Original Article

Angiotensin II–Induced Osteopontin Expression in Vascular Smooth Muscle Cells Involves $G_{q/11}$, Ras, ERK, Src and Ets-1

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Recent studies suggest that osteopontin (OPN) plays a critical role in the progression of atherosclerotic plaques and that angiotensin II (Ang II) is a potent upregulator of OPN expression. The goal of the present study was to characterize the signaling mechanisms whereby Ang II increases OPN expression in vascular smooth muscle cells (VSMC). YM-254890, a specific inhibitor of $G_{q/11}$, potently suppressed Ang II–induced OPN expression and ERK1/2 activation. Among dominant-negative (DN) mutants of small G proteins, only DN-Ras suppressed Ang II–induced OPN promoter activity. DN-MEK1 markedly inhibited Ang II–induced OPN promoter activity, while neither DN-JNK nor DN-p38 MAP kinase had any effect. DN-Src and DN-Fyn suppressed Ang II–induced OPN promoter activity. YM-254890 inhibited Ang II–induced Src and Ras activation, and PP2, a selective inhibitor for the Src kinase family, inhibited Ras activation, suggesting that the $G_{q/11}$-Src-Ras axis is the upstream signaling cascade for Ang II–induced OPN expression. Finally, small interfering RNA against Ets-1 suppressed Ang II–induced OPN expression. In conclusion, these data suggest that Ang II–induced OPN expression in VSMC is mediated by signaling cascades involving $G_{q/11}$, the Ras-ERK axis, and the Src kinase family, and by the transcription factor, Ets-1. These signaling molecules may represent therapeutic targets for the prevention of pathological vascular remodeling. (*Hypertens Res* 2008; 31: 987–998)

***Key Words*: angiotensin II, osteopontin, vascular smooth muscle cells, signal transduction, vascular remodeling**

Introduction

Osteopontin (OPN) is an acidic, secreted glycoprotein that binds to $\alpha_v\beta_3$ and other integrins *via* its arginine-glycine-aspartate (RGD) motif (*1*). It is produced by osteoblasts, macrophages, vascular smooth muscle cells (VSMC) and T cells in response to biological stimuli (*e.g.*, growth factors, glucose, and mechanical stimuli) and plays various roles in bone morphogenesis, cell transformation, immune cell activation, and bacterial resistance (*1–3*). OPN is not typically present in normal blood vessels, but its expression is increased in human

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This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T.I. and M.I.); by research grants from the Takeda Science Foundation (to T.I. and M.I.); by funds from the Japan Heart Foundation/Zeria Pharmaceutical Grant for Research on Molecular Cardiology (to T.I. and M.I.); and by research grants from the Tsuchiya Memorial Foundation (to T.I and M.I.).

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Received August 6, 2007; Accepted in revised form December 10, 2007.

atherosclerotic plaques, neointima after experimental angioplasty, and transplant arteriosclerosis (*4–6*). Studies have demonstrated that OPN promotes migration, extracellular matrix invasion, and proliferation of VSMC (*5*, *7*). Furthermore, OPN transgenic mice show exaggerated atherosclerosis and neointimal formation (*8*, *9*), while OPN deficiency attenuates atherosclerosis (*10*, *11)*. These findings indicate that OPN plays an important role in the development of atherosclerosis and restenosis after coronary intervention.

OPN expression is induced by angiotensin II (Ang II) in various cell types, including VSMC, macrophages, and epithelial cells of renal tubules, and Ang II–induced OPN expression is associated with heart failure, kidney disease, and atherosclerosis (*12*, *13*). Although the pathogenic significance of Ang II–OPN expression is increasingly recognized, the signaling mechanisms whereby Ang II induces OPN expression remain poorly understood.

We and other investigators have reported that Ang II activates various signaling molecules that possess trophic properties, including the MAP kinase family, Akt, c-Src, and the epidermal growth factor (EGF) receptor (*14*). Therefore, the goal of the present study was to characterize the signaling molecules that mediate Ang II–induced OPN expression, with a focus on heterotrimeric G proteins, small G proteins and the MAP kinase family.

Methods

Reagents

Angiotensin II and actinomycin D were purchased from Sigma-Aldrich (St. Louis, USA). PP2, PD98059, U0126, SP600125 and SB203580 were purchased from Calbiochem (San Diego, USA). YM-254890 was provided by Astellas Pharma Inc. (Tokyo, Japan). Valsartan was provided by Novartis Pharma AG (Basel, Switzerland). Monoclonal antibody against OPN ($MPIIIB10_1$) was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa. Antibodies against phospho-ERK (phospho-p44/42 MAP kinase) and phospho-Src family (Tyr416) were purchased from Cell Signaling Technology, Inc. (Danvers, USA). Antibodies against phospho-tyrosine (PY20) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). A monoclonal antibody against Src (clone 327) was purchased from Calbiochem. An antibody against Pyk2 was purchased from BD Biosciences (Franklin Lakes, USA).

Plasmids

The plasmids for kinase-inactive MEK1/MKK1 (pCMV-HA-MKK1 S222A), kinase-inactive JNK (pcDNA3-M3-JNK-APF-mut) and kinase-inactive p38α MAP kinase (TGY→AGF mutants) were provided by Dr. Y. Gotoh (University of Tokyo, Japan), Dr. R. Davis (University of Massachusetts, USA), and Dr. J. Han (The Scripps Research Institute, USA), respectively. Dominant-negative mutants of Ras and dominant-negative Rac were gifts from Dr. N. Nathanson (University of Washington, USA) and Dr. J. S. Gutkind (National Institute of Dental Research, National Institutes of Health, USA), respectively. Kinase-inactive Src and Fyn were provided by Dr. S. Courtneidge (Sugen Inc., San Francisco, USA). Kinase-inactive Pyk2/CADTK was provided by Dr. H. S. Earp (University of North Carolina at Chapel Hill, USA).

Cell Culture

VSMC were prepared from thoracic aorta of Sprague Dawley rats by enzyme dispersion and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum, as previously described (*15*). The cultures were maintained at 37°C in a 5% CO_2/95% O_2 humidified atmosphere. Cells were between passages 4–15 for all experiments.

RNA Isolation and Real-Time PCR

RNA isolation and real-time PCR were performed as described previously (*16*). In brief, cells were lysed in TRIzol (Invitrogen, Carlsbad, USA) and then mixed with chloroform. The lysate was then centrifuged to separate RNA, DNA, and protein. Total RNA was recovered, precipitated with isopropanol, washed in 80% ethanol to remove impurities, and finally dissolved in water. Next, 2.0 μg of RNA was reverse-transcribed into cDNA with random primer (Toyobo, Osaka, Japan) using a ReverTra Ace-α-TM First-Strand cDNA Synthesis Kit (Toyobo), as described in the manufacturer's instructions. The mRNA levels were analyzed by real-time PCR using a DNA Engine Opticon 2 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA) as described in the manufacturer's instructions. Specific primers for rat OPN, Ets-1, and β-actin were obtained from Takara Biomedics (Tokyo, Japan). OPN and Ets-1 mRNA expression were normalized to housekeeping β-actin expression.

Preparation of Serum-Free Conditioned Medium

Rat VSMC were seeded in complete medium and incubated to 70–80% confluence. The cells were washed twice with phosphate-buffered saline (PBS) and incubated in serum-free DMEM for another 48 h. After the serum-free medium was replaced, cells were treated with Ang II for the indicated amount of time. Then, the conditioned medium was harvested and concentrated 15-fold with a centrifugal filter (Amicon Ultra-15TM; Millipore, Bedford, USA).

Western Blot Analysis

Western blot analysis was performed as described previously (*15*, *17*). In brief, cells were lysed in radioimmunoprecipita-

tion assay (RIPA) lysis buffer (20 mmol/L Tris [pH 7.8], 25 mmol/L NaF, 25 mmol/L sodium pyrophosphate, 150 mmol/L NaCl, 10% glycerol, 5 mmol/L EDTA, 1% Triton X, 0.1% SDS, 0.5% DOC, 1 mmol/L Na_3VO_4, 10 μg/mL leupeptin, and 1 mmol/L PMSF). The cell lysates were prepared by scraping, sonication, and centrifugation. 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.

Transient Transfection and Reporter Gene Assay

The luciferase reporter construct, OPN-1500Luc, consisted of the OPN promoter region spanning −1500 to +87 linked to the luciferase reporter gene. Cells were seeded in 12-well plates and utilized for experiments on the following day at a confluence of 60–80%. Cells were transfected with OPN-1500Luc along with constructs for dominant-negative (DN) mutants of various signaling molecules using Fugene 6 transfection reagent (Roche, Basel, Switzerland). Eight hours after introduction of the transfection reagents, cells were placed in serum-free medium for 48 h. Next, cells were treated with Ang II (100 nmol/L) for 12 h and then harvested. Luciferase activity in the cell lysate was measured using a Dual-Luciferase Assay Kit (Promega, Madison, USA).

RNA Interference

Small-interfering RNA (siRNA) against Ets-1 were purchased from Dharmacon Research (Lafayette, USA). These siRNA were transfected into rat VSMC with DharmaFECT2 (Dharmacon Research) using a protocol provided by the manufacturer.

Activated Ras Affinity Assay

A Ras Activation Assay Kit, which included GST-fusion proteins containing the Raf-binding domain (GST-Raf RBD), was purchased from Upstate (Lake Placid, USA). The assay was performed as described previously (*18*). Briefly, HUVEC were lysed in MLB (25 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 25 mmol/L NaF, 10% glycerol, 0.25% Na deoxycholate, 10 mmol/L $MgCl_2$, 1% Triton X-100, 1 mmol/L EDTA, 1% NP-40, 1 mmol/L 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 1 h. Bound proteins were collected by centrifugation, and pellets were washed 3 times with MLB and then resuspended in SDS sample buffer. Bound Ras was quantified by Western blot analysis with anti-Ras antibodies.

Rat Carotid Balloon Injury Model

All animals used in this study were treated in accordance with the Guidelines of the Institutional Animal Experimental Committee of Hiroshima University. Male, 10-week-old Wistar rats (275 to 320 g) were anesthetized with sodium pentobarbital (20 mg/kg, intraperitoneally). The left common carotid artery was denuded of endothelium and stretched with a 2 Fr Fogarty balloon catheter (Baxter Healthcare, Deerfield, USA), as described elsewhere (*19*). Rats were treated with valsartan (1 mg/kg/d) or vehicle continuously for 2 weeks using osmotic minipumps (Model 2002; Alzet Corporation, Cupertino, USA) that were implanted subcutaneously at the time of the surgery. Blood pressure was measured every 7 d by the tail-cuff method. Two weeks after balloon injury, rats were euthanized and perfusion-fixed with 4% paraformaldehyde. The injured arteries were excised and embedded in paraffin.

Histomorphometric Study

Five individual sections (3 μm each) were obtained from the middle of injured segments of each rat and stained with hematoxylin-eosin (HE) and Masson's trichrome (MT) stain. The intimal and medial areas were measured with a computerized digital image analysis system (NIH Image; National Institutes of Health) and averaged from 5 independent sections.

Immunohistochemistry

A monoclonal antibody against OPN (MPIIIB10_1; 1:200 dilution) was used as the primary antibody. The sections were visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, USA), with diaminobenzidine (DAB) as the substrate. Hematoxylin was used as a counter stain. Negative controls were established using nonimmune serum in place of the primary antibody. The degree of OPN immunoreactivity was determined as the percentage of OPN-positive area in the injured vessels.

Statistical Analysis

Results are expressed as the means±SEM. The statistical significance of the difference between groups was estimated using Student's *t*-test; values of $p<0.05$ were regarded as statistically significant.

This work was partly carried out at the Analysis Center of Life Science, Hiroshima University.

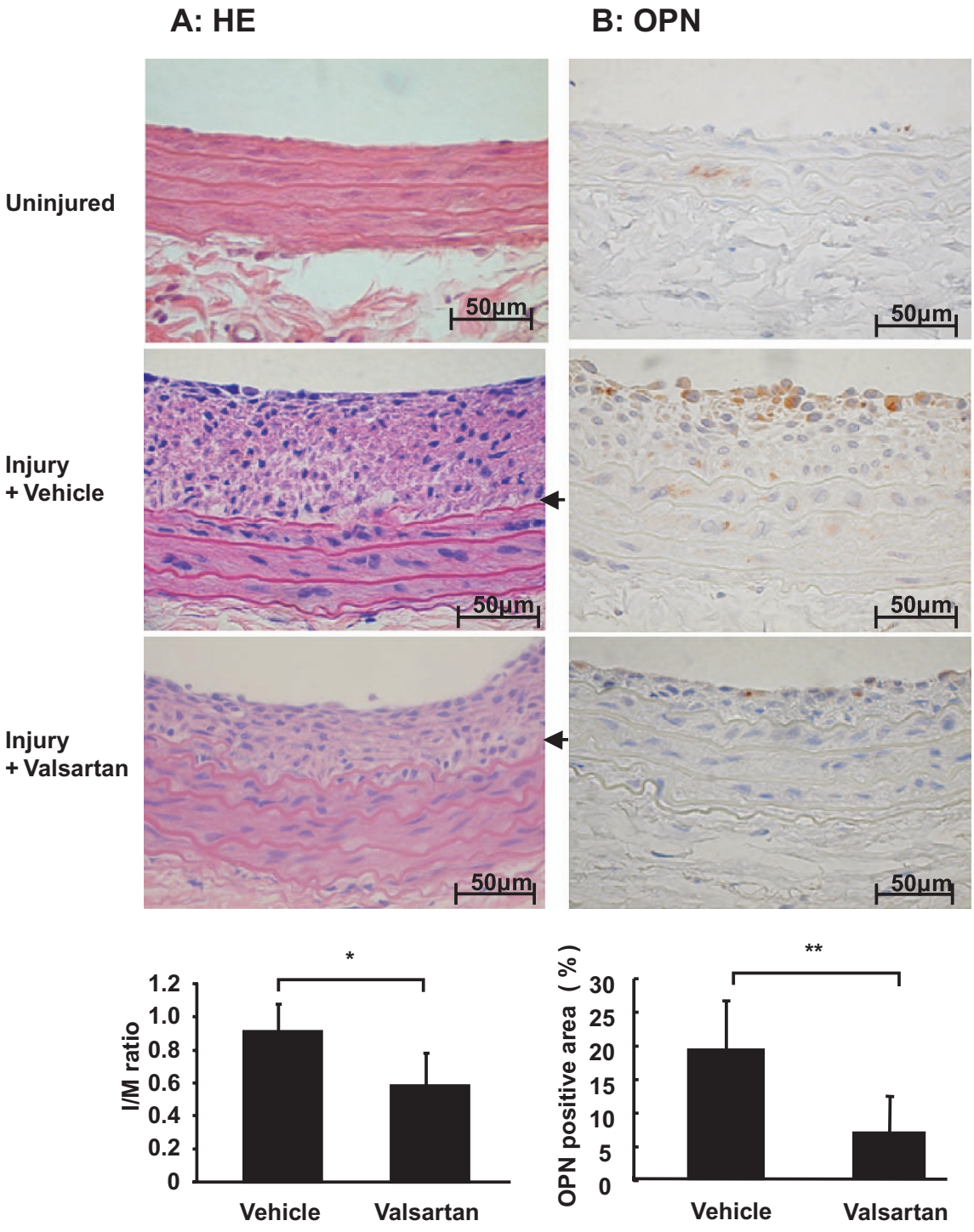


Fig. 1. *Effect of an angiotensin receptor blocker on rat carotid artery neointimal formation following balloon injury in vivo. A: Representative cross-sections of an injured carotid artery, an injured carotid artery treated with valsartan (1 mg/kg/d) for 2 weeks, and an uninjured carotid artery. Arrows indicate the internal elastic membrane. Original magnification, ×400. The graph shows morphometric analysis of the injured carotid arteries. Animals were treated with either vehicle (n=11) or valsartan (n=8). Mean ratios of intima to media (I/M ratio) are represented as the means±SEM. B: Effect of an angiotensin receptor blocker on in vivo OPN expression at 14 d after balloon injury. OPN expression in an injured carotid artery, an injured carotid artery treated with valsartan, and an uninjured artery are shown. The graph shows the percentage of OPN-positive area in the injured vessels. Animals were treated with either vehicle (n=11) or valsartan (n=8). Values are the means±SEM. *p<0.05, **p<0.01 vs. vehicle.*

Results

Role of Ang II in Neointimal Formation and OPN Expression in the Balloon-Injured Rat Carotid Artery

Previous studies have indicated that Ang II plays a critical role in neointimal formation following vascular injury. To investigate the role of Ang II in OPN expression in injured arteries, rats were treated with valsartan or vehicle for 2 weeks following balloon injury to the carotid arteries. No significant difference in blood pressure was found between these two groups (117±8 mmHg in the control group, 122±7 mmHg in the valsartan group). Two weeks after injury, a significant degree of neointimal hyperplasia was observed (Fig. 1A). Treatment with valsartan reduced the intima/media ratio by 36%. Valsartan administration also resulted in decreased OPN expression in the vessel wall following balloon injury by 64%, indicating that OPN expression in the injured artery is at least partially mediated by Ang II (Fig. 1B).

Ang II–Induced OPN Expression in Cultured VSMC

Ang II–induced OPN mRNA expression was measured by real-time PCR analysis. OPN mRNA levels increased in response to Ang II (100 nmol/L) beginning at 4 h and reaching a peak at 8–12 h in cultured rat VSMC (Fig. 2A). This induction of OPN mRNA was completely inhibited by 1 h of pretreatment with actinomycin D (data not shown). Similarly, Ang II (100 nmol/L) induced an increase in OPN protein expression in the conditioned medium of cultured rat VSMC in a time-dependent manner (Fig. 2B). An Ang II type-1 (AT_1) receptor blocker, valsartan, completely inhibited Ang II–induced OPN expression (Fig. 2C). These data indicate that Ang II–induced increases in OPN expression occur both at the mRNA and protein level, likely secondary to increases in transcription, and are mediated by AT_1 receptor.

Role of G Proteins in Ang II–Induced OPN Expression

Ang II binds to AT_1 receptor, which couples to the heterotrimeric G proteins $G_{q/11}$, G_i and $G_{12/13}$ (*20*). To determine whether $G_{q/11}$ mediates Ang II–induced OPN expression, a selective $G_{q/11}$ inhibitor, YM-254890, was utilized. Treatment of cells with YM-254890 resulted in marked dose-dependent attenuation of the Ang II–induced increases in OPN mRNA levels (by 93±2% with 100 nmol/L; Fig. 3A).

Ang II also activates multiple small GTP-binding proteins (*21*). Therefore, to study the role of the small G proteins, we performed reporter gene assays using the luciferase reporter construct for OPN, OPN-1500Luc. VSMC were transfected with OPN-1500Luc along with DN mutants of small G proteins (DN-Ras, DN-Rac, DN-Rho). DN-Ras markedly suppressed Ang II–induced OPN promoter activity, while DN-Rac and DN-Rho had no effect (Fig. 3B). These data indicate that, among the heterotrimeric and small G proteins, $G_{q/11}$ and Ras mediate Ang II–induced OPN.

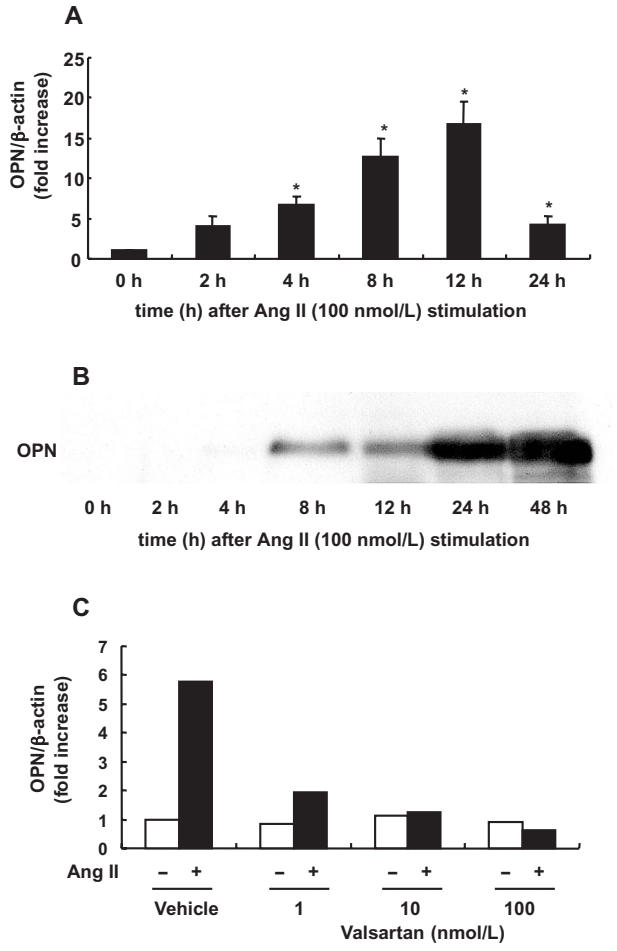


Fig. 2. *Time course of Ang II–induced OPN expression in rat VSMC. A: VSMC were treated with Ang II (100 nmol/L) for varying amounts of time following serum deprivation for 48 h. OPN mRNA levels were quantified by real-time RT-PCR and were normalized to β-actin mRNA levels. B: VSMC were treated with Ang II for varying amounts of time following serum deprivation. The conditioned medium was concentrated and assayed by Western blotting with OPN antibody, as described in the Methods section. C: Effect of valsartan on Ang II–induced OPN expression in VSMC. VSMC were pretreated with valsartan for 60 min prior to challenge with Ang II for 12 h. OPN mRNA levels were quantified by real-time RT-PCR and were normalized to β-actin mRNA levels. In all experiments, values are the means±SEM of at least three separate experiments. *$p < 0.05$ vs. the control (time-0 h).*

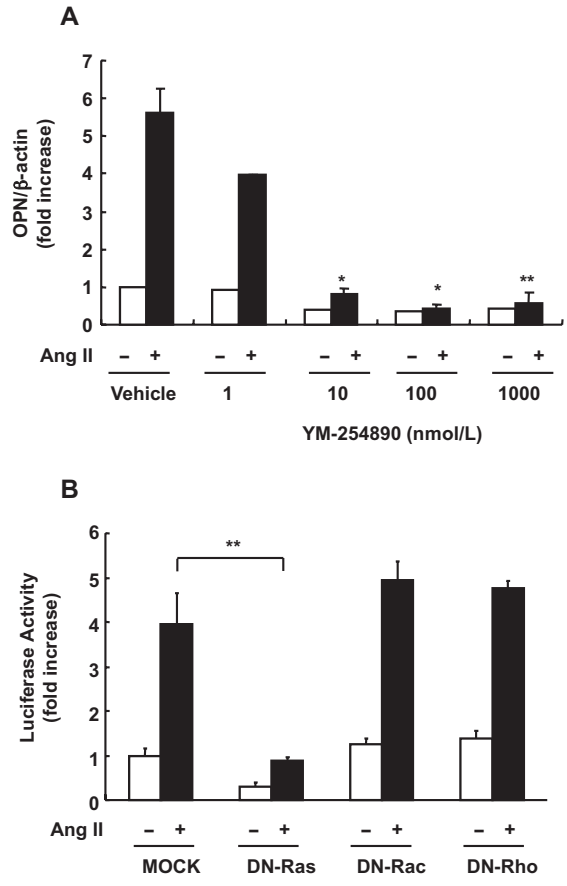


Fig. 3. *Role of G proteins in Ang II–induced OPN expression. A: VSMC were pretreated with YM-254890 for 30 min prior to challenge with Ang II for 12 h. OPN mRNA levels were quantified by real-time RT-PCR and were normalized to β-actin mRNA levels. B: VSMC were co-transfected with the OPN promoter–luciferase reporter construct and dominant-negative (DN) mutants of small G proteins. Eight hours after transfection, VSMC were serum-deprived and then treated with Ang II for 12 h. VSMC were then harvested, and luciferase activity was measured using a luminometer. In all experiments, values are the means±SEM of at least three separate experiments. *p < 0.05, **p < 0.01 vs. the control with Ang II.*

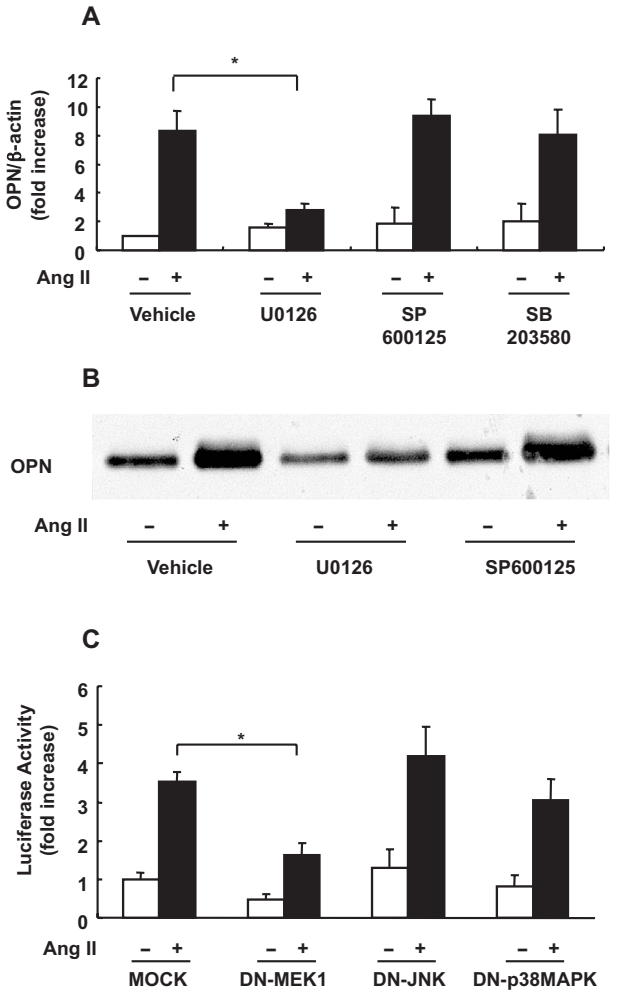


Fig. 4. *Role of MAP kinases in Ang II–induced OPN expression. A: VSMC were pretreated with U0126 (10 μmol/L), SP600125 (10 μmol/L), or SB203580 (10 μmol/L) for 1 h prior to challenge with Ang II for 12 h. OPN mRNA levels were quantified by real-time RT-PCR and normalized to β-actin mRNA levels. B: VSMC were pretreated with U0126, SP600125, or SB203580 for 1 h prior to challenge with Ang II for 48 h. The conditioned medium was concentrated and assayed by Western blotting with OPN antibody. C: VSMC were co-transfected with the OPN promoter–luciferase reporter construct and dominant-negative (DN) mutants of MEK1, JNK, and p38MAPK. Eight hours after transfection, VSMC were serum-deprived and then treated with Ang II for 12 h. VSMC were then harvested, and luciferase activity was measured using a luminometer. In all experiments, values are the means±SEM of at least three separate experiments. *p < 0.05 vs. the control with Ang II.*

ERK Mediates Ang II–Induced OPN Transcription

One of the major effectors of Ras is the Raf-MEK1-ERK cascade. We previously demonstrated that Ras is required for activation of Mnk1/2, which are kinases that are downstream of extracellular signal–regulated kinase (ERK) (*22*). To determine the role of ERK1/2 and other MAP kinase family members in Ang II–induced OPN expression, pharmacological inhibitors and kinase-inactive mutants of MEK1 (the kinase immediately upstream kinase of ERK), JNK, and p38MAPK were used. Treatment of VSMC with an MEK1 inhibitor, U0126, resulted in marked attenuation of the Ang II–induced increase in OPN mRNA levels (64±8% decrease; Fig. 4A). By contrast, treatment of cells with the JNK inhibitor, SP600125, or the p38MAPK inhibitor, SB203580, had no

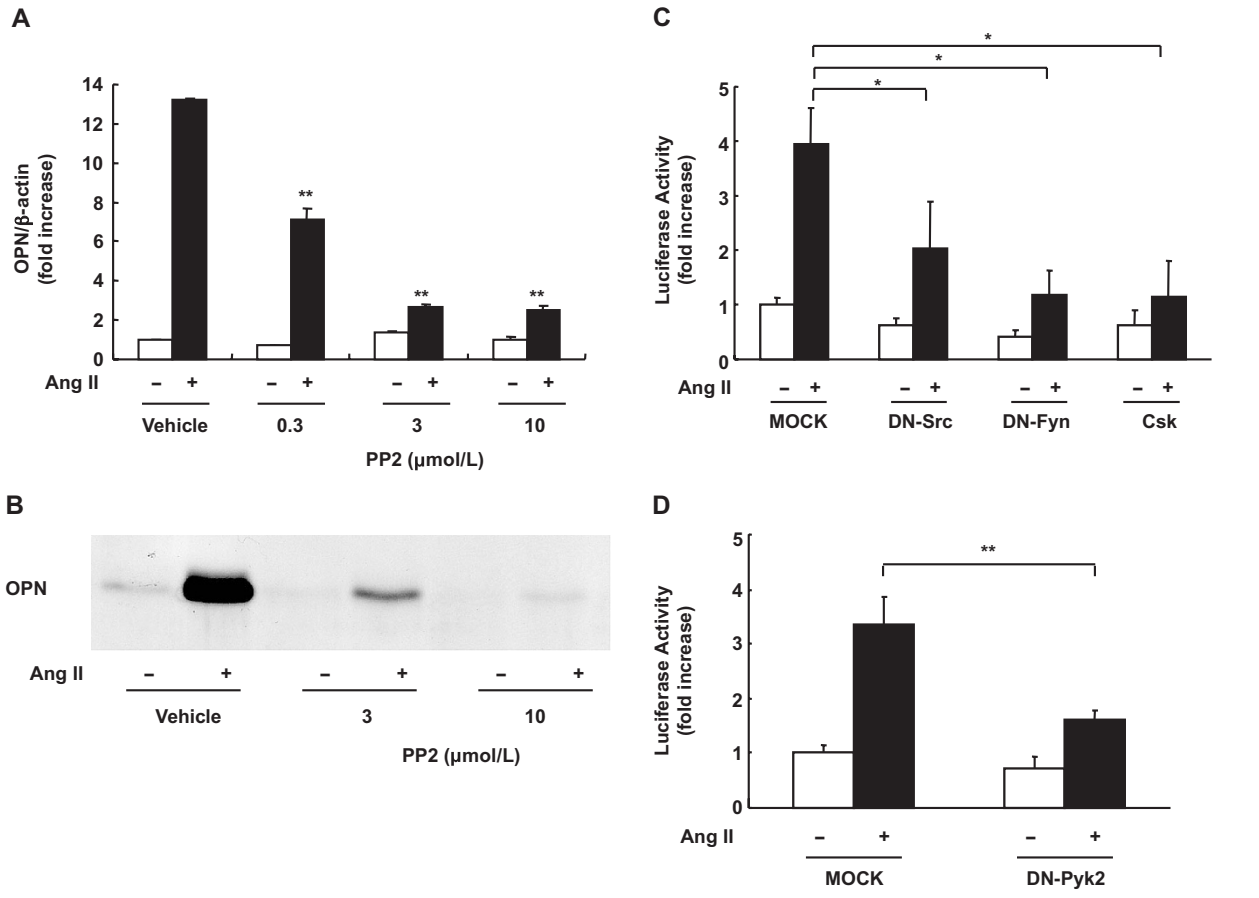
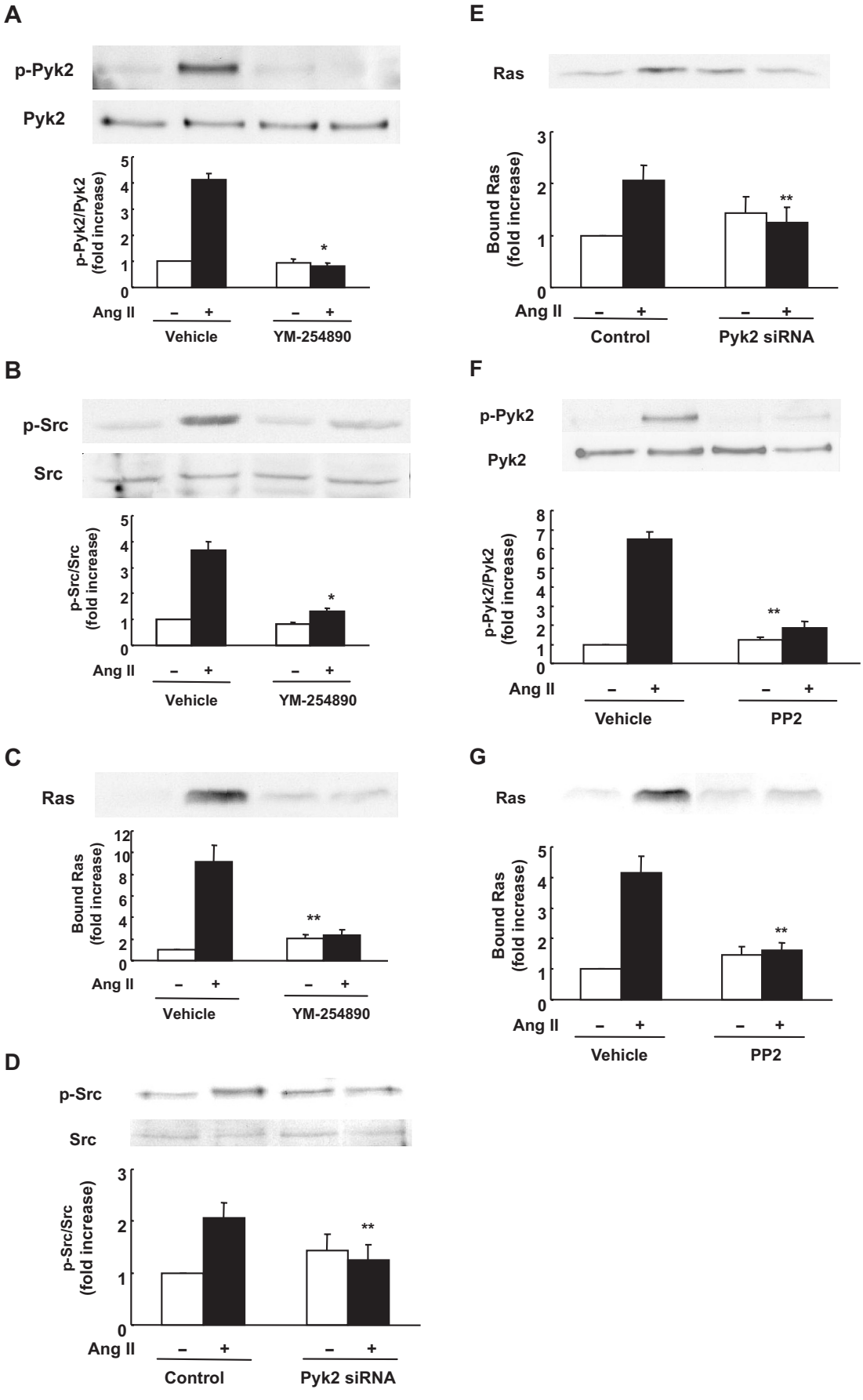


Fig. 5. *Role of the Src family in Ang II–induced OPN expression. A: VSMC were pretreated with PP2 for 1 h prior to challenge with Ang II for 12 h. OPN mRNA levels were quantified by real-time RT-PCR and normalized to β-actin mRNA levels. B: VSMC were pretreated with PP2 for 1 h prior to challenge with Ang II for 48 h. The conditioned medium was concentrated and assayed by Western blotting with OPN antibody. C: VSMC were co-transfected with the OPN promoter–luciferase reporter construct and DN-c-Src, DN-Fyn, or Csk. Eight hours after transfection, VSMC were serum-deprived and then treated with Ang II for 12 h. VSMC were then harvested, and luciferase activity was measured using a luminometer. D: VSMC were co-transfected with the OPN promoter–luciferase reporter construct and DN-Pyk2. Eight hours after transfection, VSMC were serum-deprived and then treated with Ang II for 12 h. VSMC were then harvested, and luciferase activity was measured using a luminometer. In all experiments, values are the means±SEM of at least three separate experiments. *$p < 0.05$, **$p < 0.01$ vs. the control with Ang II.*

effect on the Ang II–induced increase in OPN mRNA levels. Similarly, OPN protein expression in the conditioned medium of Ang II–treated VSMC was reduced by treatment with U0126, while SP600125 had no effect (Fig. 4B). The effect of SB203580 on OPN protein expression in the conditioned medium was not assessed, as prolonged SB203580 exposure (48 h or longer) resulted in cell toxicity (data not shown). To further delineate the role of the MAP kinase family in Ang II–induced OPN transcription, VSMC were transfected with OPN-1500Luc along with DN-MEK1, DN-JNK or DN-p38MAPK. Luciferase assay revealed that DN-MEK1 inhibited Ang II–induced OPN promoter activity by 54%, while DN-JNK and DN-p38MAPK had no effect (Fig. 4C). These data suggest that the Ras-ERK axis mediates Ang II–induced OPN transcription in VSMC.

Src and Pyk2 Are Required for Ang II–Induced OPN Transcription

c-Src is required for Ang II–induced ERK activation in VSMC (*17*). Next, therefore, we sought to determine the contribution of c-Src to Ang II–induced OPN expression. Treatment of cells with PP2, a selective inhibitor for the Src kinase family (0.3–10 μmol/L), attenuated the Ang II–induced increase in OPN mRNA levels and protein expression in a concentration-dependent manner (Fig. 5A and B). To determine which kinase among the Src kinase family is required for Ang II–induced OPN expression, VSMC were transfected with DN-Src, DN-Fyn, or Csk (a negative regulator of the Src kinase family). DN-Src, DN-Fyn, and Csk markedly suppressed Ang II–induced OPN promoter activity (Fig. 5C).


A
p-Pyk2
Pyk2
p-Pyk2/Pyk2 (fold increase)
Ang II
Vehicle
YM-254890
B
p-Src
Src
p-Src/Src (fold increase)
Ang II
Vehicle
YM-254890
C
Ras
Bound Ras (fold increase)
Ang II
Vehicle
YM-254890
D
p-Src
Src
p-Src/Src (fold increase)
Ang II
Control
Pyk2 siRNA
E
Ras
Bound Ras (fold increase)
Ang II
Control
Pyk2 siRNA
F
p-Pyk2
Pyk2
p-Pyk2/Pyk2 (fold increase)
Ang II
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Ras
Bound Ras (fold increase)
Ang II
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These data suggest that c-Src and Fyn both contribute to Ang II–induced OPN transcription.

As demonstrated above, Ang II–induced OPN expression was mediated by $G_{q/11}$ (Fig. 3A). $G_{q/11}$ activates phospholipase C, which produces inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm. To investigate whether Pyk2/CADTK, a Ca^{2+}-sensitive nonreceptor tyrosine kinase, participates in Ang II–induced OPN, VSMC were transfected with DN-Pyk2. DN-Pyk2 resulted in suppression of Ang II–induced OPN promoter activity by 52% (Fig. 5D), suggesting that Pyk2 is involved in Ang II–induced OPN expression.

Relationship among $G_{q/11}$, the Src Family, Pyk2 and Ras

Next, we determined the relationship among $G_{q/11}$, Ras, the Src kinase family and Pyk2 in Ang II–induced signaling events. YM-254890 markedly inhibited Ang II–induced activation of Pyk2, Src and Ras (Fig. 6A, B and C, respectively). Small interfering RNA (siRNA) against Pyk2 significantly suppressed Ang II–induced activation of Src and Ras (Fig. 6D and E, respectively). PP2 markedly inhibited Ang II–induced activation of Pyk2 and Ras (Fig. 6F and G, respectively). These data suggest that $G_{q/11}$ plays a role as the most upstream signaling molecule, and the Src kinase family and Pyk2 mediate Ang II–induced Ras activation at a point downstream of $G_{q/11}$. Interestingly, Src and Pyk2 appear to be required for each other (Fig. 6D and F).

Ets-1 Is Involved in Ang II–Induced OPN Expression

Multiple transcription factors, including Ets-1, Elk-1 and Sap-1, act as downstream molecules of ERK. Because the promoter region of OPN contains Ets-1–binding sites (*23*), experiments were conducted to determine whether Ets-1 is involved in Ang II–induced OPN expression. Ets-1 mRNA levels increased in response to Ang II with a peak at 2 h (Fig. 7A). YM-254890, U0126, and PP2 inhibited Ang II–induced Ets-1 mRNA expression by 86±2%, 49±5% and 47±7%, respectively (Fig. 7B), indicating that $G_{q/11}$, ERK, and the Src kinase family members are required for Ang II–induced Ets-1 expression. To investigate the role of Ets-1 in the transcription of OPN, siRNA against Ets-1 were introduced into rat VSMC. The knockdown of Ets-1 resulted in a 54±4% decrease in the Ang II–induced increase in OPN mRNA levels (Fig. 7C). These data suggest that Ets-1 plays a role in Ang II–induced OPN transcription in VSMC.

Discussion

OPN is one of the genes that is most strongly upregulated in response to Ang II (*24*). The present study demonstrates that Ang II–induced OPN expression in VSMC is mediated by $G_{q/11}$, Ras, and ERK, and also involves non-receptor tyrosine kinases, such as Pyk2/CADTK and the Src kinase family members. AT_1, the main Ang II receptor expressed in VSMC, is coupled to multiple heterotrimeric GTP-binding proteins, including $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_i$ (*20*). YM-254890, a selective $G_{q/11}$ inhibitor, completely inhibited Ang II–induced OPN expression, indicating that $G\alpha_{q/11}$ plays a crucial role in Ang II–induced OPN. In addition, it is suggested that $G\alpha_{q/11}$ is involved in the signaling mechanisms for Ang II–induced OPN expression at the most upstream point below AT_1 because YM-254890 potently inhibited the activation of Src, Pyk2 and Ras. To our knowledge, this is the first report to demonstrate that the activation of these signaling molecules by Ang II is mediated by $G_{q/11}$. A putative signaling cascade for Ang II–induced OPN expression is illustrated in Fig. 8.

DN-Ras, but not DN-Rac or DN-Rho, inhibited Ang II–induced OPN promoter activity. Among MAP kinase inhibitors, only U0126, a MEK1/2 inhibitor, reduced Ang II–induced OPN expression. In addition, only DN-MEK1 inhibited OPN promoter activity among kinase-inactive mutants of the MAP kinase family. Thus, while Ang II also activates small G proteins, Rac, Rho, and other MAP kinases (*e.g.*, JNK and p38MAPK) (*21*), the Ras-MEK1-ERK axis appears to be the primary mediator of Ang II–induced OPN expression. Interestingly, inhibition of Ras by dominant-negative Ras or pharmacological inhibitors attenuates atherosclerosis and neointimal formation after balloon injury (*25–27*).

Fig. 6. *Relationship among $G_{q/11}$, Src family, Pyk2 and Ras. A: VSMC were pretreated with YM-254890 (100 nmol/L) for 30 min prior to challenge with Ang II for 1 min. Active Pyk2 was detected by immunoprecipitation by anti-Pyk2, followed by Western blot analysis with anti-phosphotyrosine. In the graph, the phosphorylation of Pyk2 is normalized to the expression of Pyk2. B: VSMC were pretreated with YM-254890 (100 nmol/L) for 30 min prior to challenge with Ang II for 1 min. Active Src was detected by Western blot analysis with anti-phospho-Src (Tyr416) and normalized to the expression of Src in the bar graph. C: Ras activation assay. VSMC were pretreated with YM-254890 (100 nmol/L) for 30 min prior to challenge with Ang II for 2 min. D: VSMC were transfected with siRNA against Pyk2 or control siRNA (50 nmol/L). At 48 h after transfection, cells were treated with Ang II for 1 min. Active Src was detected by Western blot analysis with anti-phospho-Src (Tyr416) and normalized to the expression of Src in the bar graph. E: Ras activation assay. VSMC were transfected with Pyk2 siRNA-1 or control siRNA (50 nmol/L). At 48 h after transfection, cells were treated with Ang II for 2 min. F: VSMC were pretreated with PP2 (10 μmol/L) for 1 h prior to challenge with Ang II for 1 min. Active Pyk2 was detected by immunoprecipitation by anti-Pyk2, followed by Western blot analysis with anti-phosphotyrosine. In the graph, the phosphorylation of Pyk2 is normalized to the expression of Pyk2. G: Ras activation assay. VSMC were pretreated with PP2 (10 μmol/L) for 1 h prior to challenge with Ang II for 2 min. In all experiments, values are the means±SEM of at least three separate experiments. *$p < 0.05$, **$p < 0.01$ vs. the control with Ang II.*

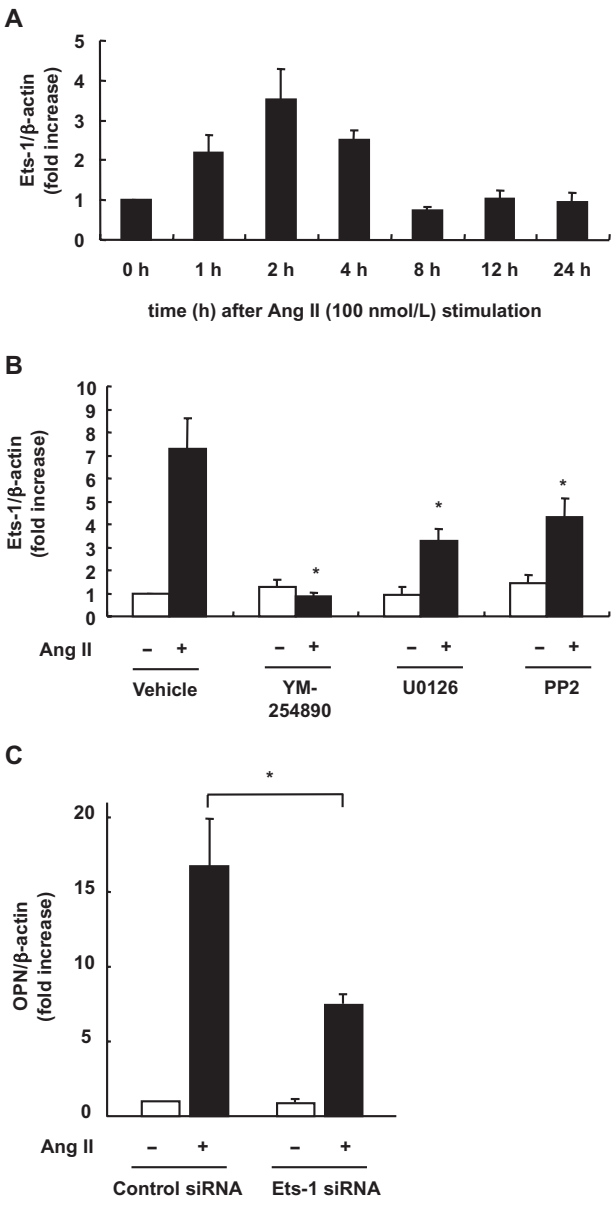

Fig. 7. *Ets-1 is involved in Ang II–induced osteopontin expression. A: VSMC were treated with Ang II for varying amounts of time following serum deprivation. Ets-1 mRNA levels were quantified by real-time RT-PCR and were normalized to β-actin mRNA levels. B: VSMC were pretreated with YM-254890 (100 nmol/L), U0126 (10 μmol/L) or PP2 (10 μmol/L). After pretreatment, cells were cultured with Ang II for 2 h. Ets-1 mRNA levels were quantified by real-time RT-PCR and were normalized to β-actin mRNA levels. C: VSMC were transfected with Ets-1 siRNA-1 or control siRNA (50 nmol/L). At 48 h after transfection, cells were treated with Ang II for 12 h. OPN mRNA levels were quantified by real-time RT-PCR and were normalized to β-actin mRNA levels. In all experiments, values are the means±SEM of at least three separate experiments. *$p < 0.05$, **$p < 0.01$ vs. the control with Ang II.*

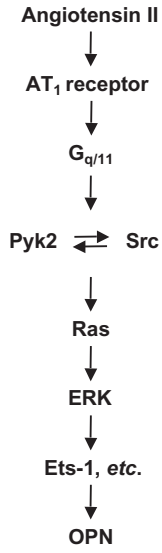

Fig. 8. *Putative signaling cascade for Ang II–induced OPN expression.*

$G_{q/11}$ activates phospholipase C, which produces IP_3 and DAG. IP_3 releases Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm, and DAG activates PKC. Eguchi *et al*. also showed that Ang II–induced ERK activation is Ca^{2+}-sensitive and PKC-independent in VSMC (*28*). Therefore, it is likely that Ca^{2+}-dependent signaling events mediate the interaction between Ang II–induced $G_{q/11}$ activation and ERK activation/OPN expression. Pyk2/CADTK is a non-receptor and Ca^{2+}-sensitive tyrosine kinase (*29*), and is rapidly activated by Ang II (*30*). In this study, siRNA against Pyk2 and kinase-inactive Pyk2 resulted in decreased Ang II–induced Ras activation and OPN promoter activity, respectively, suggesting that Pyk2 may be involved in the signaling mechanism for OPN expression at a point upstream of the Ras-ERK axis. Kinase-inactive Pyk2 inhibited Ang II–induced OPN promoter activity by only 52%. Another Ca^{2+}-dependent signaling event, such as heparin-binding–EGF shedding and the subsequent transactivation of the EGF receptor, may be involved in Ang II–induced ERK activation/OPN expression (*31*, *32*).

In the present study, Ang II–induced Ras activation and OPN expression were dependent on the Src kinase family. Previous reports have described activation of c-Src and Fyn by Ang II (*33*, *34*), and we previously described an important role for c-Src in Ang II–induced ERK activation (*17*). In this study, downregulation of Pyk2 expression by RNAi attenuated Ang II–induced Src activation, while pharmacological inhibition of the Src family resulted in decreased Pyk2 activation. Roellle *et al*. reported similar findings in the signaling mechanism initiated by another $G_{q/11}$-coupled receptor for neuropeptides (*35*). It is suggested that Pyk2 and Src depend on each other for their full activation, and the two tyrosine kinases may not be placed in a sequential order (*36*, *37*).

Ets-1 is a transcription factor that is regulated by ERK (*38*).

We and other investigators have shown that Ang II induces Ets-1 expression in VSMC (*39*). In the present study, siRNA against Ets-1 significantly suppressed Ang II–induced OPN expression, indicating that Ets-1 plays a role in Ang II–induced OPN transcription. Indeed, the promoter of the OPN gene has Ets-1 binding sites, and transcriptional regulation of the OPN gene by Ets-1 has been demonstrated in another cell type (*23*, *40*). Importantly, Ets-1 has recently been shown to play a critical role in Ang II–mediated vascular remodeling and inflammation (*41*). Thus, OPN may mediate Ang II–induced vascular remodeling as another target gene of Ets-1. In the present study, the inhibition of Ang II–induced OPN by siRNA against Ets-1 was incomplete. Perhaps Ets-1 regulates OPN expression in concert with other transcription factors, such as AP-1 in VSMC (*42*).

We and other investigators have demonstrated OPN expression in the neointima (*4*, *5*), and Ang II promotes neointimal formation in the context of atherosclerotic disease and after vascular injury (*43*, *44*). In the present study, valsartan attenuated OPN expression and neointimal formation in the balloon-injured rat carotid artery. Accumulating evidence suggest that OPN plays an important role in the development of atherosclerosis (*9*, *11*). Importantly, OPN disruption has been shown to attenuate Ang II–accelerated atherosclerosis and aneurysm formation (*10*). *In vitro* studies have demonstrated that OPN promotes migration and proliferation of VSMC, and accumulation of extracellular matrices by directly binding to collagen and fibronectin (*5*, *7*, *45*, *46*). Ang II–induced OPN expression may be involved in the development of atherosclerosis *via* these biological effects.

The present study demonstrated that Ang II–induced OPN expression in VSMC is mediated by signaling cascades involving $G_{q/11}$, the Ras-ERK axis, and the Src kinase family, and by the transcription factor, Ets-1. Other extracellular stimuli that cause VSMC proliferation, migration, and gene expression also share these signaling molecules (*15*, *47*). Thus, these signaling molecules may represent therapeutic targets for the prevention of pathological vascular remodeling in patients with atherosclerosis and hypertension.

Acknowledgements

The authors thank Lisa Hokama, Noriko Kuwaba, Mika Nobumoto, Kazuaki Neriishi, Atsuhiko Fukuto and Kumiko Takaki for their technical assistance.

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