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# Role of the JNK pathway in thrombin-induced ICAM-1 expression in endothelial cells

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**Abstract**

**Objective:** Thrombin induces leukocyte adherence to endothelial cells via increased expression of intercellular adhesion molecule-1 (ICAM-1). Although ICAM-1 expression is regulated by NF-κB, recent studies have suggested that additional signaling mechanisms may also be involved. The goal of this study was to determine whether mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAP kinase (p38), mediate thrombin-induced ICAM-1 expression in endothelial cells.

**Methods:** Western blot analysis using anti-ICAM-1 antibody and luciferase assays were performed in cultured endothelial cells after addition of signal transduction inhibitors or transfection of various gene constructs. JNK kinase activity was determined by a kinase assay using c-Jun as a substrate or by Western blot analysis with anti-phospho-JNK antibody.

**Results:** Treatment of endothelial cells with the JNK-specific inhibitors, SP600125 or JNK inhibitory peptide 1 (JNKI1), resulted in a significant decrease in thrombin-induced ICAM-1 expression as demonstrated by Western blot analysis (67 ± 3% and 72 ± 7%, respectively). In contrast, inhibitors of MEK and p38 had only minimal effect. The combination of SP600125 and the NF-κB inhibitor, BAY11-7082, resulted in complete inhibition of thrombin-induced ICAM-1 expression. The $G\alpha_q$ inhibitor, YM-254890, inhibited thrombin-induced JNK activation and ICAM-1 expression. Dominant-negative Ras and Rac1, but not Rho, inhibited thrombin-induced JNK activation and ICAM-1 promoter activity. Finally, thrombin-induced JNK activation and ICAM-1 promoter activity were inhibited by βARK1ct (a Gβγ subunit scavenger) and Csk.

**Conclusions:** These data suggest that, in concert with NF-κB, JNK regulates thrombin-induced ICAM-1 expression by a mechanism that is dependent on $G\alpha_q$, Gβγ, Ras, Rac1 and the Src kinase family.



*Keywords:* G proteins; Gene expression; MAP kinase; Signal transduction

## 1. Introduction

Thrombin is a procoagulant factor that exerts diverse effects on vascular cells via its ability to bind proteinase-activated receptor 1 (PAR1) [1], a seven transmembrane receptor that couples to heterotrimeric G proteins. Activation of this receptor results in stimulation of various downstream signal transduction elements, including the mitogen-activated protein (MAP) kinase family, nuclear factor-κB (NF-κB), Rho kinase and calcium-dependent kinases, and results in changes in transcription, cell morphology and cell migration. Thrombin may also contribute to the inflammatory state that promotes athero-

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genesis, as is evidenced by thrombin-induced increases in various proinflammatory cytokines, including interleukin (IL)-1, IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) in endothelial cells, as well as by thrombin-induced increases in expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E- and P-selectin in endothelial cells [2].

ICAM-1 mediates adhesion of inflammatory cells to endothelial cells. Binding of endothelial ICAM-1 to β integrin (LFA-1 and Mac-1) on leukocytes causes firm attachment, enabling leukocytes to transmigrate across the endothelial barrier [3]. Further, studies have demonstrated that upregulation of ICAM-1 on the surface of endothelial cells promotes atherogenesis [4]. ICAM-1 expression is induced by various stimuli, including tumor necrosis factor (TNF)-α, interferon (INF)-γ, IL-1β [5] and thrombin [6,7]. Although studies have clearly demonstrated that NF-κB is essential for ICAM-1 expression [8,9], other studies have reported that TNF-α upregulates ICAM-1 expression via activation of the c-Jun N-terminal kinase (JNK). Further, Gorgolis et al. reported that p53-mediated induction of ICAM-1 after DNA damage occurs via a NF-κB-independent mechanism [10–12]. Finally, JNK is a member of the MAP kinase family that is activated by a variety of stimuli, including cell stress and cytokines. Thrombin also activates JNK in various cell types, and thus may play a role in the inflammatory response in vascular endothelial cells [13,14].

The concordance of these data suggests that JNK may mediate thrombin-induced ICAM-1 expression. Thus, the goal of the present study was to determine whether JNK and other MAP kinase family members mediate thrombin-induced ICAM-1 expression in vascular endothelial cells and to characterize the mechanism(s) by which this regulation may occur in concert with other molecules, particularly NF-κB.

## 2. Materials and methods

### 2.1. Materials

Human thrombin and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against ICAM-1 (G-5) (1:500), JNK1 (FL) and β-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An antibody against IκB-α was purchased from Cell Signaling (Beverly, MA). An antibody against phospho-JNK was purchased from Promega (Madison, WI). An antibody against phospho-Src (pY418) was purchased from Biosource International (Camarillo, CA). An antibody against Src (clone 327) was purchased from Oncogene Research Products (San Diego, CA). PP2, PD98059, SP600125, JNKI1, SB203580, and BAY11-7082 were purchased from Calbiochem (San Diego, CA). YM-254890 was provided by Yamanouchi Pharmaceutical Co. (Ibaraki, Japan).

### 2.2. Plasmids

FLAG-tagged JNK was provided by Dr. R. J. Davis (University of Massachusetts Medical School, MA). Constitutively active Ras (Ras V14) was generated by site-directed point mutagenesis. Constitutively active $G\alpha_q$ ($G\alpha_q$ RC) and $G\alpha_{13}$ ($G\alpha_{13}$ QL) were provided by Dr. M. I. Simon (California Institute of Technology, CA). The β-adrenergic receptor kinase C-terminus (βARK-ct) was provided by Dr. R. J. Lefkowitz (Duke University Medical Center, NC). cDNA for Csk was provided by Dr. J. A. Cooper (Fred Hutchinson Cancer Research Center, WA) and was cloned into pcDNA3.1. ICAM-LUC containing 1393 bp of the 5′-regulatory region of the ICAM-1 promoter-linked firefly luciferase reporter gene was provided by Dr. C. Stratowa (Boehringer Ingelheim, Vienna, Austria). IgκB-LUC containing the κB site-integrated luciferase reporter was provided by Dr. A. Takaori (Kyoto University).

### 2.3. Cell culture and transfection

Bovine aortic endothelial cells (BAEC; purchased from Clonetec, Palo Alto, CA) and human umbilical vein endothelial cells (HUVEC; purchased from Cell Applications, Inc., San Diego, CA) were cultured in endothelial basal medium containing the specific growth supplements recommended by the manufacturer. Cells at passages 4 to 8 were plated on dishes coated with 1% gelatin. For transfection experiments, BAEC at 50% confluence were transfected using the FuGENE6 (Roche, Basel, Switzerland) method, as previously described [15]. In brief, cells were incubated for 8 h in medium containing the FuGENE reagent along with the gene constructs of interest. After transfection, cells were placed in serum-free medium overnight. Next, the cells were challenged with thrombin for the indicated periods of time and then harvested.

### 2.4. Immunoprecipitation and Western blot analysis

Immunoprecipitation and Western blot analysis were performed as described previously [16,17]. In brief, cells were lysed in gentle lysis buffer (25 mM Tris [pH 7.5], 50 mM NaF, 10 mM sodium pyrophosphate, 137 mM NaCl, 10% glycerol, 1% NP-40, 5 mM EDTA, 1 mM $Na_3VO_4$, 10 μg/mL leupeptin, and 1 mM PMSF), and centrifuged. Lysates containing equal amounts of soluble proteins were precleared and incubated with antibodies overnight at 4 °C. Antibody complexes were collected by incubation with protein G-agarose for monoclonal antibody. Precipitates were washed in lysis buffer and then resuspended in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated with a primary antibody and were visualized using the ECL system (Amersham-Pharmacia Co., Buckinghamshire, UK). Images were captured using

Adobe Photoshop, and the band intensities were quantified using NIH Image 1.61.

### 2.5. Luciferase assay

BAEC were seeded in 12-well plates at 50% confluence for transfection experiments. Cells were allowed to incubate with gene constructs (ICAM-LUC or IgκB-LUC), the Renilla luciferase vector and FuGENE6 reagent for 8 h followed by incubation in serum-free medium overnight. Next, cells were treated with thrombin (3 U/mL) for 8 h and then harvested. Luciferase activity was measured using the Dual-Luciferase Assay Kit (Promega). Firefly luciferase activity was normalized to the Renilla luciferase activity and expressed as a fold increase, in which the control value in the absence of thrombin was defined as 1.0 (control). Repetitive experiments were performed in triplicate.

### 2.6. Assays for JNK activity

JNK kinase assay was performed as described previously [18]. Briefly, approximately 250 μg of cell lysate protein was incubated with 3 μg GST-c-Jun (1-169) coupled to glutathione agarose at 4 °C for 4 h under constant rotation. Agarose beads were washed three times with buffer B (12.5 mM MOPS [pH 7.2], 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 7.5 mM $MgCl_2$, 1 mM DTT, and 0.1% Triton X-100) containing 0.25 M NaCl. Beads were then incubated in modified buffer B (standard preparation plus 10 mM $MgCl_2$, 1 mM $MnCl_2$, 50 μM ATP, and 15 μCi of [$\gamma$-$^{32}$P] ATP) for 10 min at 30 °C. For cotransfection experiments, exogenous FLAG-tagged JNK1 was immunoprecipitated with FLAG antibody, and the kinase reaction was performed in modified buffer B with 2 μg GST-c-Jun. The reaction was terminated with sample buffer, and relative protein concentrations were determined via SDS-PAGE and autoradiography. Otherwise, JNK activity was determined by Western blot analysis using anti-phospho-JNK antibody.

### 2.7. Ras activation assay

A Ras Activation Assay Kit, which included GST-fusion proteins containing Raf binding domain (GST-Raf RBD), was purchased from Upstate (Lake Placid, NY). The assay was performed as described previously [19]. Briefly, HUVEC were lysed in MLB (25 mM HEPES [pH 7.5], 150 mM NaCl, 25 mM NaF, 10% glycerol, 0.25% Na deoxycholate, 10 mM $MgCl_2$, 1% Triton X-100, 1 mM EDTA, 1% NP-40, 1 mM Na vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). After centrifugation, lysates were incubated with 10 μg of the fusion protein bound to beads, and the mixture was placed on a rocker plate at 4 °C for 45 min. Bound proteins were collected by centrifugation, and beads were washed 3 times with MLB and then resuspended in SDS sample buffer. Bound Ras was determined by Western blot analysis with anti-Ras (1 : 1000) antibodies.

### 2.8. ICAM-1 mRNA analysis

Total RNA was isolated from $1 \times 10^6$ HUVEC, which had either been left untreated or been stimulated with thrombin (3 U/ml, 6 h) ± actinomycin D (10 μg/ml, 1 h), using TRIZOL Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After isolation, 1 μg of RNA was reverse-transcribed into cDNA with an oligo $(dT)^{20}$ primer using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) as described in the manufacturer's instructions. The mRNA levels were analyzed by the LightCycler quantitative RT-PCR strategy (Roche, Basel, Switzerland). Specific primers for ICAM-1 and β-actin were obtained by Search-LC (Heidelberg, Germany). The ICAM-1 mRNA expression was normalized to housekeeping β-actin expression.

### 2.9. Statistical analysis

Statistical comparisons were made using two-tailed Student's *t* test. Experimental values were reported as mean ± S.D. Differences were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. Thrombin induces ICAM-1 expression in endothelial cells

Thrombin (3 U/ml) induced an increase in ICAM-1 protein expression in HUVEC in a time-dependent manner (Fig. 1A). Increases in ICAM-1 expression were observed beginning at 4 h after thrombin treatment with a peak between 12 and 24 h. To examine whether this increase was due to increases in ICAM-1 transcription, the ICAM-1 promoter activity was analyzed by luciferase assay using BAEC transfected with ICAM-LUC (Fig. 1B). Thrombin treatment resulted in increases in ICAM-1 promoter activity to 10.3 ± 2.1-fold of basal levels at the 8-h time point. Similarly, ICAM-1 mRNA levels increased in response to thrombin with a peak at 6 h in HUVEC (data not shown). This induction of ICAM-1 mRNA was completely inhibited by the transcriptional inhibitor, actinomycin D (Fig. 1C). These data indicate that thrombin-induced increase in ICAM-1 expression occurs secondary to increases in transcription.

### 3.2. JNK regulates thrombin-induced ICAM-1 expression in endothelial cells

To determine the role of extracellular signal-regulated kinase (ERK1/2), JNK and p38 MAP kinase in ICAM-1

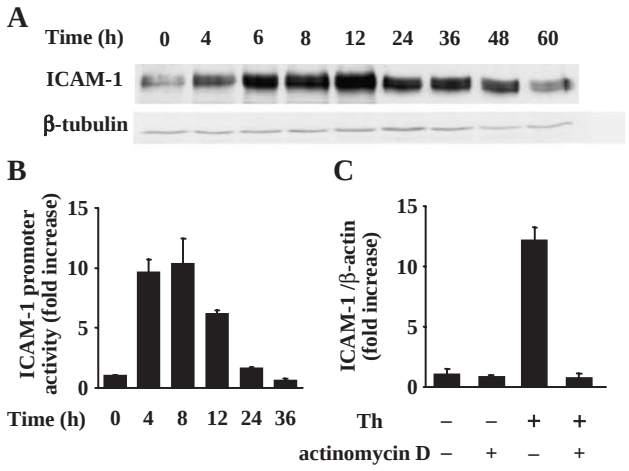


Fig. 1. Thrombin-induced ICAM-1 expression in endothelial cells. (A) Confluent HUVEC were serum-starved and challenged with thrombin (3 U/ml) for the indicated periods. Western blotting was performed with ICAM-1 antibody as described in Materials and methods. ICAM-1 protein level was normalized to that of β-tubulin. (B) BAEC, transfected with a plasmid encoding the ICAM-1 promoter linked with the luciferase reporter gene (ICAM-LUC), were placed in serum-free medium for 16 h. Cells were treated with thrombin (3 U/mL) for the indicated periods and then harvested. Luciferase activity was measured via luminometer. ICAM-1 promoter activity was normalized to Renilla luciferase activity and expressed as a fold increase. (C) HUVEC were incubated with actinomycin D (10 μg/ml) for 1 h before treatment with thrombin (th, 3 U/mL) for 6 h. ICAM-1 mRNA levels, quantitated by real time RT-PCR, were normalized to β-actin mRNA levels. In (B) and (C), values are the mean±S.D. for three separate experiments.

expression, Western blot analysis was performed to measure the change in thrombin-induced ICAM-1 expression following treatment with inhibitors of the MAP kinase family. Treatment of cells with the JNK specific inhibitors, SP600125 (3–30 μM) or a JNK inhibitory peptide (JNKI1) (3–30 μM), resulted in significant attenuation of thrombin-induced ICAM-1 protein expression in a concentration-dependent manner (67±3% and 72±7% inhibition at 30 μM, respectively; Fig. 2A). In contrast, treatment of cells with the MEK inhibitor, PD98059, or the p38 inhibitor, SB203580, had minimal effect on thrombin-induced ICAM-1 expression. Similarly, thrombin-induced ICAM-1 promoter activity was also inhibited by SP600125 or by JNKI1, while PD98059 and SB203580 had only slight inhibitory effect (Fig. 2B). These data indicate that thrombin-induced ICAM-1 expression is mediated predominantly by JNK activity in comparison to the relatively small effect of other members of the MAP kinase family in endothelial cells.

### *3.3. JNK mediates thrombin-induced ICAM-1 expression independent of the NF-κB pathway*

To determine the relative contributions of the JNK and NF-κB pathways on thrombin-induced ICAM-1 expression, Western blot analysis of thrombin-induced ICAM-1 expression was performed after treatment of cells with the JNK inhibitor, SP600125, or with the NF-κB inhibitor, BAY11-7082. Treatment of cells with either SP600125 or BAY11-

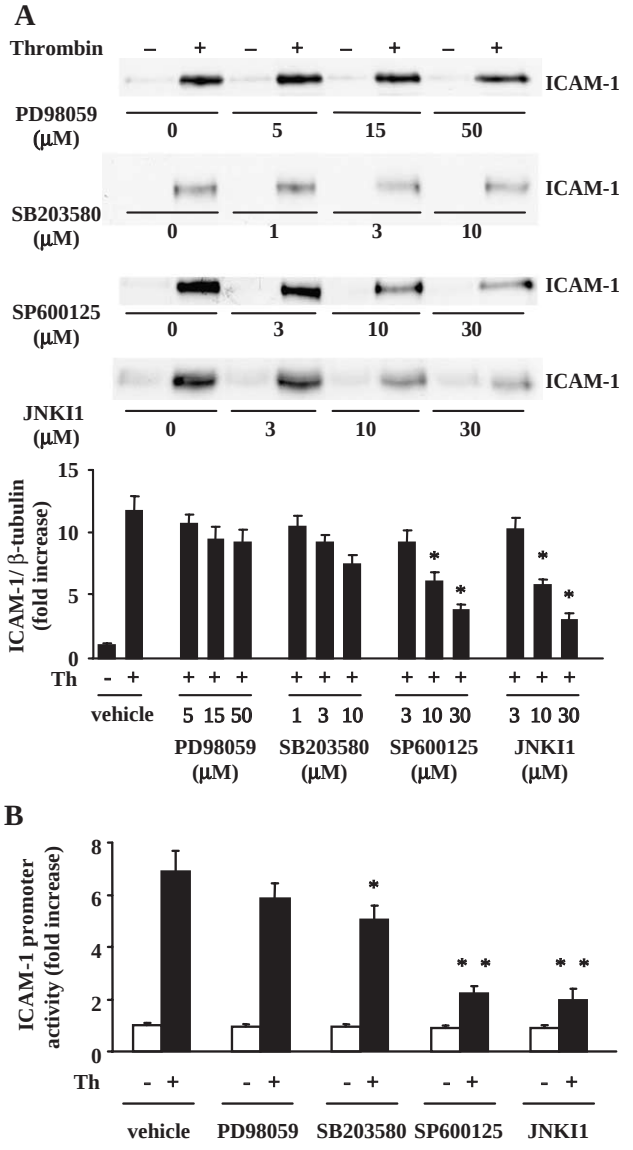


Fig. 2. Effects of MAP kinase family inhibitors on thrombin-induced ICAM-1 expression. (A) Growth-arrested HUVEC were treated with various concentrations of PD98059, SB203580, SP600125, or JNKI1 for 1 h prior to challenge with thrombin (th; 3 U/ml) for 12 h. Cell lysates were prepared and assayed for ICAM-1 and β-tubulin expression by Western blot analysis. Representative immunoblots and the corresponding bar graph are shown. In the graph, the expression of ICAM-1 was normalized to that of β-tubulin and expressed as a fold increase, in which the control ratio without thrombin was defined as 1.0 (control). (B) BAEC, transfected with ICAM-LUC, were treated for 1 h with PD98059 (50 μM), SB203580 (10 μM), SP600125 (30 μM), or JNKI1 (30 μM) prior to challenge with (filled bar) or without (blank bar) 3 U/mL thrombin for 8 h. Normalized luciferase activity was expressed as a fold increase, in which the control value without thrombin was defined as 1.0 (control). In all experiments, values are the mean±S.D. for three separate experiments. *, $p<0.05$ versus control with thrombin; **, $p<0.01$ versus control with thrombin.

7082 resulted in significant inhibition of thrombin-induced ICAM-1 expression (56±7 and 67±3% inhibition, respectively, Fig. 3A). However, treatment of cells with both agents in combination resulted in near complete inhibition (88±3% inhibition) of thrombin-induced ICAM-1 expression.

Further experiments were conducted to examine whether MAP kinases modulate thrombin-induced NF-κB activation. Phosphorylation and consequent degradation of IκB-α lead to activation of NF-κB pathway. Thrombin induced IκB-α degradation with a peak at 1 h, and this effect was not attenuated in the presence of PD98059, SP600125 or SB203580 (Fig. 3B). The MAP kinase family inhibitors did not inhibit IκB-α phosphorylation, which peaked at 10 min after thrombin stimulation (data not shown). These data indicate that JNK mediates thrombin-induced ICAM-1 expression independent of the action of the NF-κB pathway.

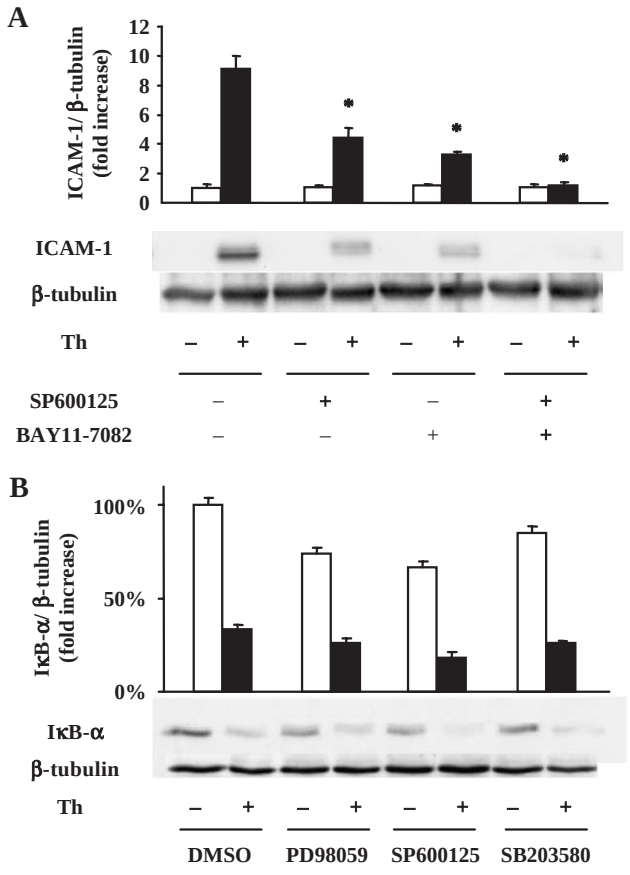


Fig. 3. Effects of JNK and NF-κB on thrombin-induced ICAM-1 expression. (A) HUVEC were treated with SP600125 (30 μM), BAY11-7082 (10 μM), or the combination of the two compounds prior to challenge with 3 U/mL thrombin (th) for 12 h. Western blot analysis was performed with anti-ICAM-1 and β-tubulin antibody. ICAM-1 expression was normalized and presented as a fold increase in the bar graph. (B) IκB-α degradation assay. HUVEC were treated with PD98059 (50 μM), SP600125 (30 μM), or SB203580 (10 μM) for 1 h before thrombin stimulation. Western blot analysis was performed with anti-IκB-α antibody. IκB-α expression was normalized to that of β-tubulin in the bar graph, in which the control ratio without thrombin was defined as 100%. Representative immunoblots and the corresponding bar graphs are shown. Values are the mean±S.D. for three separate experiments. *, $p<0.05$ versus control with thrombin.

### 3.4. Thrombin receptor-induced JNK activation and ICAM-1 expression is mediated by the $G\alpha_q$ and $G\beta\gamma$ subunits

To characterize the mechanisms by which JNK mediates thrombin-induced ICAM-1 expression, thrombin-induced JNK activity was assayed following modulation of various signal transduction elements. Thrombin (3 U/mL) induced rapid activation of JNK, with a peak at 10–20 min (Fig. 4A), and JNK activation was still detectable at 60 min.

Thrombin binds to PAR-1, which couples to $G\alpha_q$, $G\alpha_{13}$ and $G\alpha_i$ [1]. To determine whether $G\alpha_q$ mediates thrombin-induced JNK activation and ICAM-1 expression, the $G\alpha_q$-selective inhibitor, YM-254890 was utilized [20]. As shown in Fig. 4B and C, YM-254890 potently suppressed thrombin-induced JNK activation and ICAM-1 protein expression. Further, thrombin-induced ICAM-1 promoter activity was markedly inhibited by YM-254890, while overexpression of constitutively active $G\alpha_q$ resulted in induction of ICAM-1 promoter activity to levels 2.4±0.5-fold above baseline levels (Fig. 4D).

To determine the role of the released Gβγ subunit of the heterotrimeric G protein, which is also capable of signal transduction, JNK activity and ICAM-1 expression were assessed following treatment of cells with a specific scavenger for Gβγ subunit, βARK1ct. Transfection of βARK1ct resulted in a decreased level of thrombin-induced JNK activation and ICAM-1 promoter activity when compared to transfection of vector alone (61±6% and 68±2% inhibition, respectively; Fig. 4E, F). These data indicate that $G\alpha_q$ and Gβγ subunits play a significant role in JNK-mediated ICAM-1 expression induced by thrombin.

### 3.5. Ras and Rac1 mediate thrombin-induced JNK activation and ICAM-1 expression

Thrombin activates various small G proteins, such as Ras, Rac1 and Rho [1]. As shown in Fig. 5A, overexpression of constitutively active Ras (Ras V14) or Rac1 (Rac1 QL) resulted in a significant increase in JNK activity, while overexpression of constitutively active Cdc42 (Cdc42 V12) or Rho (Rho V14) had only minimal or no effect. In contrast, overexpression of dominant-negative Ras (Ras N19) or Rac1 (Rac1 N17) inhibited thrombin-induced JNK activity (Fig. 5B), while dominant-negative Cdc42 or Rho had no effect (data not shown). In addition, dominant-negative Ras or Rac1 inhibited thrombin-induced ICAM-1 promoter activity by 59±14% and 43±2%, respectively, whereas dominant-negative Rho had no effect on ICAM-1 promoter activity (Fig. 5C). Similarly, dominant-negative Ras and Rac1 inhibited ICAM-1 promoter activation induced by constitutively active $G\alpha_q$ (Fig. 5D). Furthermore, thrombin-induced Ras activation was potently

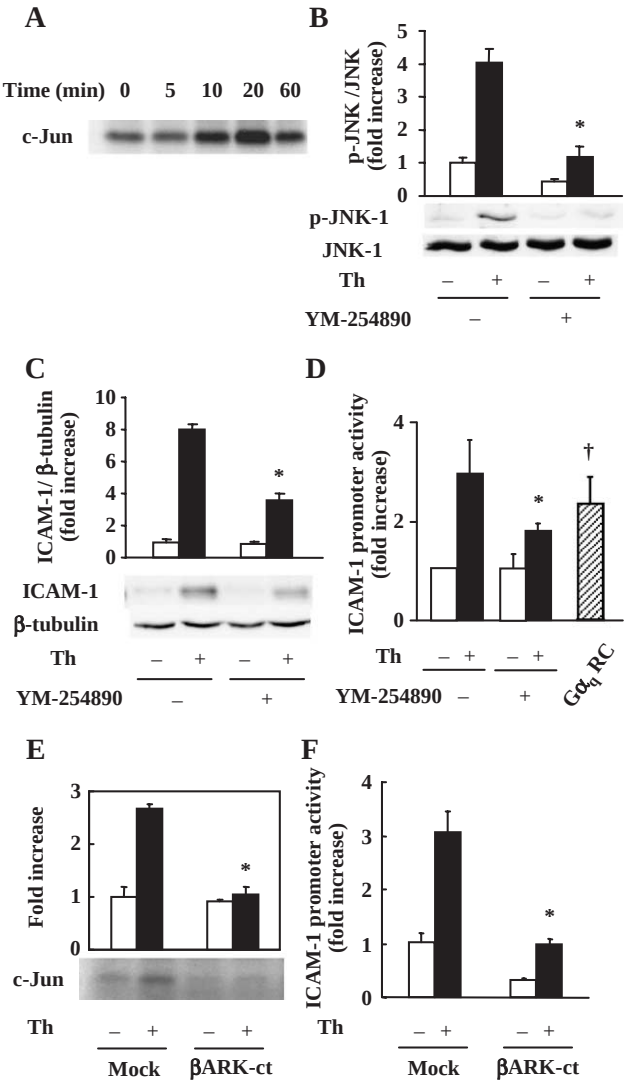


Fig. 4. Effects of $G\alpha_q$ and $G\beta\gamma$ subunits on thrombin-induced JNK activation and ICAM-1 expression. (A) Time course for thrombin-induced JNK activation. BAEC were stimulated with thrombin (3 U/mL) for indicated times. JNK activity was analyzed by a JNK kinase assay with c-*jun* as a substrate. (B) (C) Western blot analysis. HUVEC were treated with 1 μM YM-254890 for 1 h and then challenged with thrombin (th) for 10 min (B) or 1 h (C), respectively. (D) Luciferase assay. BAEC expressing ICAM-LUC were pretreated for 1 h with YM-254890 (1 μM) prior to challenge with thrombin for 8 h. BAEC cotransfected with ICAM-LUC and constitutively active $G\alpha_q$ construct were allowed to grow for 20 h. Normalized luciferase activity was expressed as a fold increase. (E) JNK kinase assay. BAEC were cotransfected with FLAG-tagged JNK and βARK-ct expression vector. Cells were stimulated with thrombin for 20 min. Exogenous JNK was immuno-precipitated with anti-FLAG antibody, and JNK activity was expressed as a fold increase, in which JNK activity in cells transfected with mock vector was defined as 1.0. (F) Luciferase assay. BAEC, cotransfected with ICAM-LUC and βARK-ct, were challenged with thrombin for 8 h. In all experiments, values are the mean±S.D. for three separate experiments. * †, $p<0.05$ versus control in the presence and absence of thrombin, respectively.

inhibited by YM-254890 (Fig. 5E), suggesting that Ras is involved in thrombin-induced JNK activation and ICAM-1 expression at a point downstream of $G\alpha_q$.

To determine whether small G proteins mediate thrombin-induced activation of NF-κB pathway, NF-κB reporter assays were performed (Fig. 5F). Preliminary data showed that NF-κB promoter activity peaked at 8 h after thrombin stimulation (7.3±0.8 fold above baseline levels, data not shown). Dominant-negative Ras, Rac and Rho had minimal or no effect on thrombin-induced NF-κB promoter. These

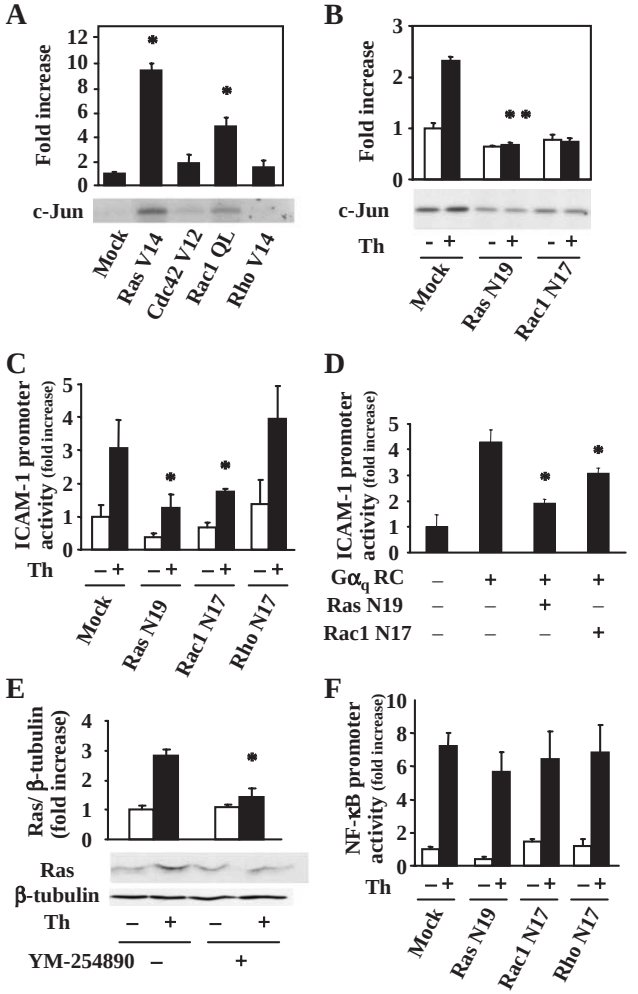


Fig. 5. Effect of small G proteins on thrombin-induced JNK activation and ICAM-1 expression. (A) (B) JNK kinase assay. In (A), BAEC were cotransfected with FLAG-tagged JNK and constitutively active small G proteins. In (B), BAEC, expressing dominant-negative small G proteins, were stimulated with thrombin (3 U/mL) for 20 min. The bar graphs represent quantified levels of JNK activity, expressed as a fold increase. (C) BAEC were cotransfected with ICAM-LUC and dominant-negative small G proteins and challenged with thrombin for 8 h. (D) BAEC were cotransfected with ICAM-LUC and constitutively active $G\alpha_q$ along with dominant-negative small G protein mutants. Cells were harvested at 36 h after transfection. (E) Ras activation assay. HUVEC were pretreated with YM-245890 (1 μM) prior to challenge with thrombin for 5 min. Ras binding activity was normalized to β-tubulin expression and expressed as a fold increase. (F) BAEC were cotransfected with IgκB-LUC and dominant-negative small G proteins and stimulated with thrombin for 8 h. Normalized luciferase activity was expressed as a fold increase. In all experiments, values are the mean±S.D. for three separate experiments. *, $p<0.05$ versus control with thrombin.

data suggest that Ras and Rac1 mediate thrombin-induced JNK activation and ICAM-1 expression in endothelial cells.

### 3.6. Thrombin-induced JNK activation and ICAM-1 expression is mediated by the Src kinase family

G-protein coupled receptors, including PAR1, activate the Src kinase family [16,17,21]. Treatment of cells with the Src kinase family inhibitor, PP2, attenuated thrombin-induced JNK activation in a concentration-dependent manner (Fig. 6A). Similarly, expression of an endogenous inactivator of the Src family kinases, Csk, inhibited thrombin-induced JNK activation and ICAM-1 promoter activity by 50±5% and 62±7%, respectively (Fig. 6B, C). Consistently, PP2 treatment resulted in decreased thrombin-induced ICAM-1 protein expression (Fig. 6D).

To examine the role of $G\alpha_q$ in thrombin-induced activation of c-Src, HUVEC were treated with YM-254890. YM-254890 significantly attenuated thrombin-induced activation of c-Src by 38±15% (Fig. 6E). Furthermore, to elucidate the relationship between c-Src and Ras, we examined the effect of PP2 on Ras activity. As a result, PP2 potently attenuated thrombin-induced Ras by 67±7% (Fig. 6F). These data, in combination with those shown in Fig. 5E, suggest that c-Src mediates the signal to JNK and ICAM-1 expression in response to thrombin at a point downstream of $G\alpha_q$ and upstream of Ras.

PP2 did not inhibit thrombin-induced degradation of IκB-α, suggesting that the Src kinase family does not affect activation of NF-κB (Fig. 6G). Consistent with these data, overexpression of Csk or dominant-negative Src and Fyn did not affect thrombin-induced NF-κB promoter activity (data not shown).

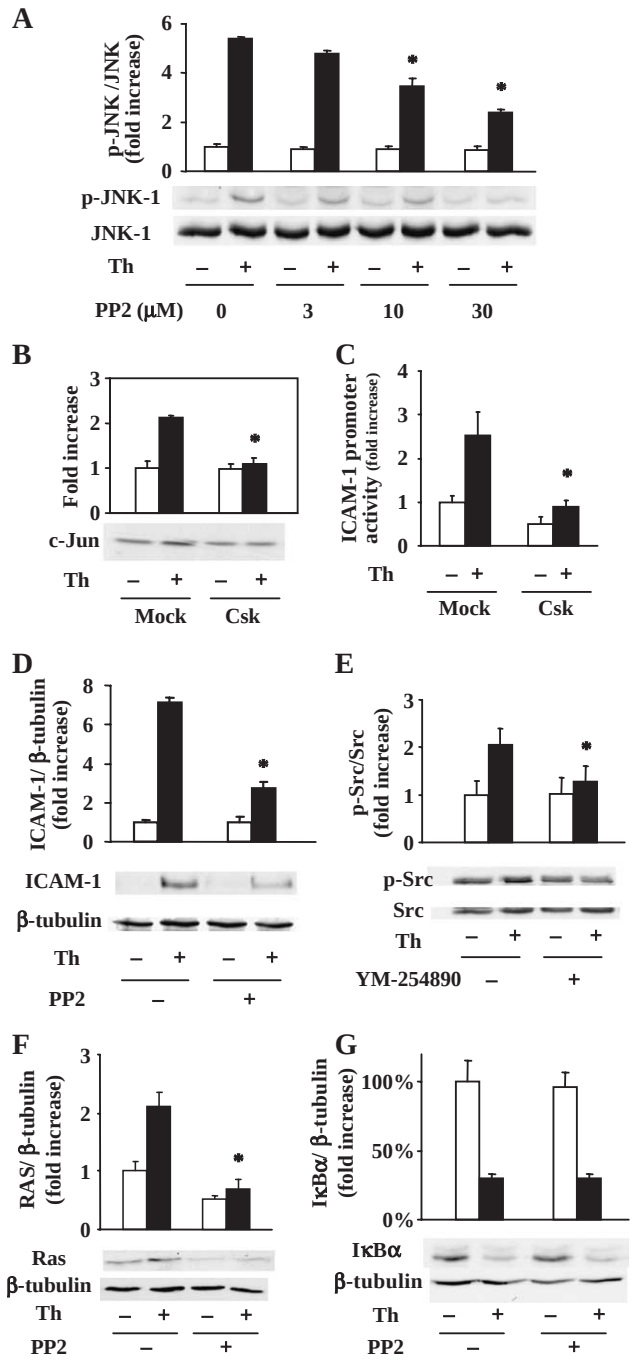


Fig. 6. Effects of Src kinase family on thrombin-induced JNK activation and ICAM-1 expression. (A) HUVEC were pretreated with PP2 (0–30 μM) for 30 min prior to challenge with thrombin (th) for 10 min. Phosphorylated JNK expression was detected by Western blot analysis and normalized to that of JNK in the bar graph. (B) JNK kinase assay. BAEC coexpressing FLAG-tagged JNK and Csk were stimulated with thrombin for 20 min. The bar graphs represent quantified levels of JNK activity as a fold increase. (C) Luciferase assay. BAEC, cotransfected with ICAM-LUC and Csk, were challenged with thrombin for 8 h. Normalized luciferase activity was expressed as a fold increase. HUVEC were pretreated with PP2 (30 μM) for 30 min prior to challenge with thrombin for 12 h (D) or 1 h (G), respectively. Expression of ICAM-1 (D) and IκB-α (G) were detected by Western blot analysis. (E) HUVEC were pretreated with YM-254890 (1 μM) for 1 h and stimulated with thrombin for 30 s. Phosphorylated Src and Src were detected by Western blot analysis. (F) Ras activation assay. HUVEC were pretreated with PP2 (30 μM) prior to challenge with thrombin for 5 min. Ras binding activity was normalized to β-tubulin expression and expressed as a fold increase. In all experiments, values are the mean±S.D. for three separate experiments. *, $p<0.05$ versus control with thrombin.

## 4. Discussion

The present study demonstrated that JNK is involved in thrombin-induced ICAM-1 expression in vascular endothelial cells. This study also presented the novel finding that the signal transduction cascade leading to JNK-dependent ICAM-1 expression involves $G\alpha_q$, $G\beta\gamma$, Ras, Rac1 and the Src kinase family. Further, inhibition of ERK and p38 MAP kinase had only slight effect on thrombin-induced ICAM-1 expression or ICAM-1 promoter activity, indicating that these kinases play only minor roles in this pathway. By contrast, Rahman et al. reported that the p38 MAP kinase inhibitor, SB203580, significantly inhibited thrombin-induced ICAM-1 expression [22]. The cause of this

discrepancy is unknown, but it is possible that the difference in HUVEC sources could result in a differential signaling mechanism for thrombin-induced ICAM-1 expression. De Cesaris et al. have demonstrated that SB203580 did not inhibit ICAM-1 expression in another setting [22,23], which is consistent with findings from the present study.

ERK and JNK are independently regulated by Ras and Rac1/Cdc42, respectively, in many cell types [24]. However, in the present study, thrombin-induced JNK activation and ICAM-1 promoter activity was inhibited by dominant-negative Ras as well as by dominant-negative Rac1. Several investigators have reported that Ras mediates JNK activation by various stimuli, including the response to v-Src and shear stress [25,26]. While the mechanism by which Ras activates JNK is largely unknown, Ras may activate Rac-guanine nucleotide exchange factors (GEF), such as Vav and Sos-1, via PI3K in several cell types [27]. It is unlikely that Ras and Rac1 independently regulate ICAM-1 expression via ERK and JNK, respectively, because inhibition of ERK by PD98059 had no effect on ICAM-1 expression in the present study. Alternatively, Ras may converge with the JNK pathway at a point upstream of Rac-GEFs.

PAR1 is the main thrombin receptor expressed in endothelial cells and is coupled to $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_i$ [1]. The present study focused on $G\alpha_q$ using the selective inhibitor, YM-254890, which does not inhibit $G\alpha_{12/13}$ or $G\alpha_i$ [20]. YM-254890 significantly inhibited JNK activation and ICAM-1 expression. In addition, the expression of constitutively active $G\alpha_q$ resulted in an increase in ICAM-1 promoter activity. These data indicate that $G\alpha_q$ plays a crucial role in thrombin-induced JNK activation and ICAM-1 expression. Further, these data are supported by a study by Nagao et al., which demonstrated that $G\alpha_{q/11}$ stimulates JNK activity through the Src family kinase [28]. It is possible that another Gα member, such as $G\alpha_{12/13}$, is also involved in thrombin-induced JNK activation and ICAM-1 expression. Indeed, constitutively active $G\alpha_{13}$ increased ICAM-1 promoter activity in endothelial cells (unpublished data), and Collins et al. demonstrated that the expression of constitutively active $G\alpha_{12}$ increased JNK activity [29]. In addition, Rahman et al. showed that the expression of constitutively active $G\alpha_q$ increased NF-κB activity [30]. Thus, $G\alpha_q$ may be involved in thrombin-induced JNK and NF-κB activation, both of which mediate ICAM-1 expression.

The βγ dimers of heterotrimeric proteins regulate the activity of many signaling molecules [31]. In the present study, expression of the carboxyl terminus of the β-adrenergic receptor kinase, which acts as a Gβγ scavenger, inhibited thrombin-induced JNK activation and ICAM-1 promoter activity. Several studies have reported involvement of βγ subunits in GPCR-induced activation of JNK [32,33] via Ras and Rac1. Further, Kiyono et al. showed that free βγ dimers activate Ras-GRFs, which serve as Ras GEF, as well as induce guanine-nucleotide exchange on Ras and Rac1 [34]. These data indicate that βγ subunits, in coordination with Gα subunits, mediate thrombin-induced JNK activation, possibly through Ras and Rac1.

Several investigators have demonstrated involvement of c-Src and other Src kinase family members in GPCR signaling [21], and we previously demonstrated that angiotensin II-induced c-Src activation regulated ERK activation and cytoskeletal reorganization in vascular smooth muscle cells [16,17,35]. In the present study, inhibition of the Src kinase family by expression of Csk resulted in marked suppression of JNK and ICAM-1 promoter activity, and YM-254890 significantly inhibited c-Src activation by thrombin stimulation. Furthermore, thrombin-induced Ras activation was inhibited by YM-254890 and by PP2. These data suggest that the thrombin-induced JNK activation and ICAM-1 expression are mediated, at least in part, via the $G\alpha_q$-Src-Ras axis, possibly in concert with Gβγ. Indeed, Kiyono et al. showed that the GEF activity of Ras-GRF1 toward Ras and Rac1 is dependent on phosphorylation by Src [36].

The present study did not elucidate the transcription mechanism by which JNK mediates thrombin-induced ICAM-1 expression. JNK phosphorylates c-Jun and ATF-2 and increases their ability to activate transcription, leading to c-*jun* induction and subsequent AP-1 activation [37,38]. ICAM-1 gene expression is also modulated by multiple *cis*-acting elements, including binding sites for AP-1, NF-κB and SP-1 [39], and thrombin stimulates AP-1 DNA binding and AP-1 mediated transactivation [40]. These data are consistent with those reported by Kobuchi et al., which showed that PMA and TNF-α induced ICAM-1 expression via the activation of the JNK pathway and AP-1 [12], and suggest that JNK-dependent ICAM-1 expression is mediated by AP-1.

Although previous studies have reported that NF-κB mediates thrombin-induced ICAM-1 expression [9,22], the present data suggest that the JNK pathway also plays a significant role in the signaling cascade leading to induction of ICAM-1 expression. NF-κB inhibition resulted in a significant, but not complete attenuation of thrombin-induced ICAM-1 expression. Further, inhibition of both JNK and NF-κB resulted in an additive inhibitory effect on ICAM-1 expression. Neither thrombin-induced IκB-α degradation nor NF-κB promoter activity was affected by inhibition of JNK, small G proteins or the Src kinase family, which suggests that the thrombin-activated JNK pathway and the NF-κB pathway are largely separate. De Cesaris et al. showed that TNF-α up-regulates ICAM-1 expression via activation of JNK, and Gorgoulis et al. reported that p53-mediated induction of ICAM-1 after DNA damage occurs via an NF-κB-independent mechanism [10,11], which is consistent with data from the present study.

In order to characterize which signaling molecules were involved in thrombin-induced ICAM-1 expression, we performed experiments with Western blot analysis and reporter assays with dominant-negative mutants. The data from both sets of experiments were comparable, except in

the case of Ras and Rac1, in which experiments could not be conducted because selective inhibitors for these molecules are not available. Thus, future studies to confirm the involvement of Ras and Rac1 in thrombin-induced increases in ICAM-1 expression are warranted.

In summary, the present study demonstrated that thrombin-induced ICAM-1 expression is mediated by JNK in vascular endothelial cells, and that this pathway likely involves $G\alpha_q$, $G\beta\gamma$, Ras, Rac1 and Src family kinases. These data support the potential clinical utility of therapeutic targeting of JNK for prevention of atherosclerosis.

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