Endothelial nitric oxide synthase (eNOS), which generates the endogenous vasodilator, nitric oxide (NO), is highly regulated by post-translational modifications and protein interactions. Heat shock protein 90 (HSP90) binds directly to eNOS, augmenting NO production. We have used purified proteins to characterize further the mechanism by which HSP90 increases eNOS activity at low (100 nM) and high (10 μM) Ca2+ levels. In the presence of calmodulin (CaM), HSP90 increased eNOS activity dose dependently at both low and high Ca2+ concentrations. This effect was abolished by the specific HSP90 inhibitor geldanamycin (GA) at both calcium concentrations. The EC50 values of eNOS for both Ca2+ and CaM were decreased in the presence of HSP90. HSP90 also significantly increased the rate of NADPH-dependent cytochrome c reduction by eNOS at both low and high Ca2+ concentrations. HSP90 bound to eNOS in a dose-dependent manner, and the amount of bound HSP90 also increased with increasing Ca2+/CaM. At 100 nM Ca2+, HSP90 promoted dose-dependent CaM binding to eNOS that was fully inhibitable by GA. At high calcium, HSP90 did not affect CaM binding to eNOS, but GA inhibited HSP90 binding to eNOS. At high Ca2+, HSP90 caused the Vmax of eNOS for l-arginine to increase by 2-fold, but the Km of eNOS was unchanged. HSP90 bound preferentially to CaM-prebound eNOS and significantly increased both its NO synthesis and reductase activities. These data support that HSP90 promotes eNOS activity by two mechanisms: (i) a CaM-dependent mechanism operative at low Ca2+ concentrations, characterized by an increase in the affinity of eNOS for CaM and (ii) a CaM-independent mechanism apparent at high Ca2+ concentrations, characterized by stimulation of eNOS reductase activity without further change in CaM binding. These studies contribute to our understanding of eNOS activation by HSP90 and provide a basis for in vitro studies of other eNOS-interacting proteins.

Nitric oxide (NO) plays a central role in the maintenance of cardiovascular homeostasis with effects on blood pressure, an- giogenesis, vascular remodeling, and platelet aggregation (1). Endothelial NO synthase (eNOS), the highly regulated enzyme responsible for physiological production of NO in the vasculature, is primarily a Ca2+/calmodulin (CaM)-dependent enzyme. However, recent studies have revealed additional regulation of eNOS activity by subcellular localization, post-translational modification such as phosphorylation and dephosphorylation, and interactions with several regulatory proteins, including heat shock protein 90 (HSP90) (2–4).

HSP90 increases the activity of eNOS in vitro, and stimulation of endothelial cells with various stimuli such as vascular endothelial growth factor, histamine, estrogen, and fluid shear stress increases an association of HSP90 with eNOS, resulting in elevation of NO production (4–6). HSP90 has no effect on eNOS dissociation from caveolin in the absence of Ca2+/CaM but facilitates CaM-induced eNOS release from eNOS-caveolin (7). Recently, the domains of eNOS and HSP90 necessary for their interaction were identified (8, 9). Brouet et al. (6) showed that HSP90 association with eNOS is a prerequisite for subsequent Akt-mediated stimulation of eNOS. Together with the data that HSP90 directly interacts with Akt (10), their results suggest that HSP90 might be a scaffold factor between eNOS and Akt. Accumulating data have indicated that HSP90 plays a crucial role in regulating eNOS activity in endothelial cells. However, the mechanism by which HSP90 directly promotes eNOS activity remains unclear.

Many studies have suggested the existence of so-called “Ca2+-independent” activation of eNOS, although it is well established that Ca2+-activated CaM is necessary for eNOS activity (11, 12). It was reported that fluid shear stress and estrogen increase NO production without mobilizing cytosolic Ca2+ in endothelial cells (13–15) and that HSP90 might mediate their effects on eNOS (4, 5). As observed in eNOS phosphorylated by protein kinases such as Akt (at Ser-1177 and -1179 for human and bovine eNOS, respectively) (16–18), these results raised the possibility that HSP90 also participates in Ca2+-independent activation of eNOS. Whether Ca2+-independent activation of eNOS can be induced by HSP90 is also a critical question that needs to be addressed. The present study was designated to clarify the mechanism of the stimulatory effect of HSP90 on eNOS activity including Ca2+/CaM requirement.

**EXPERIMENTAL PROCEDURES**

Materials—The enzymes, antibodies, and reagents used in this study and their sources are as follows: bovine brain HSP90, CHAPS, Dowex AG50Wx8, tetrahydrobiopterin, NADPH, FAD, FMN, t-LNAME, t-arginine, cytochrome c, and superoxide dismutase (SOD) (Sigma); recombinant chicken CaM, geldanamycin (GA), and protease inhibitor mixture set III (Calbiochem, La Jolla, CA); anti-eNOS antibody N30020 (BD Biosciences, San Diego, CA); CaM; calmodulin; t-LNAME, N5-nitro-t-arginine methyl ester; GA, geldanamycin; SOD, superoxide dismutase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Transduction Laboratories, Lexington, KY; anti-HSP90 antibody SP-830 (OriGene, Victoria, BC; anti-CaM antibody and protein G-agarose beads (Upstate Biotechnology, Lake Placid, NY); secondary antibodies linked to horseradish peroxidase, ECL Western blotting detection reagents, 2',5'-AMP-Sepharose 4B, CaM-Sepharose 4B, and HiTrap Q (Amersham Biosciences); Bradford protein assay kit (Bio-Rad, Hercules, CA); BENCH MARK prestained protein ladder (Invitrogen); polyvinylidene difluoride transfer membrane Immobilon-P (Millipore, Bedford, MA); calcium calibration buffer kit (Molecular Probes, Eugene, OR); L-[2,3,4-3H]arginine (45–70 Ci/mmol, PerkinElmer Life Sciences). HSP90 was reconstituted in 20 mM Tris-HCl (pH 7.5), 10 µM ATP, and 1 mM MgCl2. All other chemicals were of reagent grade.

**eNOS Purification**—Recombinant bovine wild-type eNOS, expressed in Sf9 cells, was purified from the lysate by sequential chromatography on 2',5'-AMP-Sepharose and CaM according to the method of List et al. (19). The eNOS was further purified by HiTrap Q chromatography with a linear gradient of 0.1–1 M NaCl. 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. In the case of preparing CaM-bound eNOS, purified eNOS was incubated with 1 mM Ca2+ and 300 mM CaM on ice for 30 min and then loaded to a 2',5'-AMP-Sepharose column. Following washes, CaM-bound eNOS was eluted with 10 mM NADPH.

**NOS Activity Assay**—NOS activity was measured as the conversion of L-[2,3,4-3H]arginine to L-[2,3,4-3H]citrulline as described by Bredt and Snyder (22). Specific NOS activity was determined photometrically according to previous reports (22) with minor modifications. eNOS (9.8 nM) was preincubated with HSP90 at room temperature for 5 min, because the 5-min treatment reached the maximal effect of HSP90 (data not shown). In some experiments, HSP90 was treated with 1 µM GA. eNOS was then mixed with the reaction buffer consisting of 25 mM Tris-HCl (pH 7.5), 5 mM CHAPS, 100 mM NaCl, 1 mM dithiothreitol, 1 mM L-NAME, 20 µM PDA, 20 µM FMN, 20 µM tetrahydrobiopterin, and 300 mM CaM in the presence of EGTA or Ca2+. NOS reaction was initiated by adding 100 µM L-[2,3,4-3H]arginine (1 µCi), and incubation was done at 37 °C for 10 min unless otherwise stated. The reaction was terminated by adding 50 mM HEPES-NaOH (pH 5.5) buffer containing 2 mM EGTA and 2 mM EDTA. L-[2,3,4-3H]citrulline was separated through a Dowex AG50Wx8 (Na+ form) column and then counted on a liquid scintillation analyzer. 1-NAME-inhibitable activity was determined as specific eNOS activity.

**NADPH Cytochrome c Reductase Activity Assay**—Cytochrome c reductase activity was determined photometrically according to previously reported methods (22) with minor modifications. eNOS (9.8 nM) was mixed with HSP90 (45 nM) in the presence of EGTA or Ca2+ in reaction buffer consisting of 50 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, 0.5 mM CHAPS, 200 µM cytochrome c, and 300 nM CaM. SOD was added at 1 unit. The reductase reaction was initiated by adding 100 µM NADPH, and then change in absorbance at 550 nm was measured at 25 °C for 3 min. The rate of reduction was calculated using an extinction coefficient of 0.021 µM−1 cm−1 for cytochrome c at 550 nm.

**Immunoprecipitation and Immunoblotting of eNOS, HSP90, and CaM**—eNOS (9.8 nM) was incubated at 37 °C for 10 min in the NOS reaction buffer in the presence of EGTA or Ca2+ after eNOS was preincubated with HSP90 at room temperature for 5 min. eNOS complex was then immunoprecipitated by anti-eNOS antibody (1:100–200) and protein A/G-agarose beads at 4 °C. Following washes with the corresponding buffer, the immunoprecipitates were suspended in sample buffer, separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. The blots were incubated with the primary antibody against eNOS (1:1000), HSP90 (1:1000), or CaM (1:500) and then probed with the secondary antibody linked to peroxidase. Immunoreactive proteins were visualized on x-ray films with chemiluminescent ECL reagents. The intensity of each band was measured by a densitometer and normalized to the corresponding eNOS. Relative intensity to the defined group as described in each figure legend was calculated.

**Statistical Analyses**—Data are presented as means ± S.E. Statistical differences were evaluated by Student’s t test. EC50, nM, and Vmax values were obtained from the direct fits to the data according to Hill equation on SigmaPlot 2001 software. p value of <0.05 was regarded as significant.

**RESULTS**

**HSP90 Increases eNOS Activity in a Calcium- and CaM-dependent Manner**—Recombinant bovine eNOS was purified to near homogeneity by sequential chromatographic steps (Fig. 1A, inset). The protein in our preparation was recognized by anti-eNOS antibody, but immunoblotting of the preparation with anti-HSP90 and anti-CaM antibodies were both negative (data not shown). We examined the effects of HSP90 on eNOS activity in the presence of CaM at several calcium concentrations (Fig. 1A). Depletion of free Ca2+ (<1 mM) was achieved by addition of 10 mM EGTA to the reaction. eNOS activity was negligible under these conditions, and HSP90 was unable to stimulate eNOS activity in the presence of EGTA. Presence of low (100 nM) or high (10 µM) Ca2+ caused activation of eNOS (2.2 ± 0.4 and 13.6 ± 0.6 nmol/min/nmol of protein at 100 nM and 10 µM Ca2+, respectively), which was in both cases significant in comparison to the Ca2+-free control. HSP90 also sig-
significantly augmented eNOS activity under both low and high calcium conditions. The increases in eNOS activity at 100 nM and 10 μM Ca²⁺ due to HSP90 were 7.2- and 2.3-fold, respectively, compared with the corresponding HSP90-free control. GA, a relatively specific inhibitor of HSP90, had no effect on Ca²⁺-activated eNOS activity by itself but completely inhibited the eNOS-stimulatory effects of HSP90 at both low and high calcium concentrations. The eNOS reactions at low and high calcium concentrations proceeded linearly up to 20 min both in the absence and presence of HSP90 (Fig. 1B), supporting that eNOS in the absence of HSP90 is not denatured and that the HSP90 effect is likely due to actions other than protection against thermal denaturation.

HSP90 promoted an increase in Ca²⁺-activated eNOS activity in a dose-dependent fashion (Fig. 2). The absolute activity of eNOS was greater in the presence of 10 μM Ca²⁺ than 100 nM Ca²⁺ (Fig. 1A). The overall stimulation of eNOS was substantially greater over a range of HSP90 concentrations in the presence of 100 nM Ca²⁺ (−7-fold) than in the presence of 10 μM Ca²⁺ (−2-fold). At 100 nM Ca²⁺, eNOS activity was 353% above control at 12 nM HSP90 and 697% above control at 45 nM HSP90. At maximal calcium levels (10 μM Ca²⁺), the increase in eNOS activity to increasing HSP90 was smaller (139% at 12 nM HSP90 and 254% at 45 nM HSP90) but was significant. This increase occurred with eNOS bound to maximal and constant amounts of Ca²⁺/CaM at both of these HSP90 concentrations (see Fig. 5 and results below).

Effect of HSP90 on the EC₅₀ Values of eNOS for Ca²⁺ and CaM and Maximal eNOS Activity—Ca²⁺ and CaM dependencies of eNOS activity were separately examined in the absence and presence of HSP90 (Fig. 3). eNOS activity in the presence of 300 nM CaM increased in a Ca²⁺-dependent manner in the absence and presence of HSP90 (see Fig. 3A). The EC₅₀ value of eNOS for Ca²⁺ was 14.9 nM (Fig. 3A). HSP90 enhanced the Ca²⁺ sensitivity of eNOS under these conditions. The EC₅₀ values of eNOS for Ca²⁺-dependent stimulation were 239 nM and 83 nM in the absence and presence of HSP90, respectively. HSP90 significantly increased eNOS activity at all calcium concentrations (maximal increase from 14.9 to 32.4 nmol/min/nmol of protein at 10 μM Ca²⁺). This effect of HSP90 on eNOS activity is determined by the intrinsic activity of the reductase domain of the enzyme to transfer electrons to heme (22–24). The HSP90 effect became apparent at CaM levels ≥1 nM, though HSP90 had no measurable effect on eNOS activity at lower CaM concentrations, even at maximal Ca²⁺ levels (Fig. 3B and data not shown).

HSP90 Increases NADPH Cytochrome c Reductase Activity of eNOS in a Calcium- and CaM-dependent Manner—The rate of NO synthesis by eNOS is determined by the intrinsic activity of the reductase domain of the enzyme to transfer electrons to heme (22–24). We examined whether the increase in eNOS activity in the presence of HSP90 is accompanied by enhanced reductase activity of eNOS by measuring NADPH-dependent cytochrome c reduction in the presence of 300 nM CaM. In the presence of EGTA, cytochrome c reductase activity of eNOS was minimal, and HSP90 had no effect on this activity (Fig.
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4A). In the presence of 100 nM or 10 μM Ca\(^{2+}\), reductase activity was elevated in a Ca\(^{2+}\)-dependent manner \((0.22 \pm 0.03 \text{ versus } 1.0 \pm 0.1 \text{ μmol/min/mol of eNOS protein})\). The Ca\(^{2+}\)/CaM-activated reductase activity was significantly enhanced by HSP90 at both 100 nM and 10 μM Ca\(^{2+}\) (5.9- and 1.8-fold, respectively). Next, we repeated these studies in the presence of SOD to explore differences that might have occurred due to reduction of cytochrome \(c\) by eNOS-derived superoxide (22) (Fig. 4B). SOD decreased the rate of cytochrome \(c\) reduction somewhat for control eNOS both at 100 nM and 10 μM Ca\(^{2+}\) (28.1% and 25.7%, respectively), but the majority of Ca\(^{2+}\)/CaM-dependent reductase activity was still present. The extent of uncoupling was quite similar to previously reported values determined by the same method (22). The cytochrome \(c\) reductase activity of eNOS in the presence of HSP90 was only slightly reduced by SOD, (6.7% at 100 nM Ca\(^{2+}\) and 10.3% at 10 μM Ca\(^{2+}\)) compared with the corresponding value without SOD. HSP90 still significantly promoted cytochrome \(c\) reductase activity of eNOS in the presence of SOD to degrees similar to those measured in the absence of SOD (7.3- and 2.0-fold at 100 nM and 10 μM Ca\(^{2+}\), respectively). The extents of the increases in eNOS activity (Fig. 1A) and reductase activities (Fig. 4) in the presence of HSP90 were thus of similar magnitudes. These data support that HSP90 primarily stimulates the reductase activity of eNOS and, to a much lesser extent, inhibits uncoupling of the enzyme.

HSP90 and CaM Cooperatively Potentiate Their Binding to eNOS—Binding of HSP90 and CaM to eNOS was examined by immunoprecipitation studies with anti-eNOS antibody (Fig. 5). HSP90 bound to eNOS in the absence of Ca\(^{2+}\) to a small degree, but eNOS-HSP90 complex formation was enhanced in a Ca\(^{2+}\)-dependent manner. eNOS bound more HSP90 as the concentration of HSP90 present was increased. CaM was detected in the eNOS immunopellet only in the presence of Ca\(^{2+}\). At 100 nM Ca\(^{2+}\), HSP90 dose dependently increased the amount of CaM bound to eNOS along with the corresponding increase in HSP90 bound to the synthase. The absolute amount of CaM bound in the presence of 100 nM Ca\(^{2+}\) and 45 nM HSP90 was substantially lower than the amount of CalM bound to eNOS at 10 μM Ca\(^{2+}\) even in the absence of HSP90. The level of eNOS activity at 100 nM Ca\(^{2+}\) due to the presence of HSP90 was higher than the control eNOS activity at 10 μM Ca\(^{2+}\) (Fig. 1A). In contrast, HSP90 had no further effect on CaM binding at 10 μM Ca\(^{2+}\), although HSP90 still significantly (though modestly) increased eNOS activity (cf. Figs. 1A and 2). GA potently inhibited HSP90 binding to eNOS in the absence and presence of calcium and abolished the effect of HSP90 on CaM binding at 100 nM. However, CaM binding to eNOS at 10 μM Ca\(^{2+}\) was not affected by GA. The amount of CaM bound to eNOS at 10 μM Ca\(^{2+}\) was not influenced by HSP90 or GA, indicating that Ca\(^{2+}\)/CaM binding to eNOS is saturated at 10 μM Ca\(^{2+}\).

CaM-independent Effects of HSP90 on eNOS—At 10 μM Ca\(^{2+}\), HSP90 increased both the NO synthase and cytochrome \(c\) reductase activities of eNOS. A dose-dependent increase in HSP90 binding to eNOS was also observed at high Ca\(^{2+}\) con-

![Fig. 4. Effect of HSP90 on cytochrome c reductase activity of eNOS. eNOS (9.8 nM) was incubated with vehicle (control) or HSP90 (45 nM) in the presence of 300 nM CaM and either 10 nM EGTA, 100 nM Ca\(^{2+}\), or 10 μM Ca\(^{2+}\), and then NADPH-dependent cytochrome c reductase activity was determined at 23 °C for 3 min in the absence (A) or presence (B) of SOD. Data are presented as mean ± S.E. for four determinations. * significantly different from the corresponding control.](http://www.jbc.org/)

![Fig. 5. Binding of HSP90 and CaM to eNOS. eNOS (9.8 nM) was incubated at 37 °C for 10 min with the indicated dose of HSP90 in the presence of 300 nM CaM and either 10 nM EGTA, 100 nM Ca\(^{2+}\), or 10 μM Ca\(^{2+}\). GA treatment was performed at 1 μM. Thereafter, eNOS was recovered by immunoprecipitation with anti-eNOS antibody, and the presence of eNOS, HSP90, and CaM was evaluated by immunoblotting.](http://www.jbc.org/)
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Fig. 6. Michaelis-Menten analysis of eNOS with HSP90 at 10 μM Ca2+. eNOS (9.8 nM) was incubated with vehicle (control) or HSP90 (45 nM) in the presence of 10 μM Ca2+ and 300 nM CaM, and then eNOS activity was determined as a function of L-arginine concentration. Data are presented as mean ± S.E. for three determinations.

centration. However, CaM binding was unchanged by HSP90 at this calcium concentration. To further explore the HSP90 effects at 10 μM Ca2+, we compared eNOS activities and measured the Vmax and Km values for eNOS for L-arginine at 10 μM Ca2+ in the absence and presence of HSP90 (Fig. 6). eNOS exhibited a higher velocity of NO synthesis in the presence of HSP90. The Vmax value for arginine was increased 2-fold by HSP90 (from 14.6 ± 0.15 to 28.7 ± 4.2 nmol/min/nmol of protein for eNOS in the absence or presence of HSP90, respectively). However, HSP90 did not affect the affinity for L-arginine as the Km value for L-arginine was 4.2 ± 0.3 in the presence and 4.1 ± 0.2 μM in the absence of HSP90.

We prepared eNOS in the presence of EGTA (CaM-free eNOS) or 1 mM Ca2+ (CaM-bound eNOS) (Fig. 7A) and examined the effects of HSP90 on both the synthase and reductase activities of the enzyme (Fig. 7, B and C). HSP90 bound preferentially to CaM-bound eNOS and GA treatment prevented binding of HSP90 to eNOS. In addition, both NOS and cytochrome c reductase activities of CaM-bound eNOS were significantly enhanced by HSP90. These results show that HSP90 has a direct effect on eNOS pre-bound to CaM even in the absence of added (exogenous) CaM.

DISCUSSION

We have examined the mechanism of HSP90 activation of eNOS in vitro. HSP90 appears to increase eNOS activity in the presence of Ca2+ and CaM by two mechanisms. Under conditions where eNOS is not saturated with CaM, HSP90 reduced the EC50 values for Ca2+ and CaM and dose dependently increased the amount of CaM bound to eNOS. These results indicate that HSP90 elevates the binding affinity of eNOS for CaM, resulting in an increase in NO synthesis. Gratton et al. (7) reported previously that CaM-induced dissociation of eNOS from caveolin is facilitated by HSP90 through an unknown mechanism. Our results support that an HSP90-induced increase in CaM binding might facilitate eNOS dissociation from this protein. The CaM-dependent mechanism we observed appears similar to the effect of HSP90 on nNOS, though the effect of HSP90 on eNOS activity appears to be greater than its effect on nNOS (25).

Our data support that HSP90 also activates eNOS by a CaM-independent mechanism, which was detectable at high calcium levels. The evidence for a CaM-independent HSP90 effect includes the following. (i) HSP90 dose dependently increased NO synthesis and reductase activities of eNOS in the presence of saturating levels of Ca2+ and CaM (Figs. 2–4). (ii) HSP90 binding to eNOS increases when the quantity of CaM bound to eNOS remains constant (Fig. 5), and this results in a further increase in eNOS activity (from 139% to 254% of basal eNOS activity at 12 and 45 nM HSP90, respectively; Fig. 2). (iii) Despite the fact that CaM is a critical determinant of Km, HSP90 significantly increased the Vmax for L-arginine without affecting the Km in the presence of saturating Ca2+ and CaM (Fig. 6). (iv) HSP90 association with CaM-bound eNOS results in increases in NO synthesis and reductase activities of eNOS even in the absence of added CaM or any change in CaM binding to eNOS (Fig. 7). (v) These effects of HSP90 were
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prevented by an HSP90-specific inhibitor, geldanamycin (Figs. 1, 5, and 7). The CaM-independent effect of HSP90 causes only an ~2-fold increase in eNOS activity at 45 nM HSP90 and is therefore weaker than the CaM-dependent effect of HSP90 on eNOS activity under these conditions. However, the combined CaM-dependent and -independent effects of HSP90 may both be active to varying degrees across a range of Ca\textsuperscript{2+} concentrations and cooperate in an additive or synergistic fashion to stimulate eNOS activity. Pritchard et al. (27) reported that HSP90 may prevent eNOS uncoupling and thus both inhibit superoxide production and increase NO synthesis. We observed modest eNOS uncoupling in the absence of HSP90, consistent with this prior work (27). However, uncoupling accounted for at most 20–25% of the decrease in the eNOS cytosolic Ca\textsuperscript{2+} levels. 

HSP90 promotes eNOS activity by CaM-dependent and CaM-independent mechanisms, increases the affinity of eNOS for CaM, and stimulates an increase in the intrinsic reductase activity of eNOS in the presence of a fixed amount of CaM. Since HSP90 and CaM bind to eNOS at lower Ca\textsuperscript{2+} levels, both mechanisms of HSP90-dependent eNOS activation are predicted to contribute to the increase in efficiency of NO production by eNOS at physiological Ca\textsuperscript{2+} concentrations.

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Calmodulin-dependent and -independent Activation of Endothelial Nitric-oxide Synthase by Heat Shock Protein 90
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