Thrombin Regulates Vascular Smooth Muscle Cell Growth and Heat Shock Proteins via the JAK-STAT Pathway*

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The growth-stimulating effects of thrombin are mediated primarily via activation of a G protein-coupled receptor, PAR-1. Because PAR-1 has no intrinsic tyrosine kinase activity, yet requires tyrosine phosphorylation events to induce mitogenesis, we investigated the role of the Janus tyrosine kinases (JAKs) in thrombin-mediated signaling. JAK2 was activated rapidly in rat vascular smooth muscle cells (VSMC) treated with thrombin, and signal transducers and activators of transcription (STAT1 and STAT3) were phosphorylated and translocated to the nucleus in a JAK2-dependent manner. AG-490, a JAK2-specific inhibitor, and a dominant negative JAK2 mutant inhibited thrombin-induced ERK2 activity and VSMC proliferation suggesting that JAK2 is upstream of the Ras/Raf/MEK/ERK pathway. To elucidate the functional significance of JAK-STAT activation, we studied the effect of thrombin on heat shock protein (Hsp) expression, based upon the following: 1) reports that thrombin stimulates reactive oxygen species production in VSMC; 2) the putative role of Hsps in modulating cellular responses to reactive oxygen species; and 3) the presence of functional STAT1/3-binding sites in Hsp70 and Hsp90β promoters. Indeed, thrombin up-regulated Hsp70 and Hsp90 protein expression via enhanced binding of STATs to cognate binding sites in the Hsp70 and Hsp90 promoters. Together, these results suggest that JAK-STAT pathway activation is necessary for thrombin-induced VSMC growth and Hsp gene expression.

In addition to regulating hemostasis and thrombosis, the serine protease thrombin also promotes the inflammatory response and wound healing (1) where it is mitogenic for lymphocytes, fibroblasts, vascular endothelial and smooth muscle cells (2–4). Many of the functions of thrombin are mediated via activation of protease-activated receptor(s), PAR-1, PAR-3, or PAR-4 (5–7). Thrombin cleaves the N terminus of its PAR-1 receptor between Arg^{41} and Ser^{42} to create a new N terminus $^{42}SFLLRN^{47}$ that acts as a tethered ligand and activates the receptor (5). Increased Ser/Thr kinase activity in response to the stimulation of PAR-1 and other G protein-coupled receptors is well demonstrated (8–10). Thrombin also stimulates the expression of nuclear proteins that constitute the transcription factor AP-1, which

participates in transactivation of several early growth response genes implicated in VSMC[1] proliferation (9, 11).

PAR-1, angiotensin II (Ang II) receptor, and other G protein-coupled receptors, which do not themselves possess intrinsic tyrosine kinase activity, require tyrosine kinase activity to induce mitogenesis (8, 12–14). These observations suggest that G protein-coupled receptors may utilize cytoplasmic protein tyrosine kinases such as Janus kinases (JAKs) and Src kinases to initiate mitogenesis. In fact, both JAK and Src kinases play important roles in VSMC proliferation induced by Ang II (10, 15).

JAKs are 1 of 11 mammalian nonreceptor tyrosine kinase families that were initially identified as essential mediators of cellular signaling induced by the interaction of cytokines with their cognate receptors (16). There are four members of the JAK family, JAK1, JAK2, JAK3, and TYK2. Targeted gene disruption studies in mice demonstrate that JAKs are essential for cytokine-induced signaling (17, 18). In interactions of cytokines with their cognate receptors, receptor dimerization induced by ligand binding to cell surface receptors leads to the activation of one or more of the JAK family of kinases associated with the transmembrane receptor. This, in turn, leads to phosphorylation of tyrosine residues in the receptor cytoplasmic domains, which provide docking sites for signal transducers and activators of transcription (STATs) and other proteins that contain phosphotyrosine-binding motifs (19). STATs, upon phosphorylation by JAKs on tyrosine residues, undergo homo- or heterodimerization with other STAT family members and migrate to the nucleus. Within the nucleus, STAT dimers bind to target genes to enhance transcription (20, 21). In addition to tyrosine phosphorylation, STAT proteins undergo serine phosphorylation in a mitogen-activated protein kinase (MAP kinase)-dependent manner. In fact, both serine and tyrosine phosphorylation of STAT proteins is necessary for maximal activation of transcription (22, 23). Activation of STAT proteins has been reported in cells treated with various cytokines, growth factors, insulin, and Ang II (24–26).

Heat shock proteins (Hsps), initially identified by their enhanced synthesis in cells exposed to elevated temperatures, have been subsequently shown to accumulate in response to various stresses including cardiac hypertrophy, ischemic preconditioning, oxidative stress, and aging (27). Expressed constitutively, Hsps function as molecular chaperones under physiologic conditions. During stress, Hsps prevent protein aggregation, either through refolding of denatured proteins or

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[1] The abbreviations used are: VSMC, vascular smooth muscle cells; JAK, Janus kinases; Hsp, heat shock protein; ROS, reactive oxygen species; STATs, signal transducers and activators of transcription; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP kinase/ERK kinase; DMEM, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyltransferase; Ang II, angiotensin II; PMA, phorbol 12-myristate 13-acetate; PDGF-BB, platelet-derived growth factor-BB.

by promoting their degradation through a proteolytic pathway (28). Induction of Hsps on exposure to a stressor confers protection against exposure to a subsequent stressor in various cell types (29, 30). Overexpression of individual Hsps also protects against thermal and ischemic stress and apoptosis (31, 32). Hsps may also regulate stress-responsive signaling pathways such as activation of c-Jun N-terminal kinase1 (JNK1) and p38 (33). Induction of several Hsps has been reported in VSMC (34) and may contribute to VSMC proliferation leading to the onset of vascular diseases such as atherosclerosis.

Because thrombin-activated PAR-1 requires tyrosine phosphorylation events to induce mitogenesis, we investigated the effect of thrombin on the activation of members of the JAK family and their substrates, STATs. We show that thrombin causes activation of JAK2 and tyrosine phosphorylation and nuclear translocation of STAT1, -2, and -3. We also demonstrate that inhibition of JAK2 activity attenuates thrombin-induced ERK2 activity and VSMC proliferation. Furthermore, our results show that thrombin induces Hsp70 and Hsp90 expression in VSMC via activation of the JAK-STAT pathway. Thus, the JAK-STAT pathway may be an important physiologic mediator of thrombin-induced events in VSMC.
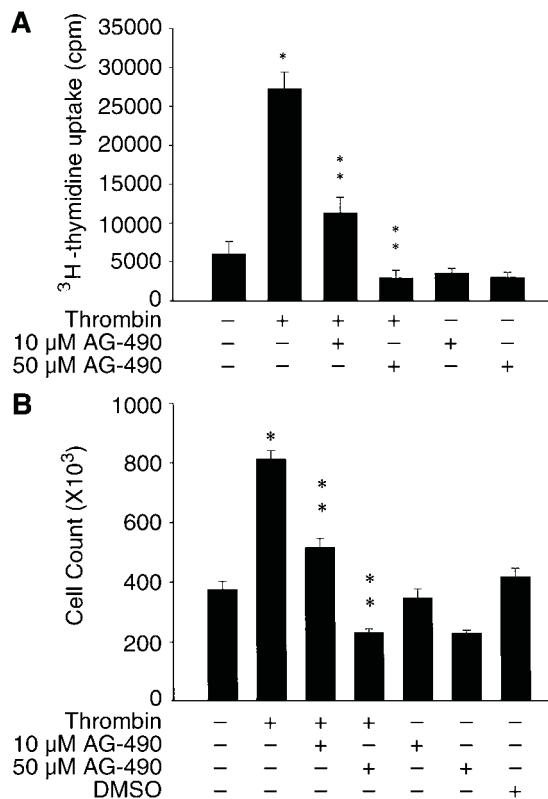
EXPERIMENTAL PROCEDURES

Materials—Thrombin and AG-490 were purchased from Calbiochem. H9C2 cells were obtained from American Type Culture Collection. Antibodies used are as follows: anti-JAK1, JAK2, TYK2, STAT1, STAT2, STAT3, FLAG, and anti-phosphotyrosine (4G10) (Upstate Biotechnology, Inc., Lake Placid, NY, and Santa Cruz Biotechnology, San Diego, CA); anti-phosphospecific JAK2 (BIOSOURCE International, Camarillo, CA); anti-phosphospecific and -nonphosphospecific ERK1/2 (New England Biolabs, Beverly, MA); anti-Hsp70 (Affinity BioReagents, Golden, CO) and anti-Hsp90 (StressGen, Victoria, Canada). [^{14}C]Chloramphenicol (55 mCi/mmol), [*methyl*-^{3}H]thymidine (70 Ci/mmol), and [γ-^{32}P]ATP (3,000 Ci/mmol) were obtained from PerkinElmer Life Sciences. Diphenyleneiodonium, *N*-acetyl-L-cysteine, and pyrrolidine dithiocarbamate were obtained from Sigma.

Cell Culture—VSMC were isolated from the thoracic aortas of 200–250-g male Harlan Sprague-Dawley rats (8). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin at 37 °C in a humidified 95% air, 5% CO_2 atmosphere. All experiments were conducted using VSMC between passage numbers 7 and 20 that were growth-arrested by incubation in DMEM containing 0.1% calf serum for 72 h.

[^{3}H]Thymidine Incorporation—VSMC, grown to ~70% confluence in 60-mm dishes, were quiesced by incubating in DMEM containing 0.1% calf serum for 72 h. Quiesced VSMC were exposed to 1.0 unit/ml thrombin for 24 h after pretreatment with a JAK2-specific inhibitor, AG-490, for 16 h. Cells were labeled with [*methyl*-^{3}H]thymidine for 4 h, and its incorporation into DNA was measured as described previously (9).

Cell Proliferation Assay—VSMC were plated in 60-mm dishes at an initial concentration of 3,500 cells/cm^2 and grown in DMEM supplemented with 10% fetal bovine serum for 48 h. Cells were growth-arrested by incubating in DMEM containing 0.1% calf serum for 48 h and then either left untreated or exposed to AG-490 for 16 h before treatment with 1.0 unit/ml thrombin for 48 h. The cells were washed with phosphate-buffered saline, trypsinized, and diluted with isotonic solution, and the increase in cell number was directly measured with a Coulter counter (model ZM, Coulter Corp., Hialeah, FL).

Immunoprecipitation, ERK2 Activity Assay, and Western Blotting—Growth-arrested VSMC were treated with 1.0 unit/ml thrombin in the presence and absence of AG-490 for the specified times at 37 °C. The cells were lysed in a buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM dithiothreitol, 50 mM β-glycerophosphate, 1% Triton X-100, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM sodium orthovana-

FIG. 1. **Thrombin-induced VSMC DNA synthesis and proliferation is inhibited by AG-490.** *A,* growth-arrested VSMC were pretreated with the JAK2-specific inhibitor, AG-490, for 16 h prior to exposure to 1.0 unit/ml thrombin for 24 h. DNA synthesis is expressed as [^{3}H]thymidine incorporation in cpm/dish. Data represent the means ± S.D. of two experiments, each with four replicates. *B,* growth-arrested VSMC were either left untreated or exposed to AG-490 for 16 h before treatment with 1.0 unit/ml thrombin for 48 h. Increase in cell number was directly measured with a Coulter counter. Data represent the means ± S.D. of two experiments, each with four replicates. The *asterisk* represents significant differences compared with control, and the *double asterisks* represent significant differences compared with thrombin treatment ($p < 0.05$). *DMSO,* dimethyl sulfoxide.

FIG. 2. **Thrombin activates JAK family kinases in rat VSMC.** VSMC were growth-arrested for 72 h and treated with 1.0 unit/ml thrombin for the indicated times. Cells were harvested in kinase lysis buffer, and lysates containing an equal amount of protein were immunoprecipitated (*IP*) with anti-phosphotyrosine antibody (4G10). Western blot (*WB*) analysis was performed with anti-JAK1, anti-JAK2, or anti-TYK2 antibody. The results presented are representative of an experiment that was repeated at least three times.

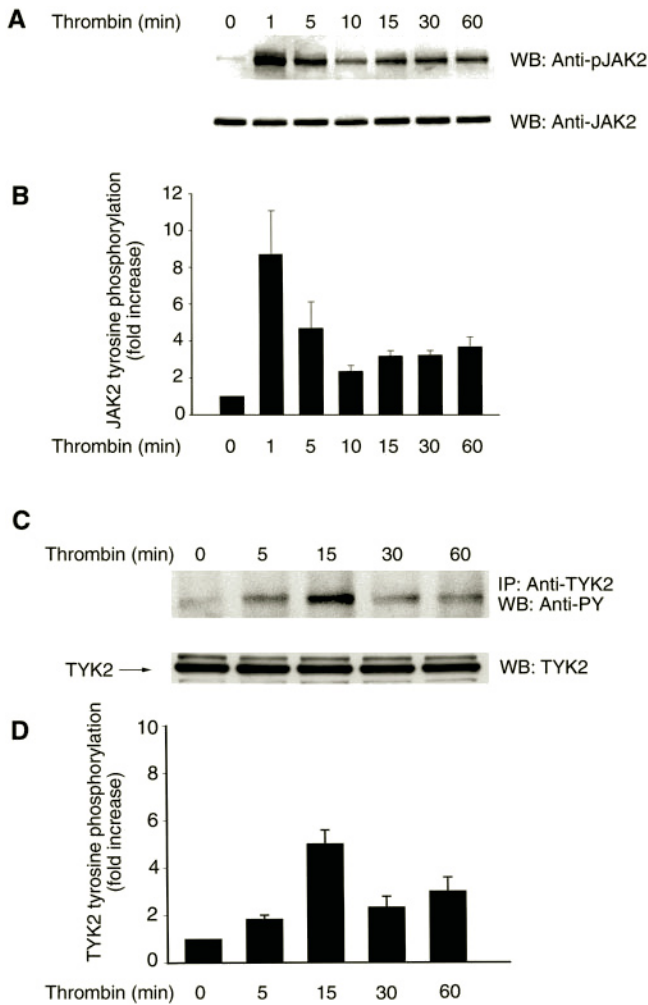FIG. 3. **Rapid tyrosine phosphorylation of JAK2 and delayed activation of TYK2 in VSMC treated with thrombin.** Growth-arrested VSMC were treated with 1.0 unit/ml thrombin for the indicated times and harvested in lysis buffer. *A,* cell lysates containing equal amounts of protein were analyzed by Western blotting (*WB*) with anti-phosphotyrosine-specific JAK2 antibody (*top*). Thrombin had no effect on JAK2 protein levels as observed in Western blot analysis with anti-JAK2 antibody (*bottom*). *B,* densitometric analysis of JAK2 tyrosine phosphorylation (mean ± S.D., $n = 3$). *C,* cell lysates containing equal amounts of protein were immunoprecipitated (*IP*) with anti-TYK2 antibody, and Western analysis was performed with anti-phosphotyrosine antibody (4G10) (*top*) or anti-TYK2 antibody (*bottom*). No difference in TYK2 protein levels was observed in the Western blot probed with anti-TYK2 antibody. *D,* densitometric analysis of TYK2 tyrosine phosphorylation (mean ± S.D., $n = 3$).

date, and 400 μM phenylmethylsulfonyl fluoride (9). For immunoprecipitation, cell lysates containing equal amounts of proteins were incubated with appropriate antibodies overnight at 4 °C. The antibody-protein complexes were incubated with protein A-Sepharose CL-4B/protein A/G plus agarose beads for 2 h at 4 °C, and antibody-protein complexes bound to the beads were pelleted at 2,000 × g for 2 min. The beads were washed three times with lysis buffer and once with phosphate-buffered saline and resuspended in Laemmli sample buffer. The samples were resolved on 7.5% SDS-polyacrylamide gels. ERK2 activity assay and Western blotting were performed as described previously (9).

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from growth-arrested VSMC that were either treated or untreated with thrombin (35). DNA binding was performed by incubating 5 μg of nuclear protein in a total volume of 20 μl of reaction mixture containing 10 mM HEPES, pH 7.9, 50 mM KCl, 4% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1 μg of poly(dI-dC), and 100,000 cpm of ^{32}P-labeled double-stranded Hsp70 ($^{-122}$GATCCGGCGAAACCCCTGGAATATTCCCCGA-CCT^{-90}) or Hsp90 ($^{-643}$GCCTGGAAACTGCTGGAAAT^{-623}) oligonucleotide for 20 min at room temperature. Canonical double-stranded oligonucleotides for SP1 (5′-ATTCGATCGGGGCGGGGCGAGC-3′) and
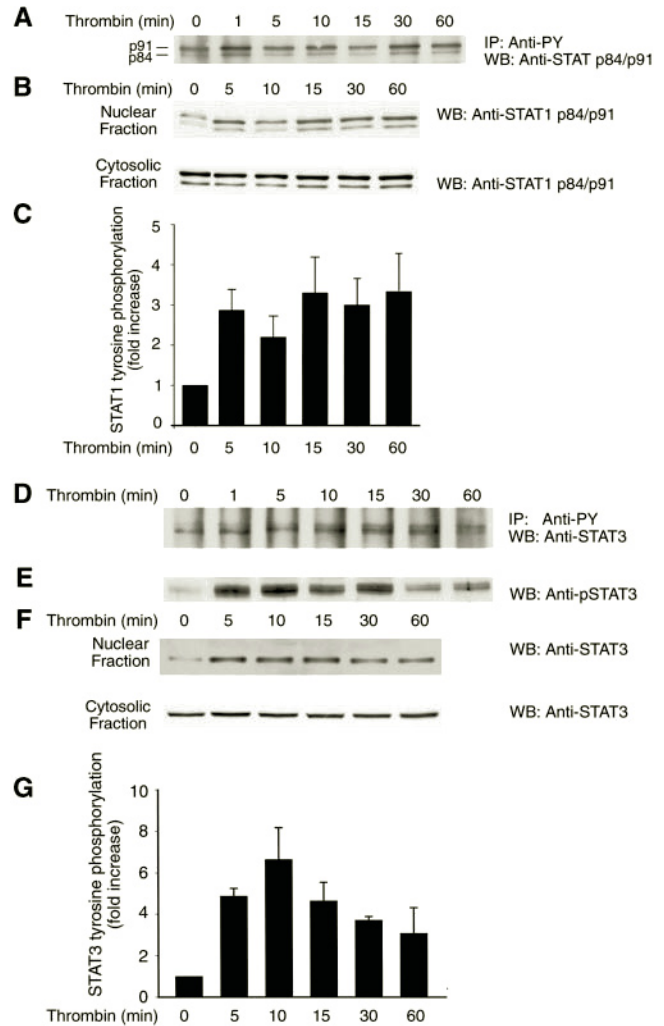

FIG. 4. **Thrombin induces tyrosine phosphorylation and nuclear translocation of STAT1 and STAT3.** Growth-arrested VSMC were treated with 1.0 unit/ml thrombin for the indicated times, and cell lysates were prepared. *A,* cell lysates with equal amounts of protein were immunoprecipitated (*IP*) with anti-phosphotyrosine antibody, and Western blot (*WB*) analysis was performed with anti-STAT1 antibody. *B,* nuclear (*top*) or cytosolic (*bottom*) fractions prepared from thrombin-treated VSMC, containing equal amounts of protein, were analyzed by Western blotting with anti-STAT1 antibody. *C,* densitometric analysis of nuclear translocated STAT1 p84/p91 protein levels (mean ± S.D., $n = 3$). *D,* lysates containing equal amounts of protein were immunoprecipitated with anti-phosphotyrosine antibody and analyzed by Western blotting with anti-STAT3 antibody. *E,* lysates containing equal amounts of protein were analyzed by Western blotting with anti-phosphotyrosine-specific STAT3 antibody. *F,* nuclear (*top*) or cytosolic (*bottom*) fractions from thrombin-treated VSMC containing equal amounts of protein were analyzed by Western blotting with anti-STAT3 antibody. *G,* densitometric analysis of nuclear translocated STAT3 protein levels (mean ± S.D., $n = 3$).

a high affinity double-stranded STAT1-binding sequence SIEm67 (5′-GATCTGATTACGGGAAATG-3′) (36) were used in competition studies. For identifying bands containing specific STAT proteins, the samples were incubated with STAT1 or STAT3 antibody for 30 min before the DNA-binding reaction was performed. Protein-DNA complexes were resolved on a 4% polyacrylamide gel, and the dried gel was exposed to X-Omat AR x-ray film with intensifying screen at −70 °C.

Transient Transfection of H9C2 Cells—Dominant negative JAK2 (ΔJAK2) that lacks the C-terminal kinase domain was kindly provided by Dr. S. Watanabe (University of Tokyo) (37). H9C2 cells were grown to 60–70% confluence in DMEM containing 10% fetal bovine serum and were transfected with either 10 μg of control vector or ΔJAK2 and FLAG-ERK2 (kindly provided by Dr. M. J. Weber, University of Virginia, Charlottesville, VA) plasmid DNA. Transient transfection was done using FuGENE (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cells were quiesced with DMEM contain-

FIG. 5. **AG-490 causes partial inhibition of thrombin-induced ERK1/2 activation.** Growth-arrested VSMC were pretreated with AG-490 for 16 h and then treated with 1.0 unit/ml thrombin for the indicated times. *A,* cell lysates containing equal amounts of protein were analyzed by Western blotting (*WB*) with phosphospecific anti-ERK1/2 antibody. *B,* lysates containing equal amounts of protein were immunoprecipitated with anti-ERK2 antibody. ERK2 activity was measured through immunocomplex kinase assay using myelin basic protein (*MBP*) as a substrate. *C,* Western blot analysis of cell lysates with anti-ERK1/2 antibody did not show any difference in ERK1/2 protein levels. Results shown represent an experiment that was repeated at least twice with similar results. *DMSO,* dimethyl sulfoxide.
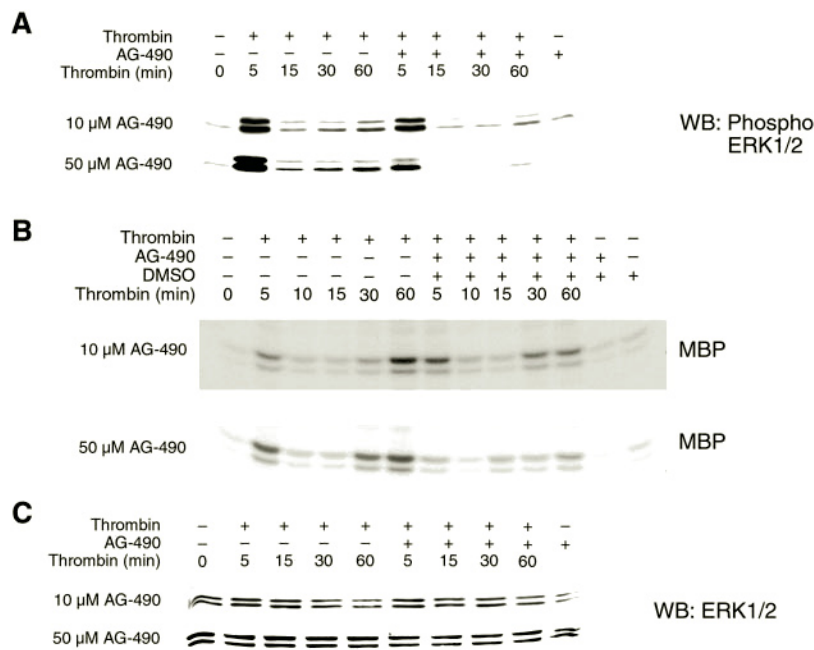
ing 0.1% calf serum 24 h after transfection. After quiescing for 20 h, cells were treated with 1.0 unit/ml thrombin for the indicated times.

Transient Transfection and CAT Assay—The Hsp70 CAT reporter constructs LSN (−188 to +1) and LSNP (−100 to +1) were kindly provided by Richard Morimoto (Northwestern University, Evanston, IL). The 5′ Hsp90β CAT reporter constructs A (−1044 to +36) and C (−299 to +36) were kindly provided by David Latchman (University College London, London, UK). For transfection, VSMC were grown to 70–80% confluence in 100-mm dishes containing DMEM with 10% fetal bovine serum. Transfection of VSMC with Hsp90β CAT reporter and ΔJAK2 plasmid DNA was performed by the calcium precipitate method (9). Cells were cotransfected with β-galactosidase expression vector to normalize for transfection efficiency. Cells were quiesced with DMEM containing 0.1% calf serum 16 h after transfection. After being quiescent for 36 h, cells were either untreated or treated with thrombin for 6 h. In experiments with AG-490, cells were treated with the inhibitor for 16 h before thrombin treatment. Cell lysates were prepared as described previously (9), and CAT activity was measured (38). In brief, 100 μg of protein was incubated with 4 μl of 40 mM acetyl-CoA and 4 μl of 50 μCi/ml [C14]chloramphenicol in a total volume of 150 μl at 37 °C for 2–4 h. Acetylated and nonacetylated chloramphenicol were extracted with ethyl acetate and separated by thin layer chromatography on Silica Gel 1B plates using chloroform/methanol mixture (19:1) as solvent. Air-dried silica plates were subjected to autoradiography, and the acetylated [C14]chloramphenicol was quantified using an Instant Imager (Packard Instrument Co.). β-Galactosidase assay was performed following manufacturer's protocol (Promega).

Statistical Analysis—Differences were analyzed with one-way analysis of variance, and post-hoc analysis was performed using Student-Newman-Keuls method. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Thrombin-induced Mitogenesis Is Inhibited by AG-490—To understand the role of tyrosine phosphorylation in thrombin-induced VSMC mitogenesis, we have investigated the effect of thrombin on the JAK-STAT pathway. Initially, growth-arrested rat VSMC were treated with 1.0 unit/ml thrombin in the presence and absence of AG-490, a specific inhibitor of JAK2. As shown in Fig. 1*A*, thrombin induced a 4.6-fold increase in DNA synthesis as measured by thymidine uptake after 24 h, an effect that was significantly inhibited in a dose-dependent manner by AG-490 ($p < 0.05$). AG-490 *per se* had no significant effect on DNA synthesis even at a concentration of 50 μM.

Measuring its effect on cell counts corroborated the inhibitory effect of AG-490 on thrombin-induced DNA synthesis. AG-490 significantly inhibited the thrombin-induced increase

in VSMC proliferation in a dose-dependent manner (Fig. 1*B*, $p < 0.05$). AG-490 alone had no significant effect on VSMC proliferation, indicating that it is not cytotoxic at the concentrations used in this experiment. AG-490 also significantly inhibited PAR-1-derived agonist peptide (SFLLRNP)-induced VSMC DNA synthesis (not shown) suggesting that the effects of thrombin-induced JAK2 activation are mediated via PAR-1 activation. The effect of AG-490 on thrombin-induced VSMC proliferation is similar to its effect on VSMC growth induced by Ang II, another G protein-coupled receptor agonist (10). These results suggest that JAK2 plays a role in VSMC proliferation induced by the activation of G protein-coupled receptors and led us to test the activation of specific members of the JAK-STAT pathway in response to thrombin stimulation.

Thrombin Stimulates JAK2 and TYK2 Kinase Activity in Rat VSMC—To assess the contributions of the different JAK kinases to thrombin-induced tyrosine phosphorylation, we immunoprecipitated tyrosine-phosphorylated proteins after thrombin stimulation and probed for the presence of phosphorylated JAK proteins (Fig. 2). By using an anti-JAK1 antibody, we found that JAK1 was transiently phosphorylated at 15 min after stimulation with thrombin (Fig. 2). In contrast, we observed a biphasic increase in JAK2 tyrosine phosphorylation following treatment with thrombin (Fig. 2). This was confirmed by Western blot analysis of thrombin-stimulated cell lysates with a phospho-specific JAK2 antibody (Fig. 3*A*), which demonstrated maximal stimulation of JAK2 at 1 min (8.7 ± 4.0-fold increase) (Fig. 3*B*). Finally, we also measured thrombin-induced TYK2 phosphorylation (Fig. 2). In contrast to the rapid activation of JAK2, peak activation of TYK2 was observed after 15 min of exposure to thrombin. These results were corroborated by additional experiments in which thrombin-stimulated VSMC lysates were immunoprecipitated with an anti-TYK2 antibody and analyzed by Western blotting with the monoclonal anti-phosphotyrosine antibody (Fig. 3*C*). TYK2 tyrosine phosphorylation was maximum (5.0 ± 1.0-fold increase) at 15 min after thrombin stimulation (Fig. 3*D*). JAK3 protein was not observed in VSMC lysates, which is consistent with reports that the expression of this protein is confined to lymphoid and myeloid cells (39, 40). The differences in the time course of tyrosine phosphorylation of various JAKs suggest that they may participate in different stress-mediated events in VSMC.
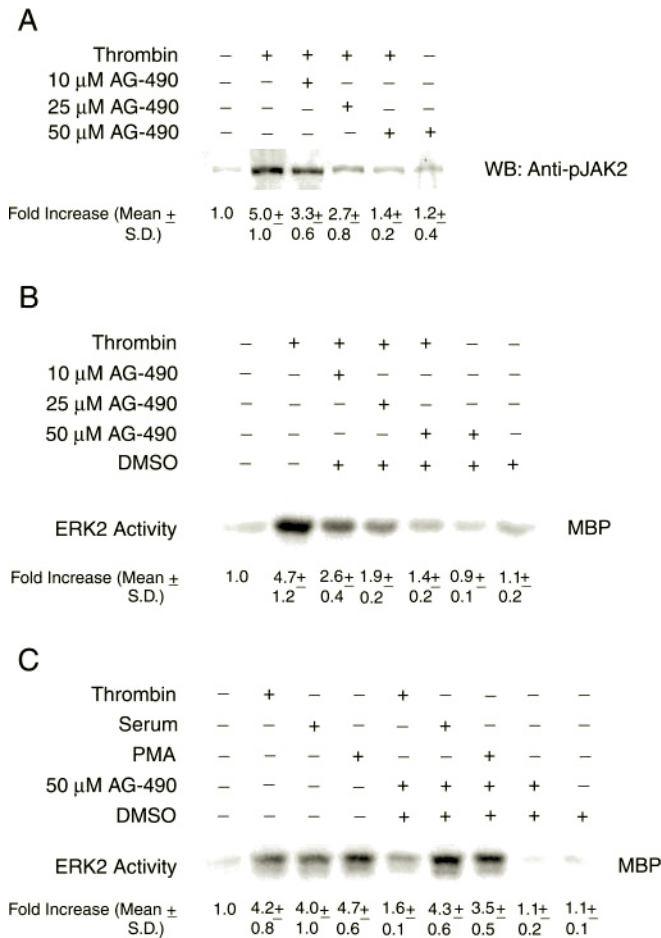
A

Thrombin	–	+	+	+	+	–
10 µM AG-490	–	–	+	–	–	–
25 µM AG-490	–	–	–	+	–	–
50 µM AG-490	–	–	–	–	+	+

WB: Anti-pJAK2

Fold Increase (Mean ± S.D.)	1.0	5.0± 1.0	3.3± 0.6	2.7± 0.8	1.4± 0.2	1.2± 0.4

B

Thrombin	–	+	+	+	+	–	–
10 µM AG-490	–	–	+	–	–	–	–
25 µM AG-490	–	–	–	+	–	–	–
50 µM AG-490	–	–	–	–	+	+	–
DMSO	–	–	+	+	+	+	+

ERK2 Activity   MBP

Fold Increase (Mean ± S.D.)	1.0	4.7± 1.2	2.6± 0.4	1.9± 0.2	1.4± 0.2	0.9± 0.1	1.1± 0.2

C

Thrombin	–	+	–	–	+	–	–	–
Serum	–	–	+	–	–	+	–	–
PMA	–	–	–	+	–	–	+	–
50 µM AG-490	–	–	–	–	+	+	+	+	–
DMSO	–	–	–	–	+	+	+	+	+

ERK2 Activity   MBP

Fold Increase (Mean ± S.D.)	1.0	4.2± 0.8	4.0± 1.0	4.7± 0.6	1.6± 0.1	4.3± 0.6	3.5± 0.5	1.1± 0.2	1.1± 0.1

FIG. 6. **AG-490 inhibits thrombin-induced JAK2 phosphorylation and ERK2 activation.** *A,* growth-arrested VSMC were pretreated with various concentrations of AG-490 for 16 h and then treated with 1.0 unit/ml thrombin for 1 min. Cell lysates containing equal amounts of protein were analyzed by Western blotting (*WB*) with anti-phosphotyrosine-specific JAK2 antibody. *B,* ERK2 activity was measured from lysates of cells treated with thrombin for 60 min in the presence and absence of AG-490 as described for Fig. 5. *C,* growth-arrested VSMC were pretreated with 50 µM AG-490 for 16 h and then treated with 1.0 unit/ml thrombin, 10% fetal bovine serum, or 1 µM PMA for 5 min. ERK2 activity was measured as described above. *MBP,* myelin basic protein; *DMSO,* dimethyl sulfoxide.

A

FLAG-ERK2	+	+	+	+	+	+	+
ΔJAK2	–	–	–	+	+	+	+
Vector	+	+	+	–	–	–	–
Thrombin, 5 min	–	+	–	+	–	–	–
Thrombin, 10 min	–	–	+	–	+	–	–

JAK2 →
ΔJAK2 →

B

IP: Anti-FLAG
WB: Anti-ERK2

C

MBP

D

FIG. 7. **Dominant negative JAK2 (Δ*JAK2*) inhibits thrombin-induced ERK2 activation.** H9C2 cells were transfected with either vector control or ΔJAK2 and FLAG-ERK2 plasmids. Cells were grown in DMEM supplemented with 10% fetal bovine serum for 36 h and growth-arrested for 20 h. Cells were treated with 1.0 unit/ml thrombin for the indicated times, and lysates containing equal amounts of protein were analyzed by Western blotting (*WB*) with anti-JAK2 antibody (*A*). *B,* lysates containing equal amounts of protein were immunoprecipitated (*IP*) with anti-FLAG antibody, and Western blotting was performed with anti-ERK2 antibody (*C*). ERK2 activity was measured in cell lysates immunoprecipitated with anti-FLAG antibody as described for Fig. 5. *MBP,* myelin basic protein. *D,* densitometric analysis of ERK2 activity (mean ± S.D., $n = 3$). The *asterisk* represents significant difference compared with control, and the *double asterisks* represent significant differences compared with thrombin treatment ($p < 0.05$).

The increase in tyrosine phosphorylation of JAK2 and TYK2 in response to thrombin treatment was not due to an increase in the levels of these protein as determined by Western blotting (Fig. 3, *A* and *C*), indicating that thrombin altered tyrosine phosphorylation of JAKs without affecting steady-state protein levels. Similar to the observation of Abe and Berk (41), pretreatment of VSMC with 50 µM AG-490 for 16 h completely inhibited thrombin-induced tyrosine phosphorylation of JAK2, whereas it had no effect on c-Src, a non-JAK cytosolic tyrosine kinase (not shown). Because JAK2 phosphorylation was most pronounced following treatment with thrombin and inhibition of the JAK2 phosphorylation with AG-490 blocked thrombin-induced proliferation of VSMC, we chose to investigate further the role of JAK2 in thrombin-induced mitogenesis.

Thrombin-induced JAK2 Activation Leads to Tyrosine Phosphorylation and Nuclear Translocation of STAT1, STAT2, and STAT3 in Rat VSMC—To determine whether thrombin-induced JAK2 activation leads to tyrosine phosphorylation of STAT proteins, the JAK substrates, we measured tyrosine phosphorylation and nuclear translocation of these reguleins. First, thrombin-treated VSMC lysates were immunoprecipitated with an anti-phosphotyrosine antibody, and the Western
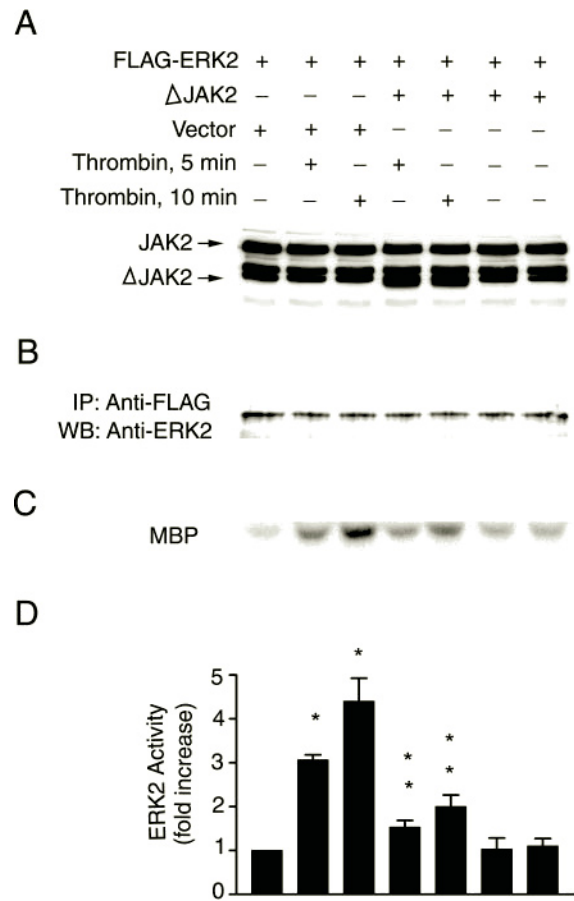
blots were probed with polyclonal antibodies against STAT1α/β, STAT2, or STAT3. All three STAT proteins were tyrosine-phosphorylated in response to thrombin treatment. STAT1α was rapidly tyrosine-phosphorylated within 1 min, an effect that was sustained for 60 min (Fig. 4*A*). The increase in tyrosine phosphorylation of STAT1β in response to treatment with thrombin was much less pronounced than that of STAT1α. To determine whether tyrosine phosphorylation of STAT proteins in response to treatment with thrombin was accompanied by translocation into the nucleus, Western blot analyses of nuclear and cytosolic fractions of thrombin-treated VSMC were performed (Fig. 4*B*). Thrombin induced nuclear translocation of STAT1 in 5 min (3.03 ± 0.25-fold increase), an increase that was sustained for 60 min (Fig. 4*C*), whereas no discernible change was observed in the protein levels in cytosolic fractions (Fig. 4*D*).

Immunoprecipitation/immunoblotting experiments revealed an increase in tyrosine phosphorylation of STAT3 within 1 min in response to treatment with thrombin that was sustained throughout the 60-min treatment period (Fig. 4, *D* and *E*). As with STAT1, STAT3 also rapidly translocated to the nucleus
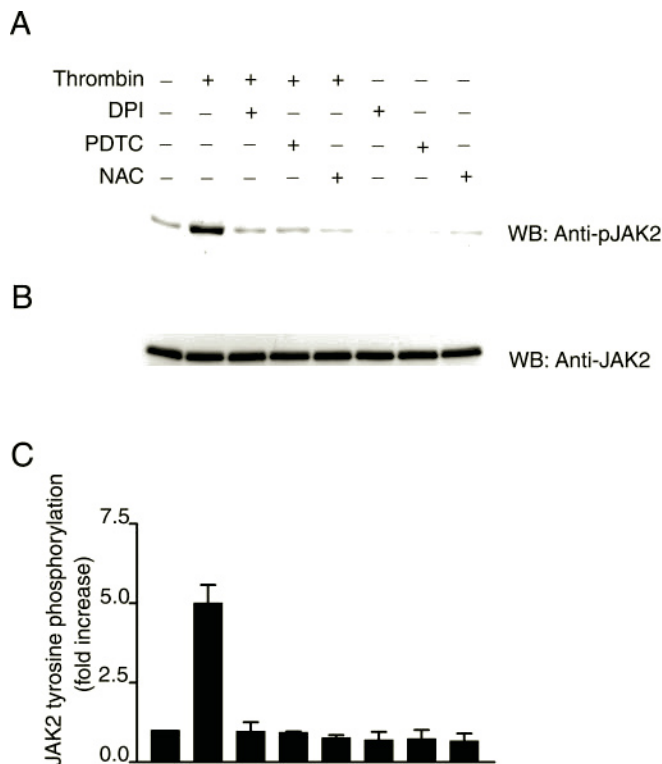
Thrombin Regulates JAK-STAT Pathway

A

Thrombin   −   +   +   +   +   −   −   −
DPI   −   −   +   −   −   +   −   −
PDTC   −   −   −   +   −   −   +   −
NAC   −   −   −   −   +   −   −   +

WB: Anti-pJAK2

B

WB: Anti-JAK2

C

FIG. 8. **ROS mediate thrombin-induced JAK2 tyrosine phosphorylation.** Growth-arrested VSMC were pretreated with 10 μM diphenyleneiodonium (*DPI*), 100 μM pyrrolidine dithiocarbamate (*PDTC*), or 10 mM *N*-acetyl-L-cysteine (*NAC*) for 30 min and then treated with 1.0 unit/ml thrombin for 1 min. Lysates containing equal amounts of protein were analyzed by Western blotting (*WB*) with either anti-phosphotyrosine-specific JAK2 (*A*) or JAK2 (*B*) antibody. *C*, densitometric analysis of JAK2 tyrosine phosphorylation (mean ± S.D., *n* = 3).

following treatment with thrombin, whereas the levels of this protein in cytosolic fractions were similar to control values (Fig. 4*F*). Peak nuclear translocation of STAT3 protein was observed at 10 min after treatment with thrombin (6.63 ± 1.52-fold increase) (Fig. 4*G*). Increased tyrosine phosphorylation and nuclear translocation of STAT2 was also observed in VSMC treated with 1.0 unit/ml thrombin (not shown). These experiments demonstrate that thrombin causes tyrosine phosphorylation and nuclear translocation of STAT proteins in VSMC. Inhibition of JAK2 tyrosine phosphorylation by pretreatment of VSMC with 50 μM AG-490 for 16 h blocked tyrosine phosphorylation and nuclear translocation of STAT1, STAT2, and STAT3 (not shown), indicating that JAK2 kinase activity is required for phosphorylation of these proteins.

Inhibition of JAK2 Kinase Activity Partially Blocks ERK1/2 Activation in VSMC—Because JAK2 has been suggested to be upstream of the MAP kinase cascade (10, 41, 42) and MAP kinase activation is required for thrombin-induced DNA synthesis in VSMC (8), we have investigated whether JAK2 activation is required for thrombin-induced stimulation of ERK1/2. ERK1/2 activation was measured in Western blots using a phospho-specific antibody and also by an immunocomplex kinase assay. Consistent with our previous work (8), thrombin activated ERK1/2 kinases in a biphasic manner with peaks at 5 and 60 min (Fig. 5, *A* and *B*). AG-490 markedly inhibited thrombin-induced activation of ERK1/2 in Western blots (Fig. 5*A*) and ERK2 activity in immunocomplex kinase assays (Fig. 5*B*) (4.3 ± 0.7 at 60 min *versus* 2.1 ± 0.6 and 1.5 ± 0.4 with 10 and 50 μM AG-490, respectively, *p* < 0.05). However, no detectable change was observed in the steady-state ERK1/2 protein levels following treatment with thrombin, either in the presence or absence of AG-490 or with AG-490 alone (Fig. 5*C*).

AG-490 caused a concentration-dependent inhibition of thrombin-induced JAK2 phosphorylation (Fig. 6*A*) and ERK2 activation (Fig. 5*B* and 6*B*). In contrast, ERK2 activation induced by 1 μM phorbol 12-myristate 13-acetate (PMA) and 10% fetal bovine serum was not significantly affected by 50 μM AG-490. ERK activation in VSMC induced by PMA and serum is consistent with the previous reports (9, 43). Together, these results suggest that thrombin-induced JAK2 is specifically inhibited by AG-490, and thrombin-induced ERK1/2 activation is partially regulated through JAK2 kinase in VSMC.

It is possible that any given inhibitor may have pleiotropic effects on cell physiology. Recently, AG-490 was shown to inhibit activation of JAK3 induced by interleukin-2 in antigen-activated human T cells (44). However, we did not detect JAK3 in VSMC with or without thrombin treatment. To provide an alternative demonstration of the role of JAK2 inhibition, H9C2 rat myoblast cells were transiently transfected with a dominant negative JAK2, which lacks the kinase domain (37) and a FLAG-ERK2. The rationale for using this clonal muscle cell line instead of VSMC is the relative ease of transfection. As expected, transfection of dominant negative JAK2 markedly inhibited thrombin-induced JAK2 phosphorylation (Fig. 7*A*), whereas a vector control had no such effect (not shown). Dominant negative JAK2 transfection also had no effect on steady-state ERK2 protein levels (Fig. 7*B*). Dominant negative JAK2 markedly reduced thrombin-induced FLAG-ERK2 activation (4.40 ± 0.53 *versus* 2.00 ± 0.27 at 60 min of thrombin treatment, *p* < 0.05) confirming the regulatory role of JAK2 in thrombin-induced ERK2 activation (Fig. 7, *C* and *D*).

Antioxidants Inhibit Thrombin-stimulated JAK2 Phosphorylation—Reactive oxygen species (ROS)-dependent activation of JAK2 has been reported in rat VSMC treated with Ang II (45). Because thrombin also generates ROS in VSMC (58), we investigated whether antioxidants inhibit thrombin-induced activation of JAK2. Pretreatment of VSMC with various antioxidants (diphenyleneiodonium, an inhibitor of flavin-containing enzymes, *N*-acetyl-L-cysteine, and pyrrolidine dithiocarbamate) significantly inhibited thrombin-induced JAK2 phosphorylation without affecting steady-state protein levels (Fig. 8), indicating that one mechanism by which thrombin induces JAK2 activation is via generation of ROS.

Thrombin-induced Heat Shock Protein (Hsp70 and Hsp90) Expression Is Mediated through the Activation of the JAK-STAT Pathway—G protein-coupled receptor agonists such as Ang II (46) and thrombin (9) and receptor tyrosine kinase agonists such as platelet-derived growth factor-BB (PDGF-BB) (47) are known to regulate VSMC proliferation through the induction of ROS. Accumulation of Hsps has been reported in cardiac tissue during ischemia and reperfusion, conditions known to produce ROS (48, 49). In addition, Hsp70 and Hsp90β promoters contain functional STAT-binding sites (50). Therefore, we investigated whether treatment of VSMC with thrombin leads to the accumulation of these proteins. Thrombin-stimulated expression of Hsp70 protein was evident at 2 h, and a 3.36 ± 0.78-fold increase was observed at 24 h (Fig. 9*A*). Pretreatment of VSMC with 50 μM AG-490 prior to exposure to thrombin abolished the agonist-induced increase in Hsp70 steady-state protein levels (3.50 ± 0.50 *versus* 1.30 ± 0.17, *p* < 0.05) (Fig. 9*B*). A biphasic increase in Hsp90 protein levels was also observed in VSMC in response to thrombin, with an initial peak at 4 h (2.46 ± 0.74-fold increase), and a second peak at 24 h (3.87 ± 0.71-fold increase) (Fig. 9*C*). As with Hsp70, increased levels of Hsp90 were ablated in response to the inhibition of JAK2 tyrosine phosphorylation with 50 μM AG-490 (3.67 ± 0.83 *versus* 1.33 ± 0.06, *p* < 0.05) (Fig. 9*D*).
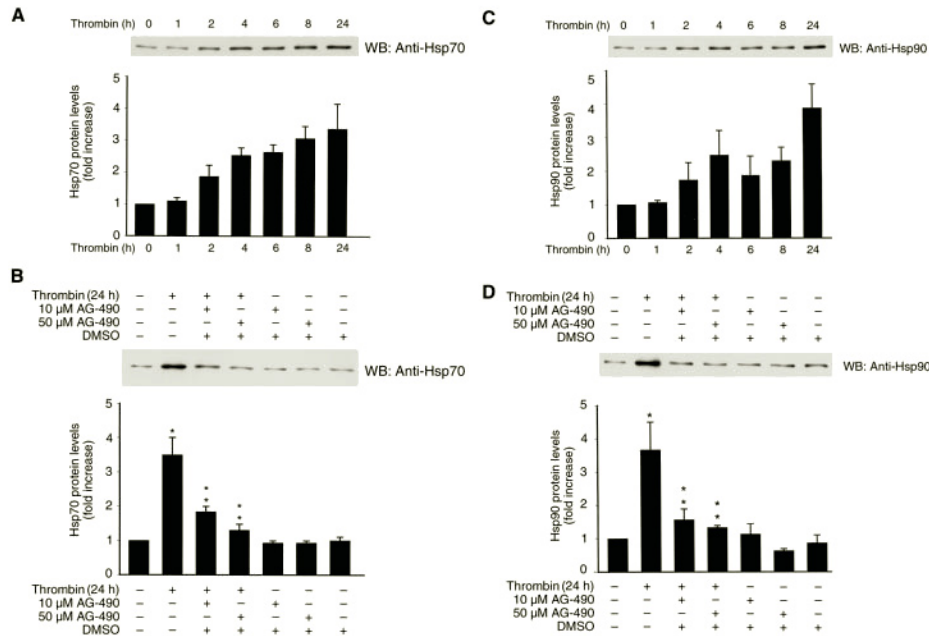
FIG. 9. **AG-490 inhibits thrombin-induced Hsp70 and Hsp90 protein levels.** *A,* growth-arrested VSMC were treated with 1.0 unit/ml thrombin for the indicated times, and Hsp70 protein levels were analyzed by Western blotting with anti-Hsp70 antibody (*top*). Densitometric analysis of Hsp70 protein levels was performed (mean ± S.D., $n = 3$) (*bottom*). *B,* cells were pretreated with AG-490 for 16 h and treated with thrombin for 24 h (*top*). Cell lysates containing equal amounts of protein were analyzed by Western blotting (*WB*) with anti-Hsp70 antibody. Densitometric analysis of Hsp70 protein levels was done (mean ± S.D., $n = 3$) (*bottom*). *C,* cell lysates from growth-arrested VSMC treated with 1.0 unit/ml thrombin for the indicated times were analyzed by Western blotting with anti-Hsp90 antibody (*top*). Densitometric analysis of Hsp90 protein levels was performed (mean ± S.D., $n = 3$) (*bottom*). *D,* cells were pretreated with AG-490 for 16 h and treated with thrombin for 24 h. Cell lysates containing equal amounts of protein were analyzed by Western blotting with anti-Hsp90 antibody (*top*). Densitometric analysis of Hsp90 protein levels was done (mean ± S.D., $n = 3$) (*bottom*). The *asterisk* represents significant difference compared with control, and the *double asterisks* represent significant differences compared with thrombin treatment ($p < 0.05$). *DMSO,* dimetyl sulfoxide.

AG-490, by itself, had no marked effect on Hsp70 and Hsp90 protein levels.

Next, we investigated whether induction of Hsp70 and Hsp90 in thrombin-treated VSMC is mediated via the transcriptional activity of the STAT proteins. For this, an electrophoretic mobility shift assay was performed by incubating nuclear proteins from thrombin-treated VSMC with a synthetic Hsp70-STAT oligonucleotide corresponding to -122 to -90 base pairs of the Hsp70 promoter. Three shifted bands were observed with nuclear extracts from thrombin-treated VSMC, each of which was competed with an excess of unlabeled specific oligonucleotide, but not with a nonspecific one (Fig. 10). The faster migrating two bands were partially abolished by preincubation of complexes with either anti-STAT1 or anti-STAT3 antibodies, indicating that these complexes likely contain STAT1/STAT3 heterodimers. The slower migrating band was abolished by anti-STAT1 antibody, but not by anti-STAT3 antibody, demonstrating the presence of STAT1 protein in this complex. Electrophoretic mobility shift assay of VSMC nuclear extracts with the STAT-binding region of Hsp90β synthetic oligonucleotide (-643 to -623 of Hsp90β promoter) demonstrated two shifted bands, the intensity of which was enhanced in response to thrombin (Fig. 11). The bands were competed with an excess of unlabeled specific oligonucleotide but not with a nonspecific one. The faster migrating band was competed with an unlabeled high affinity STAT1-binding sequence (SIEm67 oligonucleotide) from the c-*fos* promoter and was abolished by addition of an anti-STAT1 antibody. In contrast, the slower migrating band was abolished by anti-STAT3 antibody but not by anti-STAT1 antibody. These results indicate that the faster migrating band has STAT1 protein and the slower migrating band has STAT3 protein in the complex. None of the antibodies tested produced supershifts in electrophoretic mobility shift assays.

Activation of the Hsp90β Promoter by Thrombin Is JAK2-dependent—To investigate whether thrombin-induced Hsp90 expression was mediated via a direct effect of activated STAT proteins on its promoter, VSMC were transfected with an Hsp90β promoter-reporter construct either containing Hsp90 A (-1044 to $+36$) or lacking Hsp90 C (-299 to $+36$), a functional STAT-binding site. The Hsp90 A construct, besides possessing a STAT3-like binding site, also binds activated STAT1 protein (50). The reporter construct Hsp90 A was activated 2–4-fold by thrombin, whereas deletion of sequences containing the functional STAT-binding site abolished the activation of this promoter by thrombin (Fig. 12*A*). Thrombin-induced Hsp90β promoter activity was also abolished in VSMC pretreated with 50 μM AG-490, indicating that phosphorylation of STAT proteins by JAK2 kinase is necessary for maximal promoter activity (Fig. 12*B*). To confirm the role of JAK-STAT pathway in thrombin-induced Hsp90 expression, VSMC were cotransfected with Hsp90 A promoter-reporter construct and a dominant negative JAK2. Again, thrombin-induced Hsp90β promoter activity was completely abolished in the presence of dominant negative JAK2 (Fig. 12*C*). Together, these results indicate that the G protein-coupled receptor agonist thrombin causes activation of the JAK-STAT pathway in rat VSMC, and this pathway plays an important role in thrombin-induced VSMC proliferation and expression of proliferation-associated Hsps.

DISCUSSION

Thrombin-mediated tyrosine phosphorylation of various proteins such as insulin-like growth factor-1 receptor (8) and epidermal growth factor receptor (51) has been attributed to the activation of cytosolic tyrosine kinase, c-*src*. Stimulation of the Ras/MAP kinase pathway by the activation of G protein-coupled receptors has also been linked to Src kinases (14). In contrast, we show that JAK2, a non-Src family cytosolic tyro-
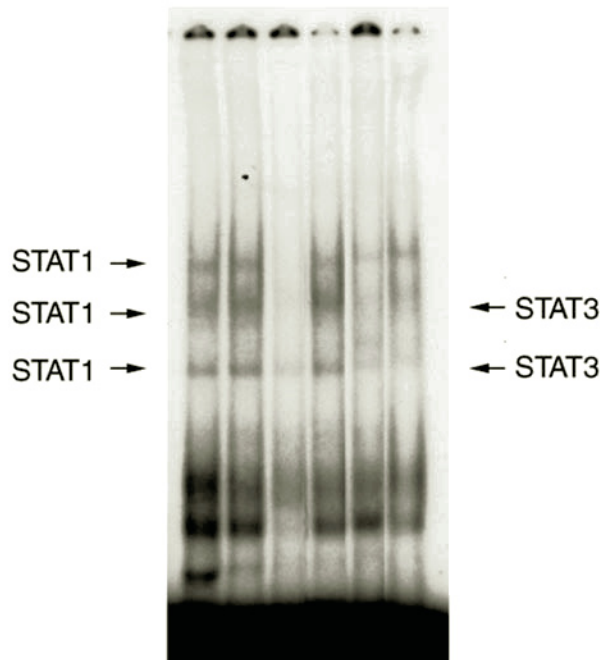
FIG. 10. **Thrombin induces DNA binding of Hsp70 STAT sequence.** Nuclear extracts from VSMC, either untreated (*1st lane 1*) or treated with 1.0 unit/ml thrombin for 10 min (*2nd* to *6th lanes*), were subjected to an electrophoretic mobility shift assay using a labeled Hsp70 STAT probe. To determine specificity of Hsp70 STAT binding complex, nuclear extracts were preincubated with unlabeled specific or nonspecific competitors. The specific competitors used were 100-fold molar excess of Hsp70 STAT (*3rd lane*), and the nonspecific competitor was 100-fold molar excess of SP1 consensus oligonucleotide (*4th lane*). For the characterization of protein components of thrombin-induced binding complex, nuclear extracts were preincubated with anti-STAT1 (*5th lane*) or anti-STAT3 (*6th lane*) antibody.
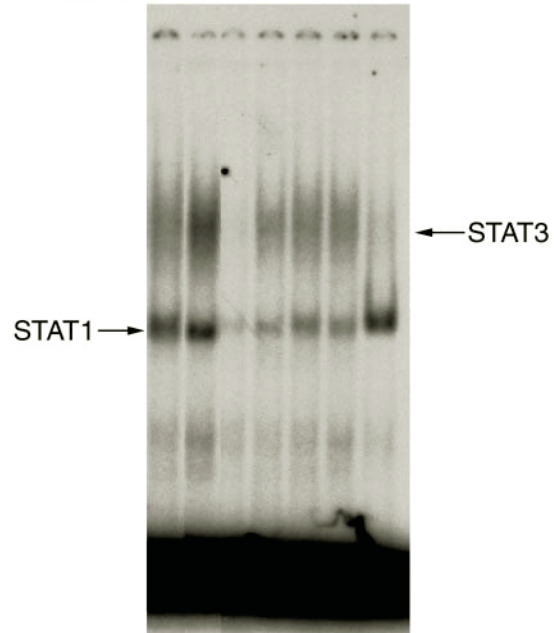
FIG. 11. **Thrombin induces DNA binding of Hsp90 STAT sequence.** Nuclear extracts from VSMC, either untreated (*1st lane*) or treated with 1.0 unit/ml thrombin for 10 min (*2nd* to *7th lanes*), were subjected to an electrophoretic mobility shift assay using a labeled Hsp90 STAT probe. To determine specificity of Hsp90 STAT binding complex, nuclear extracts were preincubated with unlabeled specific or nonspecific competitors. The specific competitors used were 100-fold molar excess of Hsp90 STAT and STAT1-inducible element (*SIE*) (*3rd* and *4th lanes,* respectively), and the nonspecific competitor was 100-fold molar excess of SP1 consensus oligonucleotide (*5th lane*). For the characterization of protein components of thrombin-induced binding complex, nuclear extracts were preincubated with anti-STAT1 (*6th lane*) or anti-STAT3 (*7th lane*) antibody (*Ab*).

sine kinase, is involved in thrombin-induced activation of ERK1/2 kinases, VSMC proliferation, and expression of Hsp70 and Hsp90.

The involvement of JAK kinases in signaling pathways induced by the activation of cytokine receptors and receptor tyrosine kinases is well documented (16, 53). Recently, it was shown that activation of the G protein-coupled receptor, Ang II AT$_1$, leads to phosphorylation of tyrosine 319 in the C-terminal intracellular domain and subsequent binding of SHP-2 phosphotyrosine phosphatase and the JAK2 tyrosine kinase complex (54). This suggests that G protein-coupled receptors possess mechanisms similar to those of cytokine and growth factor receptors for signal transduction involving cytosolic tyrosine kinases such as JAK2. Here we demonstrate that thrombin causes JAK2 and TYK2 activation in rat VSMC, and we have investigated the role of JAK2 in thrombin-induced cellular signaling using a specific pharmacologic inhibitor of JAK2. AG-490 inhibited both thrombin and PAR-1-derived peptide-induced DNA synthesis in VSMC. In addition, JAK2 coprecipitates with PAR-1 in VSMC treated with thrombin,[2] suggesting a physical association between JAK2 and PAR-1. It remains to be determined whether the association of JAK2 with PAR-1 is similar to that described between JAK2 and Ang II AT$_1$.

We found JAK2-dependent rapid tyrosine phosphorylation and nuclear translocation of STAT1, STAT2, and STAT3 proteins in VSMC. Tyrosine phosphorylation of STATs was also reported in Ang II-treated VSMC (25). In agreement with our findings, Ang II-induced STAT1 tyrosine phosphorylation is mediated by JAK2 (54). Recently, it has been shown that JAK2, TYK2, and STATs are also activated in response to oxidants in several cell types, including VSMC ((41, 55, 56).[2] We and others (47, 57, 58) have demonstrated that growth factors such as PDGF-BB and G protein-coupled receptor agonists such as Ang II and thrombin stimulate VSMC growth through the production of ROS. We also found that JAK2 activation induced by thrombin is sensitive to antioxidants. Similar results with regards to JAK2 sensitivity to antioxidants were reported recently in VSMC treated with Ang II (45). Together these results suggest that in addition to receptor-associated stimulation, another possible mechanism for the activation of JAK-STAT pathway by thrombin is via the generation of intracellular ROS.

A biphasic increase in ERK1/2 activity was observed in rat VSMC treated with thrombin. Inhibition of JAK2 activity by AG-490 pretreatment partially inhibits thrombin-induced ERK1/2 activity. This is in contrast to the complete inhibition of ERK1/2 phosphorylation by AG-490 in VSMC treated with either Ang II or PDGF-BB (10). This inhibition of ERK1/2 activity was shown to be a consequence of the inhibitory effect of AG-490 on JAK2 stimulation, blocking the association be-

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[2] N. R. Madamanchi, S. Li, C. Patterson, and M. S. Runge, unpublished data.

A

Hsp90 A (-1044)	−	+	+	−	−
Hsp90 C (-299)	−	−	−	+	+
Vector	+	−	−	−	−
Thrombin	−	−	+	−	+

Fold Activation   1.0 2.5 0.4 0.6

B

Hsp90 A (-1044)	−	+	+	+	+	+	+	+
Vector	+	−	−	−	−	−	−	−
10 µM AG-490	−	−	−	+	−	+	−	
50 µM AG-490	−	−	−	−	+	−	+	−
DMSO	−	−	−	+	+	+	+	+
Thrombin	−	−	+	+	+	−	−	−

Fold Activation   1.0 4.0 2.0 1.2 1.4 1.4 1.2

C

Hsp90 A (-1044)	−	+	+	+	+
Vector	+	+	+	−	−
∆JAK2	−	−	−	+	+
Thrombin	−	−	+	+	−

Fold Activation   1.0 3.0 0.9 0.9

FIG. 12. **JAK2 inactivation inhibits thrombin-induced Hsp90 promoter activity.** AG-490 inhibits thrombin-induced Hsp90 promoter activity. *A,* VSMC were transiently transfected with an Hsp90 CAT reporter construct containing −1044 to +36 (*A*) or lacking −299 to +36 (*C*) STAT binding region, or vector lacking any insert, growth-arrested, and were either untreated or treated with thrombin for 6 h. Cell lysates were prepared, and lysates containing equal amounts of protein were assayed for CAT activity. To normalize for transfection efficiency, cells were also cotransfected with a β-galactosidase construct. *B,* VSMC transfected with Hsp90 CAT reporter constructs were growth-arrested, pretreated with AG-490 for 16 h, and treated with thrombin in the presence and absence of AG-490. *C,* VSMC transfected with Hsp90 CAT reporter and either vector or ∆JAK2 DNA were growth-arrested and treated with thrombin for 6 h. Autoradiograms shown represent an experiment that was repeated at least twice with similar results. Fold activation shown is based on the quantitation of radioactivity measured by an Instant Imager. *DMSO*, dimethyl sulfoxide.
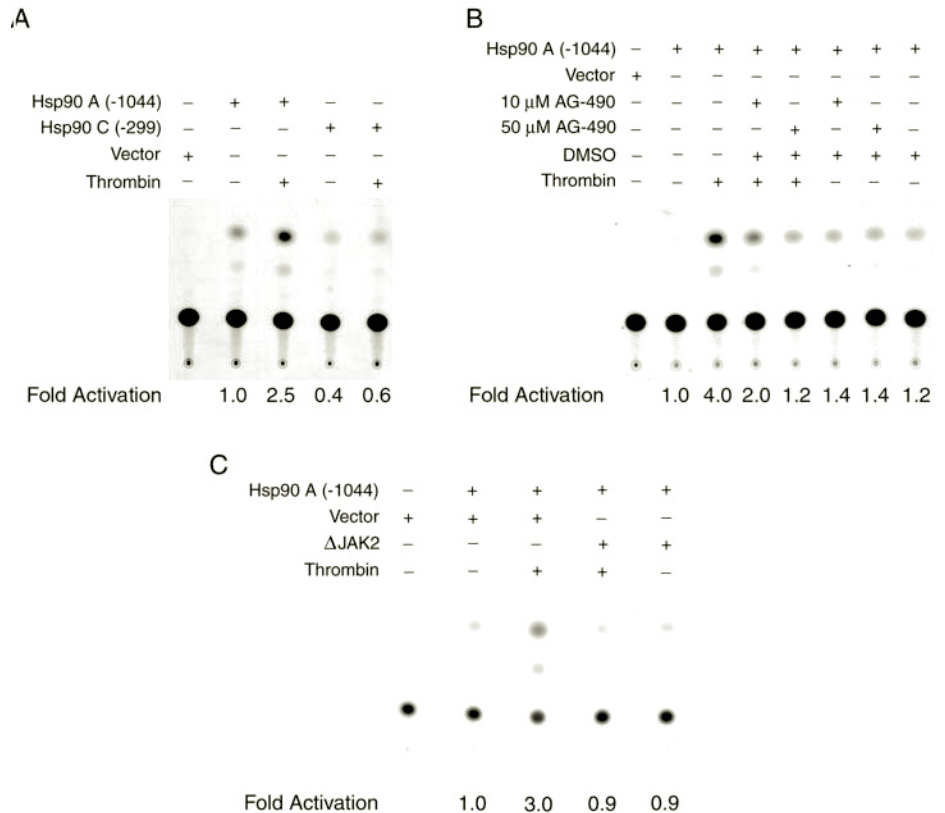
tween JAK2 and Raf1 and subsequent Raf1 tyrosine phosphorylation. Abe and Berk (41) reported that H_2O_2-mediated ERK1/2 phosphorylation is partially dependent on JAK2 activation. Taken together, these results place JAK2 upstream of Ras in the Ras/Raf/MEK/ERK pathway and, thus, implicate JAK2 in regulation of early growth response genes and cell proliferation. This argument is supported by a recent report (59) that activation of p38 MAP kinase, another member of the MAP kinase superfamily, is JAK2-dependent. Because inhibition of JAK2 stimulation only partially inhibits thrombin-induced ERK1/2 activation, a parallel pathway may exist to activate ERK1/2 in thrombin-treated cells similar to that proposed for H_2O_2-treated fibroblasts (41). Cross-talk between the JAK-STAT and ERK pathways suggests that inhibition of JAK2 activity might block not only direct tyrosine phosphorylation but also serine phosphorylation of STATs by ERK1/2. However, emerging evidence indicates that regulation of STAT proteins by ERK1/2 depends on the nature of stimulus. In contrast to the reports that maximal activation of transcription by STATs requires serine phosphorylation by MAP kinases in addition to tyrosine phosphorylation (22, 23), ERK1/2 activation has been shown to inhibit interleukin-6-induced JAK-STAT signaling (60). Further experiments are required to define the role of ERK1/2 in thrombin-induced activation of STATs.

Thrombin is known to stimulate generation of ROS, the cellular effects of which are modulated by Hsps. We therefore hypothesized that thrombin might induce Hsp production in VSMC. This hypothesis was supported by previous reports documenting the presence of functional STAT-binding sites in Hsp70 and Hsp90 promoters (50). Our results demonstrate time-dependent induction of Hsp70 and Hsp90 proteins in thrombin-treated VSMC. Pretreatment with 50 µM AG-490 inhibits the induction of these proteins suggesting that Hsp synthesis is regulated via the JAK-STAT pathway.

Although Hsps were initially characterized by their induc-

tion in response to stress, emerging evidence indicates that these proteins play a role in cellular signaling and cell proliferation (61). Abrogation of Hsp70 expression in tumor cells inhibits cell proliferation and induces apoptosis (62). Prior induction of Hsp70 has been shown to play a role in the resumption of proliferation after acute heat treatment (63). Similarly Hsp90 is known to interact with many signaling molecules, particularly kinases and ligand-regulated transcription factors (64, 65). Decreased Hsp90 protein levels have been linked to a slow rate of cell division (66). More recently it was demonstrated that Hsp90 plays a crucial role in the maturation and regulation of eukaryotic translation initiation factor kinase Gcn2 (67) and proper functioning of the centrosome (52). Induction of VSMC growth by ROS has been linked in part to autocrine/paracrine effects of proteins, including Hsp90α and cyclophilins (34). AlThough we have not addressed the role of Hsps in VSMC growth, these reports suggest that Hsps are likely to play a role in thrombin-induced VSMC proliferation.

Electrophoretic mobility shift assays demonstrate that STAT1 and STAT1/STAT3 heterodimers recognize the STAT binding domain of the Hsp70 promoter, whereas STAT1 and STAT3 are the selective proteins that bind the STAT-binding element of the Hsp90β promoter. Hsp70 and Hsp90β promoter constructs containing functional STAT-binding sites are activated by thrombin, and pretreatment with AG-490 or cotransfection with dominant negative JAK2 blocks the activity of these promoters. This strongly suggests that JAK2-mediated tyrosine phosphorylation is required for activation of the Hsp70 and Hsp90 promoters in VSMC. In close proximity to the STAT binding regions, the Hsp70 and Hsp90β promoters also contain binding sites for the stress-activated transcription factor, HSF1 (50). Activation of HSF-1 has been reported in heart tissue perfused with H_2O_2 (48). Therefore, a concomitant role for HSF1 in thrombin-induced expression of Hsp70 and Hsp90 is possible in view of the stimulation of ROS in thrombin-treated VSMC. Consistent with this hypothesis, overexpression

of STAT1 and HSF1 has an additive effect on Hsp70 promoter activity in HepG2 cells, suggesting that protein-protein interactions between these nuclear proteins may play a role in the regulation of Hsp70 transcriptional activity (50).

In summary, we have shown that the JAK-STAT pathway plays an important role in thrombin-induced VSMC proliferation. In addition, enhanced expression of Hsp70 and Hsp90 via the JAK-STAT pathway indicates that this pathway modulates cellular responses to generation of ROS in VSMC treated with thrombin. Together with the extensive work done on the JAK-STAT pathway in VSMC mitogenesis (10, 34, 41), our results suggest that this pathway plays a significant role in the progression of pathophysiologic vascular diseases such as atherosclerosis.

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