect the expression of mammalian targets of rapamycin and rictor, which phosphorylate Akt at Ser473. In contrast, depletion of Hsc70 enhanced the expression of 3-phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates threonine 308. On the other hand, Hsc70 siRNA downregulated production of PI3K p110 subunits to less than 40%, and a modest decrease in p85 was observed (Figure 6A). A similar result was obtained with KNK437 treatment, which reduced the production of the PI3K p110 subunits (Figure 6B). As shown in Supplemental Figure II, a significant decrease in the amount of p110 protein and mRNA was not observed under an Hsp40 or an Hsp72 knockdown condition, although both of them were inhibited by KNK437 (Supplemental Figure VIII). The knockdown of Hsp72 blocked VEGF-induced phosphorylation of Akt, whereas PI3K p110 production was not changed (Supplemental Figure IXA). Likewise, geldanamycin, an Hsp90 inhibitor, failed
Hsp70s participate in cellular stress responses by binding and refolding damaged proteins or removing consistently misfolded proteins through the ubiquitin-proteasome system or autophagy. In addition to their chaperone functions, the Hsp70s have also been reported to act as signaling molecules and regulators in intracellular trafficking and regulation of diverse signaling proteins. Recently, Madeddu et al reported that PI3K p110β, which is constitutively expressed in vascular ECs, also functions as a scaffold protein for mammalian target of rapamycin complex 2 and Akt. However, an immunoprecipitation analysis revealed that Hsc70 reduction did not change the amount of Akt bound to rictor (data not shown). Although it is also reported that Akt Ser473 and threonine 308 are phosphorylated by PDK1, we did not detect PDK1 binding or changes in PDK1 phosphorylation (data not shown). Moreover, the inhibition of Hsc70 gene expression did not reduce the expression of mammalian targets of rapamycin, rictor, or PDK1 (Supplemental Figure V). Finally, we evaluated the expression of PI3K, which functions upstream of Akt in the signaling cascade. The data revealed that Hsc70 siRNA significantly reduced p110 protein production (Figure 6). Furthermore, we investigated the relative abundance of mRNAs encoding p110α, which form heterodimers with p85; and p110γ, which do not form heterodimers with p85. The data indicated that knockdown of Hsc70 gene expression inhibited the steady-state levels of p110γ mRNA to a greater degree than p110α mRNA. This result is consistent with the knowledge that PI3K p110γ participates in control of Akt phosphorylation. Recently, Madeddu et al reported that PI3K p110γ modulates angiogenesis, arteriogenesis, and vasculogenesis. Hsc70 may regulate the expression of PI3K by being involved with the control of stabilization and the degradation of PI3K. There is report that Hsc70 plays an interesting role in the cytokine-mediated posttranscriptional regulation of the proapoptotic Bcl-2 (B-cell lymphoma/leukemia-2) family member, Bim (B-cell lymphoma 2 interacting mediator of cell death), in human blood cells. Hsc70 may be recognized as an active component constitutively involved in many cellular processes, including stabilization and degradation of mRNA.

**Discussion**

Herein, we provide evidence showing that Hsp70s, especially Hsc70, are essential for EC function, including angiogenesis, via regulation of the Akt pathway. First, we demonstrated that pharmacological inhibition of Hsp70s attenuates Akt activity, leading to suppression of EC migration and capillary-like tube formation. Moreover, the inhibition of Hsp70s abrogated blood vessel recruitment in a mouse hind limb model of vascular insufficiency, as evaluated by laser Doppler flow and capillary density analysis. These observations show that Hsp70s are essential for angiogenesis via activation of PI3K-Akt signaling. KNK437 is not specific for Hsc70, but it inhibited the expression level of Hsc70 at each time point of the assay (1.5 and 6 hours). Therefore, we presumed that these results were caused by downregulation of Hsc70. Hsc70 knockdown significantly suppressed EC migration and tube formation in vitro. Furthermore, the depression of Hsc70 decreased the activation of Akt and its downstream effector molecules, including eNOS. Because activation of Akt is a trigger and is essential for endothelial function and postnatal angiogenesise, further elucidated the mechanism of Akt activation in Hsc70-silenced HUVECs. The data presented herein suggest that the molecular mechanism is due to inhibition of PI3K protein production by Hsc70 knockdown. The attenuation of Hsc70 could abrogate endothelial functions by blocking PI3K/Akt activation. Thus, the Akt pathway might regulate angiogenic responses by several distinct and possibly counterbalancing mechanisms.

Hsp70s participate in cellular stress responses by binding and refolding damaged proteins or removing consistently misfolded proteins through the ubiquitin-proteasome system or autophagy. In addition to their chaperone functions, the Hsp70s have also been reported to act as signaling molecules and regulators in cellular processes such as apoptosis. Hsp90β, which is constitutively expressed in vascular ECs, also functions as a scaffold protein for eNOS and Akt, and coordinates the trafficking and regulation of diverse signaling proteins. Recently, it has been shown that Hsp702 is identified as a rictor-binding protein and a component of the mammalian target of rapamycin complex 2. Activation of mammalian target of rapamycin complex 2, which consists of rictor, leads to the phosphorylation of Akt at Ser473, resulting in full activation of Akt. Thus, the observation that Hsp70s regulate kinase function is not unique. Therefore, we considered the possibility of Hsc70 functioning as a scaffold protein for mammalian target of rapamycin complex 2 and Akt. However, an immunoprecipitation analysis revealed that Hsc70 reduction did not change the amount of Akt bound to rictor (data not shown). Although it is also reported that Akt Ser473 and threonine 308 are phosphorylated by PDK1, we did not detect PDK1 binding or changes in PDK1 phosphorylation (data not shown). Moreover, the inhibition of Hsc70 gene expression did not reduce the expression of mammalian targets of rapamycin, rictor, or PDK1 (Supplemental Figure V). Finally, we evaluated the expression of PI3K, which functions upstream of Akt in the signaling cascade. The data revealed that Hsc70 siRNA significantly reduced p110 protein production (Figure 6). Furthermore, we investigated the relative abundance of mRNAs encoding p110α, which form heterodimers with p85; and p110γ, which do not form heterodimers with p85. The data indicated that knockdown of Hsc70 gene expression inhibited the steady-state levels of p110γ mRNA to a greater degree than p110α mRNA. This result is consistent with the knowledge that PI3K p110γ participates in control of Akt phosphorylation. Recently, Madeddu et al reported that PI3K p110γ modulates angiogenesis, arteriogenesis, and vasculogenesis. Hsc70 may regulate the expression of PI3K by being involved with the control of stabilization and the degradation of PI3K. There is report that Hsc70 plays an interesting role in the cytokine-mediated posttranscriptional regulation of the proapoptotic Bcl-2 (B-cell lymphoma/leukemia-2) family member, Bim (B-cell lymphoma 2 interacting mediator of cell death), in human blood cells. Hsc70 may be recognized as an active component constitutively involved in many cellular processes, including stabilization and degradation of mRNA.

Hsc70 is considered an essential housekeeping gene, and Hsc70-knockout mice are embryonically lethal as a result of the essential role of Hsc70 in cell survival. Transfection with Hsc70 siRNA results in massive cell death in various cell types. As a consequence of this inhibition, we observed a decrease in cell number. Akt also contributes to cell survival, and Akt blockade by Hsc70 may trigger cell death. Indeed, depression of Hsc70 downregulated Akt-driven phosphorylation of eNOS (data not shown). These data clearly implicate Hsc70 as a critical facilitator of Akt-mediated cell survival in response to VEGF. In the present study, knockdown of Hsc70 for 48 hours caused cell death of less than 40% in ECs (Figure 4A). However, the inhibition of angiogenesis by blocking Hsc70 did not result in cell death because we did not detect any remarkable cell death during the tube formation and migration assay (Figure 4B and C, control versus Hsc70 siRNA treatment).

PI3K/Akt signaling regulates multiple steps in angiogenesis, including EC survival, migration, and capillary-like structure formation. Furthermore, this signaling pathway also regulates cardiovascular homeostasis and vessel integrity at least in part by controlling NO synthesis. Several groups have shown that inhibition of PI3K or its downstream target Akt blocked VEGF-stimulated migration in ECs. Hsc70 is constitutively expressed in ECs and may promote endothelial health and may, therefore, be important in treating disorders that result from endothelial dysfunction. Thus, one potential mechanism by which knockdown of Hsc70 induces endothelial dysfunction is regulation of the PI3K/Akt pathway. This
study also provides data supporting a role for Hsp70s in regulating ischemic and postnatal angiogenesis in vivo. The loss of eNOS,49,50 Akt,41 or PI3K49 in response to tissue injury or exogenous angiogenic factors results in severe peripheral vascular disease. Treatment of KNK437 attenuated postsischemic mechanical and endothelial functions, similar to the results obtained with Akt knockout mice.41 These data indicate that there are distinct regulatory mechanisms that involve the Hsp70s, including Hsc70, in blood vessel recruitment during normal tissue development and in response to pathological stimuli, such as ischemic stress. Collectively, these findings suggest that Hsc70 plays a fundamental role in maintaining homeostasis in ECs. Herein, it was found that knockdown of Hsc70 downregulates PI3K p110 expression, leading to Akt inactivation. This is consistent with the hypothesis that Hsc70 downregulation promotes endothelial dysfunction via inactivation of the PI3K/Akt pathway. Therefore, we hypothesize that Akt activation by Hsc70 is one of many regulatory mechanisms needed to maintain endothelial homeostasis. However, further study would be required to clarify the mechanistic details.

In conclusion, the present study demonstrates that Hsc70 is essential for multiple EC functions via the PI3K/Akt pathway. Hsc70 may promote endothelial health and may, therefore, be important in the treatment of disorders in which there is evidence of endothelial dysfunction. Angiogenesis has been implicated in the pathophysiology of a number of diseases, and regulation of vasculogenesis could be an important therapeutic strategy for those diseases. Thus, further elucidation of the mechanism of Akt activation via Hsc70 may reveal important information on which the development of novel therapeutic interventions and new therapeutic medicines can be based.

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Disclosures

None.

References


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Supplemental data

Supplemental Methods

Immunoblotting

Antibodies were obtained from the following sources: anti-Akt (p-Ser473), Akt (p-Thr308), Akt1, eNOS (p-Ser1177), PDK1 (p-Ser241), PDK1, mTOR, rictor, Hsp72, and PI3kinase p85 antibodies from Cell Signaling; anti-PI3kinase p110 antibody from Santa Cruz; anti-eNOS antibody from BD Biosciences; anti-Hsc70 antibody from StressMarq; and anti-β-actin antibody from SIGMA. Our detailed method has been described previously. 1, 2

Protein extracts were obtained from homogenized HUVECs or skeletal muscles. Cells were washed with PBS and lysed in RIPA lysis buffer consisting of 50 mM HEPES, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% CHAPS, 10% glycerol, 100 mM NaF, 0.2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Nacalai Tesque). Lysates for immunoblotting (20 µg of protein) were separated on 10% or 7.5% SDS-polyacrylamide gels under reducing conditions, followed by electrophoretic transfer to polyvinylidine difluoride membranes (Immobilon-P; Millipore). After blocking, the membranes were probed with the appropriate primary antibodies. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with horseradish peroxidase. Immunoblots were detected with LAS-3000 (FUJIFILM) using the enhanced chemiluminescence technique (Immobilon Western HRP Substrate; Millipore). Immunoblots were quantified using Multi Gauge software (FUJIFILM).

Migration assay
The transwell inserts (Nunc) were coated with 0.1 mg/mL collagen. VEGF at 10 ng/mL dissolved in EBM-2 medium containing 0.25% bovine serum albumin (BSA) was added to the bottom chamber of the Boyden apparatus. HUVECs (1x10^5 cells) suspended in 100 µl aliquot of EBM-2 containing 0.25% BSA were added to the upper chamber. After 6 hours incubation, the nonmigrating cells in the upper part of the chamber were mechanically removed, and the remaining cells in the lower part fixed with methanol. For quantification, cells were stained by Diff-Quik solutions (Kokusaishiyaku). Cells migrating into the lower chamber were counted in six random microscopic fields.

**In vitro Angiogenesis**

The formation of vascular-like structures by HUVECs was assessed on growth factor–reduced Matrigel (BD Biosciences). HUVECs were plated at 1x10^4 cells/well in a 96-well-multidish precoated with 30 µL of Matrigel in the absence or presence of VEGF (10 ng/mL). After 6 h of incubation in 5% CO₂-humidified atmosphere at 37°C, cells were stained by Calcein-AM (5 µg/mL). Then, the length of completed tube-like structures in a center field (10-fold magnification) was quantified. Images were captured with a CCD color camera attached to the microscope and tube length was measured using a Micro Analyzer (JPD).

**RNA interference**

All siRNAs against human Hsc70 (HSPA8), Hsp72 (HSPA1A), HSP40 (DNAJB1), and Silencer negative control number 1 siRNA (NC) were obtained from Ambion. Lipofectamin RNAiMAX (Invitrogen) was used to transfec siRNAs into the HUVECs (final concentration 10 nmol/L) as suggested in the manual. The sense sequences of Hsc70 siRNAs
are #1; 5’-CCUAAAUCGUAGCAAAUUt3’, #2; 5’-GGAGGUGUCUUCUAUGGUUtt-3’, #3; 5’-UGAUGCUGUUGUCCAGUCUGAAtt-3’.

After 48 h of reverse transfection, quantitative RT-PCR and immunoblotting were carried out to examine the knockdown of targeted molecules.

**RNA preparation and Real-time reverse transcriptase-polymerase chain reaction analysis**

Total RNA was isolated from KNK437- or siRNA-treated HUVECs using the Rneasy kit (QIAGEN). Reverse transcription was then performed using 1 µg of RNA and the ReverTra Ace (TOYOBO). Real–time PCR analysis was done with the 7500FAST system using the TaqMan Gene Expression Assay kit (Applied Biosystems). RNA level was normalized by GAPDH. All data were analyzed by comparative C_T using the 7500 Software ver.2.0.1 (Applied Biosystems).

**Plasmid construction and transfection**

pDNOR201 and pcDNA-DEST40 vector were purchased from Invitrogen. The V5-tagged cDNA for human Hsp40, Hsp72, Hsp90α, Hsp90β and Hsp110 constructs were generated by Gateway cloning methods. Full-length human Hsp40, Hsp72, Hsp90α, Hsp90β and Hsp110 were amplified using PCR from a HUVEC reverse transcription product. The PCR products were cloned into pDONR201 vector performing the BP reaction and confirmed by sequencing. Then, the gene was further subcloned into the pcDNA-DEST40 (tagged with V5 at the C-terminus) vector by LR recombination reaction according to the manufacturer’s protocol. The EGFP plasmid was kindly provided by Shinya Yamanaka (Kyoto University).
HUVECs were plated in 35-mm dish at 80% confluence and then transfected with 2.5 µg of plasmids. Transfection was performed using the Lipofectamine LTX and PLUS Reagent (Invitrogen) according to the manufacture’s protocol. Cells were incubated with the DNA transfection reagent complexes at 37 °C for 6 hour, and this was followed by recovery to new medium.

Figures and legends

Fig.1

(A) Quantitation assessment of the extent of tube formation. HUVECs were seeded on Matrigel-coated culture dishes in the absence or presence of VEGF (10 ng/mL) with or without KNK437 (100 µmol/L) for 6 h. The length of capillary-like structure was measured by a Micro Analyzer image analysis program. Data are presented as means ± SD. *P < 0.05 vs. control, **P < 0.05 vs. VEGF (+), n = 3. (B) Endothelial cell migration was assessed using a modified Boyden Chamber. Quiescent Vehicle or KNK437 were added to the upper chamber, VEGF was added to the lower chamber. After 6 h of incubation at 37°C, migrated cells on the lower side of the membrane were counted. The values are means ± SD. *P < 0.05 vs. control, **P < 0.05 vs. VEGF (+), n = 3. (C) Effect of KNK437 on eNOS phosphorylation. Serum starved HUVECs were pretreated with KNK437 (100 µmol/L) for 1 h, and stimulated with VEGF (10 ng/mL) for 30 min. The phosphorylation of eNOS was examined using immunoblotting. Intensity of eNOS phosphorylation was quantified using Multi Gauge software. Quantitative data are shown as the means ± SD. *P < 0.05 vs. control, **P < 0.05 vs.
VEGF (+), n = 3.

Fig.2
(A) Upper panel shows a representative LDBF image. A low perfusion signal (dark blue) was observed in the ischemic hindlimb of a KNK437-treated mouse, whereas a high perfusion pattern (green) was detected in a vehicle-treated mouse. The lower panel shows the computer-assisted quantitative analysis of hindlimb blood perfusion that demonstrated a significant reduction in the ischemic/normal hindlimb blood flow ratio in the KNK437-treated mice (n=4) compared with the vehicle-treated animals (n=4). Each bar represents mean ± SD. *P<0.05 vs. vehicle. (B) Representative photomicrographs of tissue immunostained with anti-CD31 Ab (upper panel) and capillary density (lower panel) on postoperative day 5 (n=4 in each group). Each bar represents mean ± SD. *P<0.01 vs. WT.

Fig.3
(A) The extent of Akt phosphorylation was detected by anti-phosphorylation serine 473 residue of Akt1 specific antibody. HUVECs were pretreated with KNK437 (100 μmol/L) for 1h, before 30 min stimulation with 10 ng/mL VEGF. Equal amounts of protein (20 μg) from the control and each treatment were analyzed by immunoblotting for phosphorylated Akt (Akt-p) levels using their phospho-specific antibodies. As a loading control, the blots were reproved with β-actin antibodies. Densitometric quantitation of phospho-protein to total protein for Akt. Data are presented as means ± SD. *P < 0.05 vs. control, **P < 0.05 vs. VEGF (+), n = 3. (B) Akt and eNOS phosphorylation at 1 h after hindlimb ischemia in vehicle and KNK437-treated mice. Mice were injected intraperitoneally with 50 μg/g of KNK437 or
DMSO for 3 h, and hindlimb ischemia was induced by resecting the left femoral arteries and veins. After 1 h, ischemic tissues were excised. N, non-ischemia; I, ischemia.

**Fig.4**

HUVECs were transfected with the negative control (NC) and siRNA to Hsc70. (A) After 48 h of transfection, cell number was assessed by trypan blue staining. Data are presented as means ± SD. *P < 0.05 vs. NC; NC, negative control. (B) HUVECs tube formation assay. At 48 h after transfection, cells were trypsinized, and seeded on growth factor-reduced Matrigel in the absence or presence of VEGF (10 ng/mL) for 6 h. The tube length was measured by a Micro Analyzer image analysis program. Data are presented as means ± SD. *P < 0.05 vs. NC, **P < 0.05 vs. VEGF (+), n = 3. (C) Quantification of migration cells in Boyden chamber assays. The role of Hsc70 in the migration of HUVECs was assessed using Boyden chamber assays. HUVECs were transfected with either control or Hsc70 siRNAs, incubated for 48 h, and subjected to Boyden chamber assays in the presence or absence of VEGF (10 ng/mL). After 6 h, the migrated cells on the lower side were counted. Data are presented as means ± SD *P < 0.05 vs. NC, **P < 0.05 vs. VEGF (+), †P < 0.05 vs. Hsc70 siRNA, n = 4. NC, negative control.

**Fig.5**

Immunoblot analysis showing siRNA-mediated Hsc70 knockdown in HUVECs. HUVECs transfected with either NC or Hsc70 siRNA were serum-starved for 12 h and incubated with or without VEGF (10 ng/mL) for 30 min. Thereafter, lysates were immunoblotted with phospho-Akt antibodies. Densitometric quantitation of phospho-protein (phosphorylation of
Akt Ser473) to total protein for Akt. Data are presented as means ± SD. *$P < 0.05$ vs. NC, **$P < 0.05$ vs. VEGF (+), $n = 3$, NC, negative control.

**Fig. 6**

(A) Immunoblot analysis showing siRNA-mediated Hsc70 knockdown in HUVECs. After 48 h of siRNA transfection, total protein was prepared from the HUVECs. Equal amounts of protein (20 µg) from the control and each treatment were analyzed by immunoblotting for PI3K p110 and PI3K p85 levels using their specific antibodies. As a loading control, the blots were reprobed with β-actin antibodies. Densitometric quantitation of each protein to β-actin. Data are presented as means ± SD. *$P < 0.05$ vs. NC, **$P < 0.05$ vs. VEGF (+), $n = 3$, NC, negative control. (B) Effect of KNK437 on the expression of PI3K p110. Serum-starved HUVECs were pretreated with KNK437 (100 µmol/L) for 1 h, and stimulated with VEGF (10 ng/mL) for 30 min, lysed, and immunoblotted against PI3K p110 and PI3K p85 antibodies. Data represent three repartee experiments. Results from two other experiments were similar. (C) Quantitative RT-PCR analysis showing siRNA-mediated Hsc70 knockdown in HUVECs. Total RNA were isolate from HUVECs transfected with either NC or Hsc70 siRNA for 48 h. The mRNA contents of PI3K p110α and PI3K p110γ that were examined by quantitative RT-PCR as described in Methods were normalized to the amount of GAPDH mRNA. Data are presented as means ± SD. *$P < 0.05$ vs. NC, $n=3$, NC, negative control.

**Supplemental Figures and legends**

**Supplemental Fig. 1**

The effect of KNK437 on Akt and eNOS phosphorylation. Serum starved HUVECs were
pretreated with KNK437 (100 µmol/L) for 1 h, and stimulated with VEGF (10 ng/mL) for the indicated periods, lysed, and immunoblotted against phospho-Akt, Akt, phospho-eNOS, eNOS antibodies.

**Supplemental Fig. 2**

The mRNA levels in KNK437-treated HUVECs. Total RNA was prepared from KNK437-treated HUVECs for 1.5 h or 6 h. The mRNA contents of the indicated Hsps examined by quantitative RT-PCR as described in Methods were normalized to the amount of GAPDH mRNA. Data for Hsps are expressed as means ± SD of three independent experiments, each determined in triplicate. *P < 0.05 vs. NC, NC; negative control.

**Supplemental Fig. 3**

The inhibitory effect of Hsc70 siRNA. HUVECs were transfected with the negative control (NC) and siRNA to Hsc70. The total cell lysates from HUVECs transfected with either control or Hsc70 siRNA that target different sequences in Hsc70 (si1, si2, si3) were analyzed by immunoblotting using anti-Hsc70 and anti-β-actin antibodies. Immunoblot analysis shows siRNA-mediated Hsc70 knockdown in HUVECs.

**Supplemental Fig. 4**

The degree of Caspase3 activation. After 48 h of transfection, HUVECs were lysed, and immunoblotted against cleaved-Caspase3 and β-actin antibodies. NC; negative control.

**Supplemental Fig. 5**

The mRNA levels of Hsps, which is affected on KNK437, in Hsc70-silenced HUVECs. Total RNA was prepared from Hsc70 siRNA-transfected HUVECs. The mRNA contents of the
indicated Hsps examined by quantitative RT-PCR as described in Methods were normalized to the amount of GAPDH mRNA. Data for Hsps are expressed as means ± SD of three independent experiments, each determined in triplicate. *$P < 0.05$ vs. NC, NC; negative control.

**Supplemental Fig. 6**

Effect of siRNA for Hsc70 on Akt and eNOS phosphorylation. After 48 h of transfection, serum starved HUVECs were treated with VEGF (10 ng/mL) for the indicated periods, lysed, and immunoblotted against phospho-Akt, Akt, phospho-eNOS, eNOS antibodies.

**Supplemental Fig. 7**

Immunoblot analysis showing siRNA-mediated Hsc70 knockdown in HUVECs transfected with either NC or Hsc70 siRNA. The cells were serum-starved for 12 h and incubated with or without VEGF (10 ng/mL) for 30 min. Thereafter, lysates were immunoblotted with phospho-Akt (S473), mTOR, Rictor, phospho-Akt (T308), phospho-PDK, PDK1, Hsc70, and β-actin antibodies.

**Supplemental Fig. 8**

(A) Immunoblot analysis showing siRNA-mediated Hsp40 and Hsp72 knockdown in HUVECs. After 48 h of siRNA transfection, total protein was prepared from the HUVECs. Equal amounts of protein (20 µg) from the control and each treatment were analyzed by immunoblotting for PI3K p110 levels using their specific antibodies. As a loading control, the blots were reprobed with β-actin antibodies. Densitometric quantitation of each protein to β-actin. Data are presented as means ± SD. $n = 5$, NC, negative control. (B) Quantitative
RT-PCR analysis showing siRNA-mediated Hsp40 and Hsp72 knockdown in HUVECs. Total RNA were isolate from HUVECs transfected with either NC, Hsp40, nor Hsp72 siRNA for 48 h. The mRNA contents of PI3K p110α and p110γ that were examined by quantitative RT-PCR as described in Methods were normalized to the amount of GAPDH mRNA. Data are presented as means ± SD. NC, n=5, NC, negative control.

**Supplemental Fig. 9**

(A) Immunoblot analysis showing siRNA-mediated Hsp72 knockdown in HUVECs transfected with either NC or Hsc70 siRNA. The cells were serum-starved for 12 h and incubated with or without VEGF (10 ng/mL) for 30 min. Thereafter, lysates were immunoblotted with phospho-Akt (S473), Akt, phospho-eNOS (S1177), eNOS, PI3K p110, Hsp72 and β-actin antibodies. (B) Immunoblot analysis showing geldanamycin-treated HUVECs. The cells were treated by geldanamycin (1 µmol/L) for 24 h and incubated with or without VEGF (10 ng/mL) for 30 min. Thereafter, lysates were immunoblotted with phospho-Akt (S473), Akt, phospho-eNOS (S1177), eNOS, PI3K p110, and β-actin antibodies.

**Supplemental Fig. 10**

Immunoblot analysis showing overexpressed V5-tagged Hsp40, Hsp72, Hsp90α, Hsp90β, and Hsp110 in HUVECs. After 24 h of transfection, total protein were prepared from the HUVECs. Equal amounts of protein (20 µg) from the control and each treatment were analyzed by immunoblotting for PI3K p110 levels using their specific antibodies. As a loading control, the blots were reprobed with β-actin antibodies.

**Supplemental References**

Supplemental Figures

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(-)     | KNK437
Supplemental Figure 2

![Graph showing relative mRNA level for different genes under different conditions](image1)

Supplemental Figure 3

![Image showing Western blot for Hsc70 and β-actin](image2)
Supplemental Figure 4

NC siRNA  +  -  
Hsc70 siRNA -  +  
Cleaved-Caspase-3

\[ \text{\beta-actin} \]

Supplemental Figure 5

\[ \text{Relative mRNA level} \]

NC  
Hsc70

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Supplemental Figure 8

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B.  
![Graph showing relative mRNA levels for p110α and p110γ across NC, Hsp40, and Hsp72 treatments.](image)

Supplemental Figure 9

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B.  
![Graph showing relative mRNA levels for Akt S473p, Akt, eNOSp, eNOS, PI3K p110, and β-actin across GA, VEGF, Akt S473p, Akt, eNOSp, eNOS, PI3K p110, and β-actin treatments.](image)