Regulation of contractility by Hsp27 and Hic-5 in rat mesenteric small arteries

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Regulation of contractility by Hsp27 and Hic-5 in rat mesenteric small arteries. *Am J Physiol Heart Circ Physiol* 294: H961–H969, 2008. First published December 14, 2007; doi:10.1152/ajpheart.00939.2007.—The regulation of small artery contractility by vasoconstrictors is important for vascular function, and actin cytoskeleton remodeling is required for contraction. p38 MAPK and tyrosine kinases are implicated in actin polymerization and contraction through heat shock protein 27 (Hsp27) and the cytoskeletal protein paxillin, respectively. We evaluated the roles of downstream targets of p38 MAPK and tyrosine kinases in cytoskeletal reorganization and contraction and whether the two signaling pathways regulate contraction independent of each other. We identified the expression of the paxillin homologue hydrogen peroxide-inducible clone-5 (Hic-5) and showed its activation by norepinephrine (NE) in a Src-dependent manner. Furthermore, we demonstrated a NE-induced interaction of proline-rich tyrosine kinase-2 (PYK2) but not Src or p125 focal adhesion kinase with Hic-5. This interaction was Src dependent, suggesting that Hic-5 was a substrate for PYK2 downstream from Src. The activation of Hic-5 induced its relocalization to the cytosol. The parallel activation of Hsp27 by NE was p38 MAPK dependent and led to its dissociation from actin filaments and translocation from membrane to cytosol and increased actin polymerization. Both Hsp27 and Hic-5 activation resulted in their association within the same time frame as NE-induced contraction, and the inhibition of either p38 MAPK or Src inhibited the interaction between Hsp27 and Hic-5 and the contractile response. Furthermore, combined p38 MAPK and Src inhibition had no greater effect on contraction than individual inhibition, suggesting that the two pathways act through a common mechanism. These data show that NE-induced activation and the association of Hsp27 and Hic-5 are required for the reorganization of the actin cytoskeleton and force development in small arteries.

smooth muscle; cytoskeleton; cell signaling; subcellular localization; α-adrenergic

VASOCONSTRICTOR HORMONE REGULATION of small artery contractility is an important determinant of vascular resistance and blood pressure. The initial activation of the phosphoinositide signaling pathway is the major mechanism for smooth muscle contraction via increased cytoplasmic calcium and myosin light chain phosphorylation (48). However, contraction is also regulated by additional mechanisms of which the reorganization of the actin cytoskeleton plays an important role (57). In cells in culture, the cytoskeleton is involved in regulating processes such as growth and migration, and recent evidence suggests that the reorganization of the actin cytoskeleton and

its interaction with the contractile machinery may be necessary for smooth muscle responses (17).

Two pathways have been identified in agonist-induced cytoskeletal reorganization: p38 MAPK and nonreceptor tyrosine kinases such as Src family, p125 focal adhesion kinase (p125FAK), and proline-rich tyrosine kinase-2 (PYK2) (15, 17, 38, 42, 59). However, whether these two pathways act independently of each other or through a common mechanism is not clear. p38 MAPK regulates the actin cytoskeleton through heat shock protein 27 (Hsp27), a member of the family of small heat shock proteins that also includes Hsp20 and crystallins (30). Hsp27 is thought to regulate actin filament dynamics in cells by binding to and capping actin filaments and inhibiting F-actin polymerization, a property relieved by Hsp27 phosphorylation (42). Furthermore, the overexpressing non-phosphorylatable mutant of Hsp27 inhibits actin stress fiber formation (28). Nonreceptor tyrosine kinases regulate the actin cytoskeleton through the tyrosine phosphorylation of the cytoskeletal scaffold protein paxillin (9). In association with Src, p125FAK subsequently binds to and phosphorylates paxillin, an event associated with the assembly of focal adhesions and stress fiber formation (9, 44). Vasoactive peptides, via G protein-coupled receptors (GPCRs), activate nonreceptor tyrosine kinases, and in airway and vascular smooth muscle tissues, the tyrosine phosphorylation of paxillin increases after contractile stimuli in parallel with force generation (37, 41, 54). Furthermore, its membrane association appears to be important for ACh-induced contraction (39, 50), suggesting that a tyrosine kinase/paxillin/cytoskeleton pathway is an important component of smooth muscle contractility.

Hydrogen peroxide-inducible clone-5 (Hic-5), originally identified from mouse osteoblastic cells (46), shares considerable sequence homology with paxillin, although unlike the latter, it is neither activated by integrin engagement nor phosphorylated by p125FAK. However, it is phosphorylated by PYK2, a calcium-dependent homologue of p125FAK (16, 51), suggesting different cellular functions for the two proteins. Hic-5 was shown to be a substrate for PYK2 (23), and in COS cells and rat fibroblasts, it was shown to interact with PYK2 (31), whereas in rat kidney epithelial cells (24), Hic-5 was shown to associate with Hsp27. Hic-5 has been identified in smooth muscle tissues (58), and PYK2, also present in smooth muscle, is activated by ANG II (7, 59) and norepinephrine (NE) (38), causing its translocation from cytosol to the cytoskeleton. In its ability to interact with both Hsp27 and PYK2, Hic-5 may be important in the regulation of smooth muscle actin dynamics.

Given the importance of α-adrenergic signaling in the regulation of small artery contractility and evidence that during agonist-stimulated contraction of smooth muscle tissue actin polymerization occurs and is necessary for force development (4, 11, 33), we investigated the activation of Hsp27 and Hic-5 and studied their interaction in rat mesenteric small arteries (RMSAs) after NE stimulation.

METHODS

The investigation was carried out in accordance with the University of Manchester Animal Experimentation Guidelines and the United Kingdom Animals (Scientific Procedures) Act of 1986. The experiments were performed with the approval of the Review Board of the University of Manchester and the Home Office.

Agonist treatment and preparation of small arteries. Adult Sprague-Dawley rats (200–300 g) were used for all experiments. The mesentery was excised and kept in ice-cold physiological salt solution until dissection (38). Mesenteric small arteries <400 μm in internal diameter were cleaned of adjoining fat and connective tissues and equilibrated in culture medium (medium 199; Invitrogen) for 45 min at 37°C before the addition of NE (15 μM) or vehicle [distilled H$_2$O (dH$_2$O)]. For p38 MAPK inhibition, SB-203580 (final concentration, 1 μM) or Src family tyrosine kinase inhibition herbimycin A (final concentration, 1 μM), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyra-zolo[3,4-D]pyrimidine (PP2; final concentration, 10 μM), or vehicle (DMSO; 0.01%) was added 15 min before agonist stimulation. The vessels were removed at various time points, as specified in the text, and homogenized manually in 50 μl ice-cold homogenization buffer containing 20 mM MOPS (pH 7.2), 0.25 M sucrose, and 1 mM dithiothreitol with proteinase inhibitors (Complete Mini tablet; Roche Applied Science) and phosphatase inhibitors sodium orthovanadate (1 mM) and sodium pyrophosphate (200 μM). An aliquot was removed from each sample for protein determination using the method of Bradford (6), and the protein amounts in each sample were equalized before subcellular fractionation or immunoprecipitation, as described in *Subcellular fractionation of small artery homogenates* and *Western blot analysis and immunoprecipitation.*

Subcellular fractionation of small artery homogenates. Differential centrifugation was used to prepare subcellular fractions as described previously (38, 52). Briefly, tissue homogenate was centrifuged at 800 *g* in a Beckman TL100 ultracentrifuge for 10 min at 4°C to pellet nuclei and unhomogenized tissue. The supernatant was then centrifuged at 150,000 *g* to yield the membrane pellet and final cytoplasmic supernatant (49). The pellet was washed twice with homogenization buffer to remove residual cytosolic proteins. The Triton X-100-insoluble fraction was prepared by resuspending the membrane pellet in homogenization buffer containing 1% (wt/vol) Triton X-100 and by mixing for 30 min at 4°C. These samples were then centrifuged again at 150,000 *g* to separate the solubilized proteins from the nonsolubilized material. All fractions were boiled for 5 min in Laemmli sample buffer (26) and used immediately or stored frozen at −20°C.

Western blot analysis and immunoprecipitation. The analysis of protein phosphorylation was carried out using phosphospecific antibodies (Hsp27) or phosphotyrosine antibodies of immunoprecipitates (Hic-5) as described previously (36, 52). Briefly, equivalent amounts of total tissue extracts (20 μg proteins), subcellular fractions, or immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed for phosphoSer82Hsp27 or phosphotyrosine. The membranes were stripped and reprobed for Hsp27 or Hic-5. Signals were developed using the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody and chemiluminescence reaction. Band intensities were measured using densitometry (GS800 densitometer; Bio-Rad), and the phosphoantigen signal was corrected for total antigen. Images showing saturation were not used in the analysis.

Immunoprecipitates were prepared as described previously (52). Briefly, small artery homogenates containing equal amounts of protein were precleared with IgG-agarose (50% solution) before incubation with the appropriate antibody for 1 h, followed by a 30-min incubation with protein G-agarose. Immune complexes were washed three times with TBS containing (in mM) 50 Tris·HCl (pH 7.4) and 150 NaCl, resolved by SDS-PAGE, and processed for Western blot analysis as described above.

Analysis of G-actin-to-F-actin ratios. G-actin-to-F-actin ratios in smooth muscle tissue were measured as described previously (43). Briefly, smooth muscle tissue was homogenized in F-actin buffer containing (in mM) 50 PIPES (pH 6.9), 50 NaCl, 5 MgCl$_2$, 5 EGTA, and 1 ATP and centrifuged at 100,000 *g* for 40 min. Pellets were resuspended in cytochalasin-D/dH$_2$O to dissociate F-actin. The supernatant of the resuspended pellets was collected after centrifugation at 2,300 *g* for 2 min. Equal amounts of protein from the first supernatant (G-actin) and second supernatant (F-actin) were subjected to an analysis by immunoblot using actin antibody. The total amounts of G-actin and F-actin from the original soluble and insoluble fractions were calculated based on the total protein in each fraction.

Small artery contraction. Contractile responses were determined from segments of the small artery (~320 μm id) by pressure myography as described previously (36). Vessel segments were incubated with vehicle, 0.2% DMSO, 1 μM SB-203580, 10 μM PP2, or 1 μM SB-203580 plus 10 μM PP2 for 15 min before the recording of cumulative concentration response curves to NE (0.1–15 μM). The lumen diameter was measured 2 min following the addition of the agonist, immediately before the addition of the next concentration. NE concentration response curves in the presence of vehicle and inhibitor were obtained from a single vessel segment.

Data and statistical analysis. For inhibition experiments, the arteries from each animal were divided into four samples: vehicle control, vehicle plus NE, inhibitor control, and inhibitor plus NE, and data were then normalized with respect to the vehicle control with each animal acting as its own control. For the effect of PP2 on PYK2/Hic-5 association, a greater amount of homogenate than could be obtained from tissue from a single animal was required and, therefore, the NE-stimulated data were normalized to their respective vehicle or PP2-treated controls. Data were analyzed by Student's *t*-test or repeated-measures ANOVA with Dunnett correction for multiple comparisons or Bonferroni posttest using GraphPad Prism software. $P < 0.05$ was considered statistically significant with n = number of experiments as indicated.

Materials. Many of the reagents used were obtained from sources described previously (38). Herbimycin A, SB-203580, and PP2 were purchased from Calbiochem, and NE was from Sigma. The antibodies

Table 1. *The effect of p38 MAPK and Src family kinase inhibitors on NE-induced Hsp27 and Hic-5 phosphorylation*

Treatment	Fold Change from Control	
	Hsp27 Phosphorylation	Hic-5 Phosphorylation
NE	1.95±0.29 (*n* = 5)	1.55±0.21 (*n* = 6)
SB-203580 + NE	1.08±0.28* (*n* = 5)	1.63±0.20 (*n* = 6)
NE	3.43±0.32 (*n* = 5)	1.50±0.11 (*n* = 5)
PP2 + NE	3.63±0.71 (*n* = 5)	1.00±0.06* (*n* = 5)

Values are means ± SE; *n*, number of experiments. Hsp27, heat shock protein 27. Small arteries were stimulated with 15 μM norepinephrine (NE) for 10 min in the presence of vehicle (0.01% DMSO), 1 μM SB-203580 (p38 MAPK inhibitor) (36), or 10 μM 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo(3,4-D)pyrimidine (PP2; Src family tyrosine kinase inhibitor) (19), and phosphoSer82Hsp27 phosphorylation or tyrosine phosphorylation of hydrogen peroxide-inducible clone-5 (Hic-5) immunoprecipitates were determined by immunoblot analysis, as described in METHODS. The data are expressed as fold change from control, where control =1 and *P < 0.05 NE compared with inhibitor + NE.
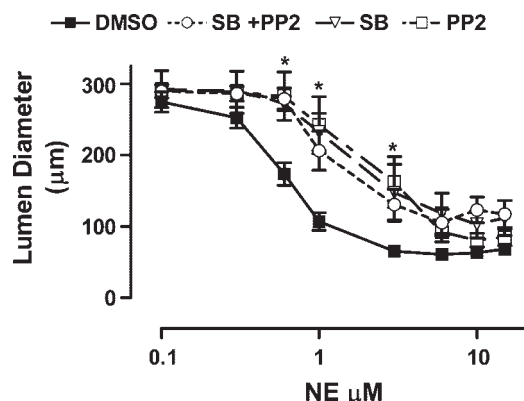
Fig. 1. Effect of nonreceptor tyrosine kinase and p38 MAPK inhibition on norepinephrine (NE)-induced contraction. Segments of small artery were mounted in a pressure myograph, and lumen diameter was monitored continuously as described in METHODS. Vessels were treated with 0.01% DMSO ($n = 15$ experiments), 10 μM 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-D]pyrimidine (PP2; $n = 6$ experiments), 1 μM SB-203580 (SB; $n = 8$ experiments), or 10 μM PP2 + 1 μM SB ($n = 9$ experiments) for 15 min before construction of a cumulative concentration response curve to NE. The data are means ± SE. *$P < 0.05$ by ANOVA and Bonferroni posttest.

used were monoclonal anti-phospho-Hsp27(Ser82) (13), anti-Hsp27 polyclonal (Upstate, NY), anti-PYK2 monoclonal and anti-Hic-5 monoclonal (BD Transduction), anti-actin monoclonal (Sigma), and anti-phosphotyrosine (P-Tyr-100; Cell Signaling Technology). HRP-conjugated secondary antibodies were from Jackson Laboratories. The chemiluminescence reagent was from Pierce.

RESULTS

p38 MAPK and nonreceptor tyrosine kinases modulate NE-induced contraction through a common mechanism. We have previously shown that vasoconstrictor hormones activate p38 MAPK and nonreceptor tyrosine kinases such as Src family kinases in RMSAs and that the inhibition of these pathways reduces contraction (36, 37). However, to determine whether these pathways act separately or in concert to modulate contractility, we investigated the contractile response of RMSAs to NE in the presence of p38 MAPK or Src family kinase inhibition either singly or in combination. To confirm the selectivity of the inhibitors, the effect of SB-203580, p38 MAPK inhibitor (12, 36) on Hic-5 tyrosine phosphorylation and PP2, a Src family tyrosine kinase inhibitor (3, 19) on Hsp27 phosphorylation was determined (Table 1). SB-203580 (1 μM) had no effect on NE-induced tyrosine phosphorylation of Hic-5, indicating that at this concentration it had no nonspecific effect on the activation of cytosolic tyrosine kinases.
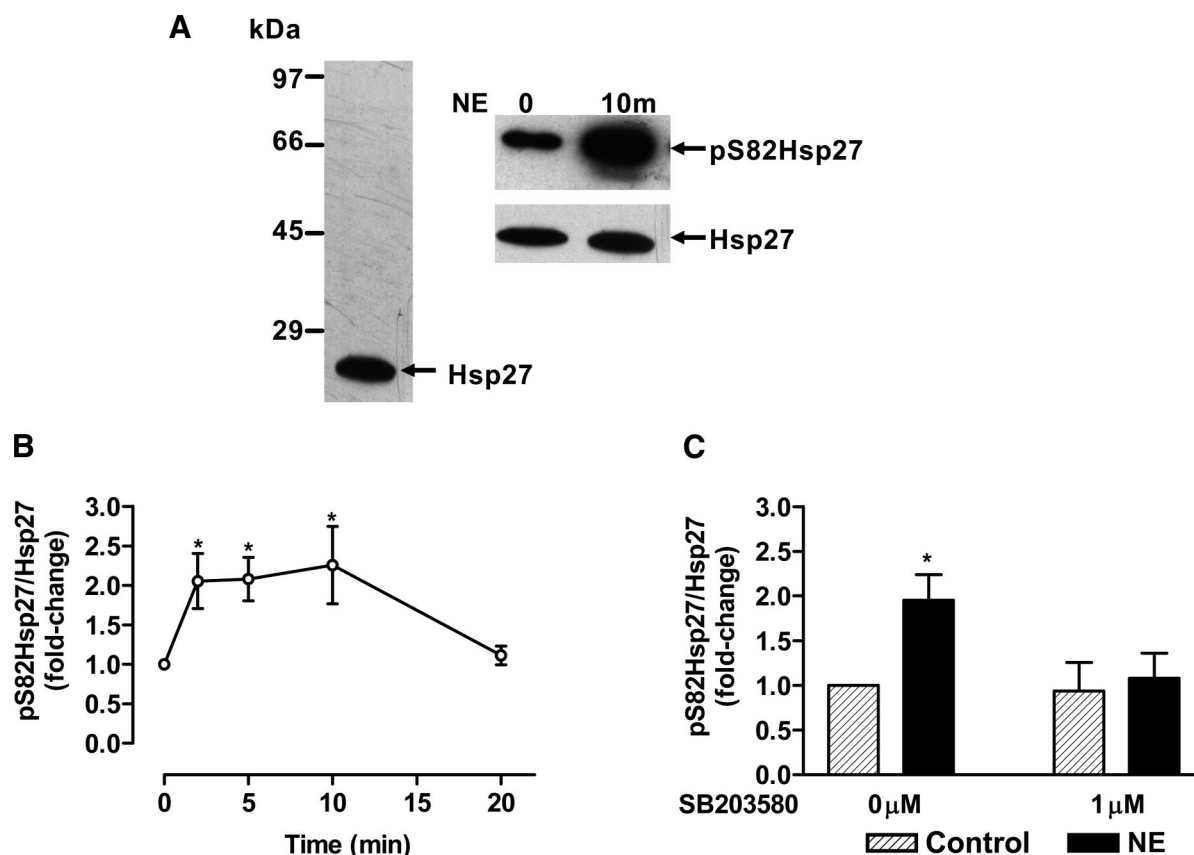

Fig. 2. Immunodetection of heat shock protein 27 (Hsp27) in rat mesenteric small arteries and p38 MAPK-dependent activation by NE. *A*: small artery homogenates were analyzed with antibodies against Hsp27 or phosphoSer82Hsp27 (pS82Hsp27) as described in METHODS. Immunoblots show the presence of Hsp27 and an increase in pS82Hsp27 following 15 μM NE for 10 min. Small arteries were stimulated with 15 μM NE for various times up to 20 min (*B*) or stimulated with 15 μM NE for 10 min in the presence of vehicle (0.01% DMSO) or 1 μM SB (*C*) and pS82Hsp27 phosphorylation measured as detailed in METHODS. Results are means ± SE for densitometric data of the phosphoantigen signal corrected for total antigen expressed as fold change from control (Cont), where Cont = 1 (*B*, $n = 5$ experiments and *$P < 0.05$ compared with 0 s; and *C*, $n = 5$ experiments and *$P < 0.05$ compared with Cont).

Conversely, PP2 at 10 μM had no effect on NE-induced Hsp27 phosphorylation, an effect downstream of p38 MAPK in RMSAs (36), but did completely inhibit Hic-5 tyrosine phosphorylation. In the presence of 1 μM SB-203580, there was a significant rightward shift of the concentration response curve (Fig. 1), confirming our previous observation that p38 MAPK activity is involved in NE-induced contraction (36). Treatment with 10 μM PP2 also induced a significant rightward shift in the concentration response curve (Fig. 1), implicating Src tyrosine kinase activity in the response. When the inhibitors were combined, there was no further shift in the concentration response curve (Fig. 1), suggesting that although both p38 MAPK and Src tyrosine kinase pathways modulate NE-induced contraction, they most likely do so through a common mechanism.

Hsp27 is present in RMSAs and activated by NE. We next investigated the downstream signaling molecules that might be involved. Recently, we have shown that p38 MAPK activation in response to NE leads to the stimulation of a Hsp kinase activity (36). Accordingly, we investigated whether Hsp27 was activated by NE. Immunochemical analysis showed the presence of Hsp27 in small artery extracts (Fig. 2A). Using an antibody that recognizes activated Hsp27 when phosphorylated on Ser82 (27), we detected the rapid activation of Hsp27 (Fig. 2A) within 1 to 2 min that returned toward basal by 20 min (Fig. 2B). This NE-induced activation of Hsp27 precisely resembled the increased Hsp kinase activity induced by NE in RMSAs that we described previously (36). The NE-induced phosphorylation of Hsp27 was completely blocked by SB-203580 (Fig. 2C) but not by PP2 (Table 1).

NE causes Hsp27 redistribution. The phosphorylation of Hsp27 is associated with a decrease in its actin-capping ability and dissociation to dimers and monomers (45). In addition, Hsp27 binds actin in a phosphorylation-dependent manner in smooth muscle cells (5). To investigate whether Hsp27 was associated with the actin cytoskeleton in small arteries, we examined its subcellular distribution in the presence and absence of NE stimulation. In nonstimulated tissues, ∼50% of Hsp27 was present in the cytosol and the remaining 50% was divided evenly between Triton X-100 soluble and -insoluble (cytoskeleton) fractions (Fig. 3A). Phosphorylated Hsp27 was similarly distributed (Fig. 3B). After 10 min of NE stimulation, the peak time point of Hsp27 activation (Fig. 2B), Hsp27 moved from both membrane fractions to the cytosol (Fig. 3A). Phosphorylated Hsp27 also increased in the cytosol but only decreased in the Triton X-100 soluble membrane fraction. Therefore, the pool of Hsp27 in the Triton X-100 insoluble (cytoskeleton) fraction was more highly phosphorylated (∼35% increase) during NE stimulation.

NE-stimulated actin reorganization is dependent on p38 MAPK/Hsp27 pathway. In RMSAs, p38 MAPK inhibition (Fig. 1B) (36) or cytoskeleton disruption (38) reduces NE-induced contraction. Given that Hsp27 is an actin-capping protein and its phosphorylation alleviates its inhibitory action on actin polymerization (45), we investigated whether NE-induced actin filament formation required p38 MAPK/Hsp27 activation. NE stimulation favored F-actin formation and reduced G-actin-to-F-actin ratios (Fig. 4, A and B), and the inhibition of p38 MAPK attenuated NE-induced F-actin formation without any effect on basal levels (Fig. 4B). To investigate whether the changes in F-actin levels reflect changes in
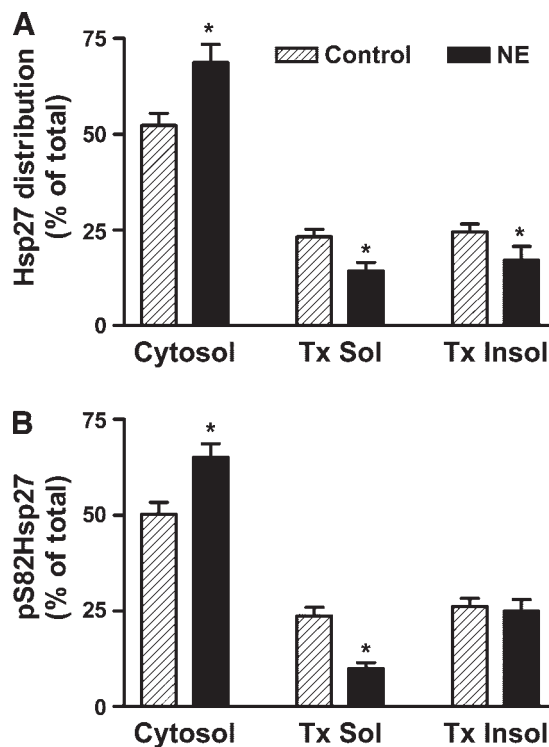

Fig. 3. NE induces redistribution of Hsp27 and pS82Hsp27. Arteries were treated with vehicle (dH$_2$O, Cont) and NE (15 μM) for 10 min. Cytosol, Triton X-100 insoluble (Tx Insol) membrane (cytoskeleton), and Triton X-100 soluble (Tx Sol) membrane fractions were prepared and immunoblotted with antibodies against Hsp27 (A) and pS82Hsp27 (B) as described in METHODS. Densitometric analysis of distributions is shown. Results are expressed as percentages of total antigen and represent means ± SE (A, n = 6 experiments; and B, n = 9 experiments and *P < 0.05 compared with Cont).

Hsp27 F-actin interaction, we determined levels of Hsp27 in G- and F-actin fractions by immunoanalysis. In nonstimulated vessels, Hsp27 was present in both G- and F-actin fractions, with ∼25% associating with F-actin (Fig. 4, A and C). This was in good agreement with its subcellular distribution where, again, ∼25% was found in the cytoskeletal, i.e., F-actin fraction (Fig. 3A). The stimulation of the RMSA with NE caused dissociation of Hsp27 from the F-actin fraction with an equivalent increase in the G-actin fraction. Again, this is in good agreement with the shift of Hsp27 from the Triton X-100-insoluble membrane fraction (cytoskeleton) to the cytosol observed in Fig. 3A. This redistribution was blocked by the inhibition of p38 MAPK (Fig. 4C), suggesting that dissociation of Hsp27 from F-actin is necessary for NE-induced F-actin formation and contraction.

Identification of Hic-5 and its activation by NE. Having shown that NE activates Hsp27, causing it to dissociate from F-actin and move to a cytosolic compartment, we investigated whether Hic-5, a substrate for cytosolic tyrosine kinases and known binding partner of Hsp27 (24), was also present and activated within a similar time frame. Hic-5 antibody detected a single band at ∼55 kDa (Fig. 5A) in small artery extracts, demonstrating the abundant expression in these tissues. Furthermore, probing of Hic-5 immunoprecipitates with antiphosphotyrosine antibodies showed an increase in the phosphorylation following NE stimulation that was inhibited by Src tyrosine kinase inhibitors Herbimycin A and PP2 (Fig. 5B). NE
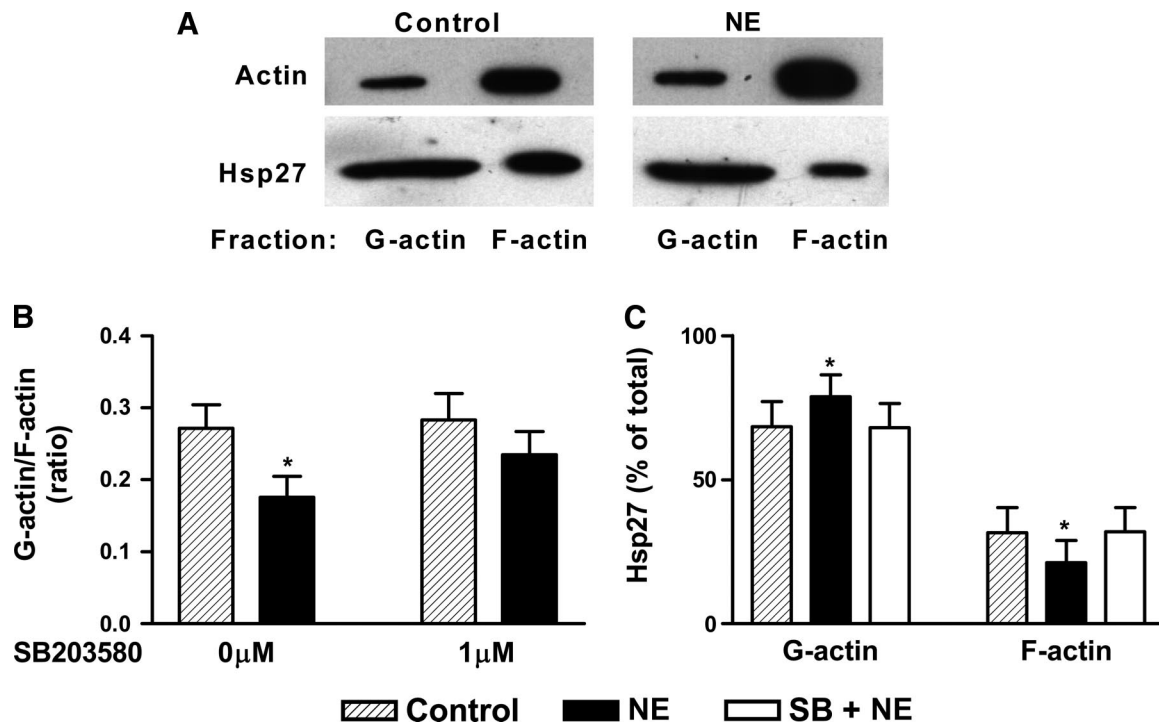
Fig. 4. NE induces p38 MAPK-dependent F-actin formation and dissociation of Hsp27. Small arteries were stimulated with 15 μM NE for 10 min in the presence of vehicle (0.01% DMSO) or 1 μM SB; G- and F-actin fractions were prepared as described in METHODS. *A*: immunoblot showing the distribution of actin and Hsp27 in the G- and F-actin fractions before and after NE treatment. *B*: densitometric analysis shown as G-actin-to-F-actin ratios ($n = 7$ experiments, $*P < 0.05$ compared with Cont). *C*: distribution of Hsp27 in G- and F-actin fractions. Densitometric analysis of distributions is shown. Results are expressed as percentage of total antigen and represent means ± SE ($n = 4$ experiments, $*P < 0.05$ compared with Cont).

activates Src and PYK2 in RMSAs (36, 38); therefore, to further investigate the kinase responsible for Hic-5 tyrosine phosphorylation, we probed Hic-5 immunoprecipitates for c-Src and PYK2. In nonstimulated arteries, PYK2 (Fig. 5*C*) but not c-Src (data not shown) was associated with Hic-5, and this association increased following NE stimulation (Fig. 5*C*). In addition, following treatment with PP2, NE no longer induced an increased association between PYK2 and Hic-5 (Fig. 5*C*). In contrast, the PYK2 homologue p125FAK did not associate with Hic-5 either under basal or stimulated conditions (data not shown). Inhibition of p38 MAPK had no effect on NE-induced Hic-5 activation (Table 1). This strongly suggests that NE induces Hic-5 tyrosine phosphorylation through PYK2, in agreement with studies in cells (23), and that Src kinases are upstream activators of this pathway. Additionally, the stimulation of RMSAs by NE causes Hic-5 to move from the membrane fraction to the cytosol (Fig. 5*D*).

NE induces Hsp27 and Hic-5 association. Our data show that NE stimulation activates Hsp27 and Hic-5 by parallel independent pathways of p38 MAPK and Src tyrosine kinases, respectively, resulting in both translocating to a cytosolic compartment. We used coimmunoprecipitations to determine any interactions between the two following stimulation. Hic-5 was present in Hsp27 immunoprecipitates from nonstimulated arteries, and its levels increased following NE stimulation (Fig. 6, *A* and *B*). We were unable to detect Hsp27 in Hic-5 immunoprecipitates due to interference from the IgG light chains (data not shown). However, phosphoSer82Hsp27 was detected (Fig. 6*A*), confirming an interaction between these proteins. Furthermore, the levels of phosphoSer82Hsp27 co-

immunoprecipitating with Hic-5 increased following NE stimulation (Fig. 6*A*), suggesting phosphorylation was important for the interaction. To further investigate these interactions, we inhibited p38 MAPK (SB-203580) and Src kinases (PP2) to inhibit NE-induced Hsp27 and Hic-5 phosphorylation, respectively. In both instances, the inhibition blocked NE-induced Hsp27/Hic-5 association (Fig. 6, *A* and *B*).

DISCUSSION

The regulation of vascular smooth muscle contractility is central to maintaining blood flow and peripheral vascular resistance, and vasoconstrictor-induced reorganization of the actin cytoskeleton is an important component of the contractile response (17). Previous studies in smooth muscle cells and tissues have implicated nonreceptor tyrosine kinases (17) or small heat shock proteins, Hsp20 and Hsp27 (32, 53), although whether these pathways act independently or together has not been addressed. Here we present the first evidence that the activation of Hsp27 and Hic-5 and their subsequent phosphorylation-dependent interaction is important for NE-induced actin filament formation and contraction (see Fig. 7 for a diagram of the proposed pathway).

Hsp27 is a molecular chaperone whose phosphorylation state determines its localization and function (2, 42, 45). In smooth muscle, GPCR agonists induce Hsp27 phosphorylation (1, 17, 21) by multiple kinases including MAPKAPK2/3, PKC, and protein kinase D (14). MAPKAPK2/3 is downstream of p38 MAPK and has been shown to have Hsp kinase activity. The activation of p38 MAPK is complex and dependent on
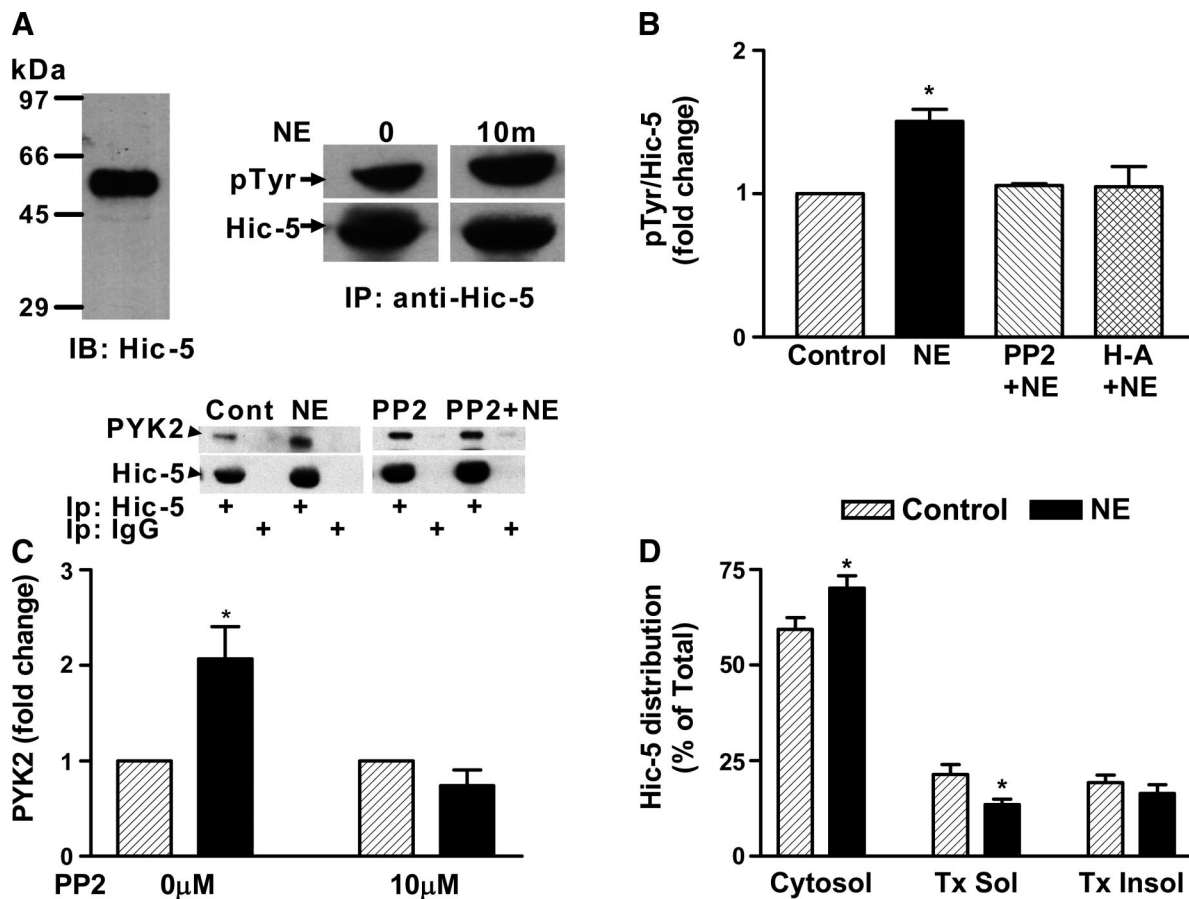
Fig. 5. Hydrogen peroxide-inducible clone-5 (Hic-5) is expressed and activated by NE in small arteries. *A*: small artery homogenates were analyzed with antibodies against Hic-5 or Hic-5 immunoprecipitates (IPs) and were probed with antiphosphotyrosine (pTyr) antibody. Membranes were stripped and probed with anti-Hic-5, confirming equivalent pulldown, as described in METHODS. Immunoblots show the presence of Hic-5 and an increase in pTyr following 15 μM NE for 10 min. *B*: small arteries were stimulated with 15 μM NE for 10 min in the presence of vehicle (0.01% DMSO), 10 μM PP2, or 1 μM herbimycin-A (H-A). Shown are densitometric data of the pTyr signal corrected for Hic-5 in the IPs expressed as fold change from Cont, where Cont = 1 (PP2, $n = 5$ experiments; and H-A, $n = 4$ experiments and *$P < 0.05$ compared with Cont). *C*: small arteries were stimulated with 15 μM NE for 10 min in the presence of vehicle (0.01% DMSO) or 10 μM PP2. Hic-5 or IgG control IPs were prepared and probed for proline-rich tyrosine kinase 2 (PYK2) and Hic-5 as detailed in METHODS. *C, top*: representative immunoblots. *C, bottom*: results are means ± SE for densitometric data of PYK2 expressed as fold change from Cont, where Cont = 1 (vehicle, $n = 3$; and PP2, $n = 4$ and *$P < 0.05$ compared with Cont). *D*: arteries were treated with vehicle (dH$_2$O, control) or 15 μM NE for 10 min. Cytosol, Tx Insol membrane (cytoskeleton), and Tx Sol membrane fractions were prepared and immunoblotted with antibodies against Hic-5 as described in METHODS. Densitometric analysis of distributions is shown. Results are expressed as percentage of total antigen and represent means ± SE (vehicle, $n = 7$ experiments; and NE, $n = 6$ experiments and *$P < 0.05$ compared with Cont). IB, immunoblot.

both cell type and the agonist used. In smooth muscle cells, it has a requirement for PKC to thrombin and bombesin (22, 55) but not to ANG II (34), whereas Src family kinases are also implicated to endothelin and not NE (36). Here we show that NE induces Hsp27 phosphorylation in RMSAs within the same time frame as Hsp27 kinase activity (36) that is dependent on p38 MAPK but independent of Src family kinases.

In many smooth muscles, p38 MAPK activation is linked to contraction (10, 29, 34, 36, 56), and in vascular tissues and gastrointestinal smooth muscle cells, Hsp27 phosphorylation is important for sustained contraction (32, 40). Although in rat aorta smooth muscle cells, the inhibition of p38 MAPK and Hsp27 showed a significant reduction in contraction to ANG II but not NE (34). In colonic smooth muscle cells, Hsp27 binds to and modulates the interactions of myosin, tropomyosin, and caldesmon (5, 47). However, Hsp27 also binds to actin filaments, and the remodeling of the latter may be associated with smooth muscle contractility (4, 11, 33). Reorganization of the

actin cytoskeleton is known to occur during smooth muscle contraction (17), and we have previously shown that NE activates p38 MAPK/Hsp27 kinase in RMSAs (36) and that the inhibition of this pathway or disruption of the cytoskeleton blocks contraction (38). NE stimulation induced a phosphorylation-dependent dissociation of Hsp27 from actin filaments in RMSAs, shown by a decrease in the cytoskeleton and F-actin fractions and accumulation in the cytosol and G-actin fractions. Concomitant with Hsp27 dissociation, NE induced an increase in F-actin, demonstrating lengthening of actin filaments. During NE stimulation, the pool of Hsp27 remaining in the cytoskeleton was more heavily phosphorylated compared with that in basal. This suggests that reorganization of the actin cytoskeleton during NE-induced contraction is regulated by Hsp27 phosphorylation, a conclusion further supported by our observation that inhibition of p38 MAPK reduced NE-induced F-actin formation and contraction. These data support a model where, in the absence of stimulation, Hsp27 acts as a capping
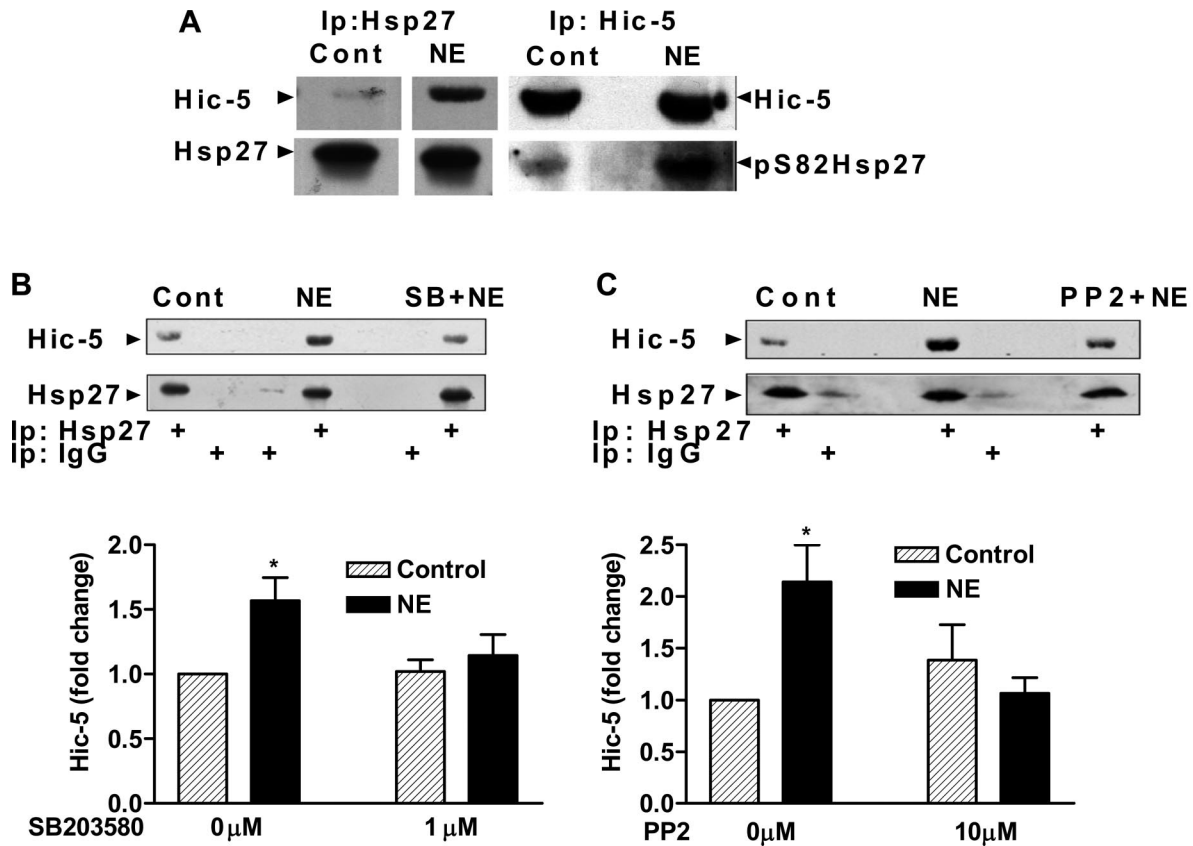
Fig. 6. NE stimulates Hsp27 and Hic-5 interaction and dependence on p38 MAPK and nonreceptor tyrosine kinase activity. Small arteries were stimulated with 15 μM NE for 10 min. Hsp27 or Hic-5 IPs were prepared and probed for Hic-5 and pS82Hsp27, respectively, or Hsp27 and Hic-5 to confirm equivalent pulldown as described in METHODS. A: immunoblots showing the presence of Hic-5 in Hsp27 IPs and pS82Hsp27 in Hic-5 IPs. B and C: small arteries were stimulated with 15 μM NE for 10 min in the presence of vehicle (0.01% DMSO), 1 μM SB (B), or 10 μM PP2 (C). Hsp27 or IgG control IPs were prepared and probed for Hic-5 and Hsp27 to confirm equivalent pulldown as described in METHODS. Top: representative immunoblots. Bottom: results are means ± SE for densitometric data of Hic-5 expressed as fold change from Cont, where Cont = 1 (SB, $n = 5$ experiments; and PP2, $n = 4$ experiments and *$P < 0.05$ compared with Cont).

protein binding to barbed ends and agonist stimulation induces phosphorylation and displacement of Hsp27, allowing F-actin polymerization. This is consistent with reports in smooth muscle cells showing that during PDGF-induced cell spreading, nonphosphorylated Hsp27 acts as an actin-capping protein at the leading edge of the cell, whereas phosphorylated Hsp27 accumulates at the base of lamellipodia, where stable actin filaments are located (2, 42).

However, in addition to p38 MAPK/Hsp27, NE also activates the nonreceptor tyrosine kinases Src and PYK2, induces paxillin tyrosine phosphorylation, and causes translocation of PYK2 and paxillin from cytosol to the cytoskeleton (38, 54), indicating that actin cytoskeletal dynamics may be additionally regulated through this pathway. During the current study, we identified in small arteries a high expression of Hic-5, a homologue of paxillin (51). Hic-5, in addition to having a similar domain structure as paxillin, also localizes to the same sites and shares binding partners with paxillin (16, 51). However, they must not entirely duplicate each others functions since paxillin knockout is embryonic lethal (18), and unlike paxillin, Hic-5 is not activated by integrin engagement or phosphorylation by p125FAK (16, 51). Furthermore, Hic-5 has unique binding partners such as Hsp27 (24) and opposes some of the effects of paxillin such as growth promotion and cell

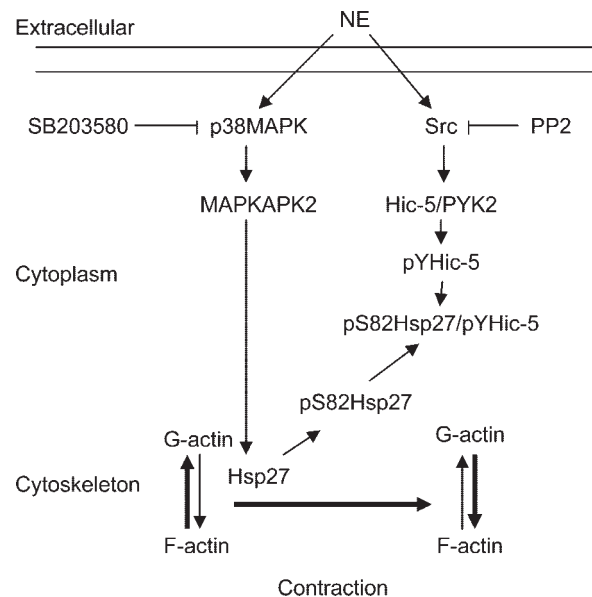

Fig. 7. Proposed schematic of NE signaling through p38 MAPK/Hsp27 and Src/Hic-5 to reorganization of the actin cytoskeleton. NE stimulates p38 MAPK and Src family kinases independently, leading to Hsp27 and Hic-5 phosphorylation, respectively. Upon phosphorylation, Hsp27 moves from the cytoskeleton to the cytosol and interacts with Hic-5, resulting in the formation of F-actin and contraction.

spreading (35). Like paxillin, Hic-5 is also regulated by phosphorylation (8), for instance by osmotic stress in a PYK2-dependent manner (16) or by growth factors (20). Our data demonstrate that in RMSAs, NE stimulation induced tyrosine phosphorylation of Hic-5 and promoted an interaction between Hic-5 and PYK2. Hic-5 phosphorylation and its subsequent association with PYK2 were inhibited by Src kinase inhibition. Under the same conditions, there was no interaction between paxillin and PYK2 or Hic-5 and p125FAK (data not shown), suggesting that NE selectively induces Src-dependent PYK2 phosphorylation of Hic-5. During NE stimulation, Hic-5 moved away from the membrane and accumulated in the cytosol. The inhibition of Src family kinases reduced NE-induced contraction, as did p38 MAPK inhibition. The inhibition of Src family kinases following p38 MAPK inhibition was unable to produce any additional decrease in contractility, suggesting that although these two pathways are activated independently of each other, they regulate smooth muscle contractility through a common target.

Hic-5 and Hsp27 are both scaffolding proteins that are implicated in the assembly of signaling complexes (14, 51). In addition, Hic-5 has been shown to bind Hsp27 (24) in rat glomerular cells, although whether such an interaction occurs in smooth muscle has not been explored. Here we show that in nonstimulated arteries, there was weak interaction between Hsp27 and Hic-5; however, NE induced a strong association between the two, an interaction that required serine phosphorylation of Hsp27 and tyrosine phosphorylation of Hic-5. Paxillin, also phosphorylated within the same time frame by NE, did not interact at all with Hsp27. This suggests that paxillin and Hic-5 have different effects on contractility. We have previously shown that NE activates paxillin, causing it to move from the cytosol to the actin cytoskeleton (38). Here we show that NE activates Hic-5, causing it to move from the actin cytoskeleton to the cytosol. At the same time, Hsp27 also moves from the actin cytoskeleton to the cytosol and associates with Hic-5. This suggests that NE may modulate these two adaptor proteins to convey signals between the cytosol and actin cytoskeleton during contraction, namely tyrosine phosphorylation of Hic-5 and association with Hsp27, removing an inhibitory effect on actin polymerization and contraction and tyrosine phosphorylation of paxillin and its association with the actin cytoskeleton promoting contraction. This is in agreement with a recent study where the expression of Hic-5 in fibroblasts reduced contraction, whereas paxillin expression increased contractility (25).

In summary, NE-induced contraction is associated with the regulation/reorganization of the actin cytoskeleton, as seen by filament extension (increased F-actin formation). Here we describe the dual and independent activation by NE of Hsp27 and Hic-5, leading to their subcellular relocalization and association within the same time frame as the contractile response. These events appear to be important in the regulation of the actin cytoskeleton and contraction in vascular tissues.

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