0026-895X/01/6004-656–665$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 60:656–665, 2001

Vol. 60, No. 4
900/923912
Printed in U.S.A.

Quercetin Inhibits Shc- and Phosphatidylinositol 3-Kinase-Mediated c-Jun N-Terminal Kinase Activation by Angiotensin II in Cultured Rat Aortic Smooth Muscle Cells

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Received February 20, 2001; accepted May 23, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Angiotensin II (Ang II) induces vascular smooth muscle cell (VSMC) hypertrophy, which results in various cardiovascular diseases. Ang II-induced cellular events have been implicated, in part, in the activation of mitogen-activated protein (MAP) kinases. Although it has been proposed that daily intake of bioflavonoids belonging to polyphenols reduces the incidence of ischemic heart diseases (known as "French paradox"), the precise mechanisms of efficacy have not been elucidated. Thus, we hypothesized that bioflavonoids may affect Ang II-induced MAP kinase activation in cultured rat aortic smooth muscle cells (RASMC). Our findings showed that Ang II stimulated rapid and significant activation of extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), and p38 in RASMC. Ang II-induced JNK activation was inhibited by 3,3′,4′,5,7-pentahydroxyflavone (quercetin), a major bioflavonoid in foods of plant origin, whereas ERK1/2 and p38 activation by Ang II were not affected by quercetin. Ang II caused a rapid tyrosine phosphorylation of Src homology and collagen (Shc), which was inhibited by quercetin. Quercetin also inhibited Ang II-induced Shc·p85 association and subsequent activation of phosphatidylinositol 3-kinase (PI3-K)/Akt pathway in RASMC. Furthermore, LY294002, a PI3-K inhibitor and a quercetin derivative, inhibited Ang II-induced JNK activation as well as Akt phosphorylation. Finally, Ang II-induced [^{3}H]leucine incorporation was abolished by both quercetin and LY294002. These findings suggest that the preventing effect of quercetin on Ang II-induced VSMC hypertrophy are attributable, in part, to its inhibitory effect on Shc- and PI3-K–dependent JNK activation in VSMC. Thus, inhibition of JNK by quercetin may imply its usefulness for the treatment of cardiovascular diseases relevant to VSMC growth.

Angiotensin II (Ang II) is the main effector peptide of the renin-angiotensin system that plays an important role in several cardiovascular diseases associated with vascular smooth muscle cell (VSMC) growth and inflammation, including hypertension, atherosclerosis, and myocardial infarction (Baker et al., 1992). Ang II has been shown to stimulate protein synthesis and induce cellular hypertrophy in VSMC by acting through the G protein-coupled AT1 receptor (Griendling et al., 1994). A growing body of evidence suggests that the stimulation of the G protein-coupled AT1 receptor causes activation of not only phospholipase C, leading to increases in diacylglycerol and intracellular calcium, but also multiple signal-transduction cascades (Macrez-Lepretre et al., 1996). Ang II treatment of VSMC caused activation of p70S6K, p90 ribosomal S6 kinase, and phosphorylation of multiple protein tyrosine residues, including focal adhesion kinase, paxillin, janus kinase 2–signal transducers and activators of transcription 1, c-Src, proline-rich tyrosine kinase 2, and so on (Kim and Iwao, 2000). Furthermore, Ang II has recently been reported to activate extracellular signal-regulated kinases 1/2 (ERK1/2) (Tsuda et al., 1992), c-Jun N-terminal kinase (JNK) (Viedt et al., 2000), and p38 (Ushio-Fukai et al., 1998), which are members of mitogen-activated protein (MAP) kinases in VSMC.

The MAP kinases are a family of serine-threonine protein kinases that are activated in response to a variety of extracellular stimuli (Nishida and Gotoh, 1993). ERK1/2, JNK, and p38 constitute three major subfamilies of MAP kinases and seem to mediate cellular responses, including proliferation, differentiation, and apoptosis (Davis, 1993). Generally, ERK1/2 has been recognized to play a major role in cell proliferation and differentiation, as well as survival by var-

ABBREVIATIONS: Ang II, angiotensin II; VSMC, vascular smooth muscle cell; AT1, angiotensin type 1 receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; quercetin, 3,3′,4′,5,7-pentahydroxyflavone; RASMC, rat aortic smooth muscle cell; Shc, Src homology and collagen; PI3-K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; PKC, protein kinase C; K-H, Krebs-Henseleit bicarbonate buffer; PMA, phorbol 12-myristate 13-acetate; Grb2, growth factor receptor bound protein 2; ROS, reactive oxygen species; EGF, epidermal growth factor.

ious growth factors (Cobb and Goldsmith, 1995). On the other hand, various inflammatory cytokines and environmental stress that lead to cellular apoptosis or hypertrophy (Kyriakis and Avruch, 1996) activate JNK and p38. However, the precise roles of these three major MAP kinase signaling pathways in regulation of cellular phenotypic modulation are still unclear and may be cell type-specific (Liu et al., 1996).

Bioflavonoids are polyphenolic compounds that are ubiquitously present in foods of plant origin (Hollman and Katan, 1999). These constituents of the diet are believed to be important in the maintenance of health, especially to maintain the integrity of the cardiovascular system. Recently, much interest in flavonoids has been spurred from the findings of the "French paradox." This paradox refers to the correlation of a high-fat and high-cholesterol diet with a lower incidence of coronary heart disease found in Mediterranean cultures contrasted with a higher incidence of coronary heart disease among most Western cultures (Renaud and de Lorgeril, 1992). It has been shown that the French paradox may be attributable to regular consumption of red wine and that the unique antiatherogenic effects of red wine reside in the action of polyphenols (St. Leger et al., 1979). 3,3′,4′,5,7-Pentahydroxyflavone (quercetin) is one of the most widely distributed bioflavonoids, which are abundant in red wine, tea, and onions (Formica and Regelson, 1995). Like other bioflavonoids, quercetin has been shown to have biological properties consistent with its sparing effect on the cardiovascular system. Quercetin has been shown to modify eicosanoid biosynthesis (antiprostanoid and anti-inflammatory responses), protect low-density lipoprotein from oxidation (prevent atherosclerotic plaque formation), prevent platelet aggregation (antithrombin effects), and promote relaxation of cardiovascular smooth muscle (antihypertensive, antiarrhythmic effects) (Formica and Regelson, 1995). However, the effect of quercetin on MAP kinase activities in VSMC and resultant cellular phenotypic modulations, such as proliferation, hypertrophy, and apoptosis, has not yet been elucidated.

In the present study, we examined the effect of quercetin on Ang II-induced MAP kinase activation in cultured rat aortic smooth muscle cells (RASMC). In addition, we compared the effects of quercetin with LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3-K) originally developed from quercetin (Vlahos et al., 1994). The findings of the present study showed that quercetin inhibited Ang II-induced JNK activation, but not ERK1/2 and p38 activation in RASMC. Quercetin also inhibited tyrosine phosphorylation of Src homology and collagen (Shc) adapter protein, its association with p85, a component of the regulatory subunit of PI3-K, and activation of the PI3-K/Akt pathway by Ang II. LY290042, an inhibitor of PI3-K and a quercetin derivative, also inhibited both Akt phosphorylation and Ang II-induced JNK activation. Finally, the Ang II-induced increase in [^{3}H]leucine incorporation was abolished by both quercetin and LY290042 in RASMC. Thus, inhibition of JNK by quercetin may imply its usefulness for relief of cardiovascular diseases relevant to VSMC hypertrophy.

Materials and Methods

Chemicals. Human Ang II was purchased from Peptide Institute, Inc. (Osaka, Japan). Quercetin, Phorbol 12-myristate 13-acetate (PMA), staurosporine, and calphostin C were from Sigma Chemical Co. (St. Louis, MO). LY294002 was obtained from Calbiochem (Darmstadt, Germany). Fluo 3-acetoxymethyl ester for intracellular Ca^{2+} measurement and phosphatidylinositol and phosphatidylserine for PI3-kinese assay were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phospho-ERK1/2 antibody (Thr202/Tyr204), phospho-p38 MAP kinase antibody (Thr180/Tyr182), phospho-Akt (Ser473), and stress-activated protein kinase/JNK assay kit were purchased from New England Biolabs, Inc. (Beverly, MA). All other chemicals were reagent grade from commercial sources and were used without further purification.

Culture of Rat Aortic Smooth Muscle Cells. Thoracic aortae of male Sprague-Dawley rats were excised rapidly and immersed in Dulbecco's modified Eagle's medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Connective tissue and adherent fat were cleaned. Isolated arteries were cut open, and the endothelium was removed by gently rubbing off the intimal surface with sharp scissors. Denuded aortae were cut into ~3-mm pieces and placed with the intimal face down into three 35-mm culture dishes (Iwaki, Osaka, Japan). Dulbecco's modified Eagle's medium containing 10% fetal calf serum and penicillin/streptomycin was gently added to the dishes to cover the tissues without disturbing the orientation of the explants. Vascular smooth muscle cells were allowed to grow from the tissue (7–10 days), and the tissues were removed using sterilized fine forceps and washed with culture medium. After reaching confluence in three 35-mm dishes, cells were harvested by brief trypsinization and grown in T-75 flasks (Iwaki) (passage 1). Early subcultured cells in 35-mm dishes (from passages 2–5) with 48 h of serum starvation were used in all experiments. Purity of the vascular smooth muscle cells was estimated to be >90% by cell morphology and by the immunoexpression of myosin as described previously (Ohlstein et al., 1993). Cell viability was >98%, as determined by exclusion of 0.2% trypan blue.

Immunoprecipitation and Western Blot Analysis. After treatment, the cells were washed once with cold PBS containing sodium orthovanadate (1 mM). Cells were lysed (30–60 min at 4°C) with cell-lysis buffer consisting of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After being frozen and then thawed, lysed cells were sonicated (Handy Sonic UR-20P, Tomy Seiko Co., Ltd., Tokyo, Japan) on ice for 15 s and were transferred to microcentrifuge tubes and centrifuged at 16,000*g* for 20 min at 4°C. The protein concentrations of the supernatants were measured using a protein assay kit (Pierce Chemical, Rockford, IL). For immunoprecipitation, cell lysates were incubated with rabbit anti-Shc antibody (Upstate Biotechnology, Lake Placid, NY) for 3 h at 4°C and then incubated with 20 μl of protein G-Sepharose (Invitrogen, Carlsbad, CA) for 1 h on a roller system in 1 ml of LiCl wash buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, and 1 mM dithiothreitol) and twice in 1 ml of washing buffer (20 mM HEPES, pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1% Triton X-100). For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech, Buckinghamshire, England) as described previously (Yoshizumi et al., 2000). The membrane was blocked for 1 h at room temperature with a commercial blocking buffer (Amersham). The blots were then incubated for 12 h at 4°C with antiphosphotyrosine (P-Tyr-100, New England Biolabs) or anti-p85 antibody (Upstate Biotechnology), followed by incubation for 1 h with secondary antibody (horseradish peroxidase conjugated). Previously, we measured each MAP kinase activity using an in-gel kinase assay with specific substrates. However, we found that the activation of ERK1/2 or p38 by in-gel kinase assay and immunoblotting for phospho-ERK1/2 or phospho-p38 were highly correlated ($R^2 = 0.90$) in many type of cells (Abe et al., 2000). Therefore, we used immunoblotting for phosphospecific ERK1/2 and p38 to evaluate ERK1/2 and p38 activation

as described previously (Yoshizumi et al., 2000). For ERK1/2 and p38 activation, the blots were incubated for 12 h at 4°C with anti-phosphospecific ERK1/2 or p38 antibodies (New England Biolabs). Akt phosphorylation was also evaluated with anti-phosphospecific Akt antibody (New England Biolabs). Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham) and were quantified by densitometry in the linear range of film exposure using an Astra 2200 scanner (UMAX, Fremont, CA) and National Institutes of Health Image (ver. 1.60; http://rsb.info.nih.gov/nih-image/)

JNK Activity Assay. JNK activity was measured with the use of a commercially available kit based on phosphorylation of recombinant c-Jun (New England Biolabs). After treatment, cells were rinsed twice with ice-cold PBS, scraped off the plates into lysis buffer (included in the kit), and sonicated three times on ice. After removing the cell debris by centrifugation (16,000*g*, 20 min, 4°C), the protein content in the supernatant was measured using a protein assay kit (Pierce). Equal amounts of protein (300 μg) were then immunoprecipitated with c-Jun (1–89) fusion protein beads overnight. After washing the beads, kinase assays were performed according to the manufacturer's instructions. The beads were loaded onto a 10% SDS-polyacrylamide gel, and immunoblotting was performed using an antibody against phosphospecific c-Jun (Yoshizumi et al., 2000).

Measurement of Intracellular Ca^{2+} ($[Ca^{2+}]_i$) Using Confocal Laser Microscopy. For measurement of $[Ca^{2+}]_i$ in RASMC, cells cultured in 35-mm tissue culture dishes with cover-slip attachment were loaded with 2 mM fluo 3-acetoxymethylester at 37°C as described previously (Yoshizumi et al., 1998). After loading for 30 min, the solution was exchanged for a dye-free Krebs-Henseleit bicarbonate buffer solution (K-H solution) (135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.2 mM $CaCl_2$, and 10 mM glucose, adjusted with HCl to pH 7.40), and the cells were allowed to de-esterify the indicator for an additional 10 min. The fluorescence intensity of the dye, which represents $[Ca^{2+}]_i$, was analyzed using confocal laser microscopy as described previously (Yoshizumi et al., 1998). Briefly, a confocal imaging system (RCM 8000; Nikon, Tokyo, Japan) with an Argon-ion laser was attached to an inverted microscope (TMD300, Diaphot; Nikon). A culture dish containing cells in 1 ml of K-H solution was placed on the stage of the microscope, and the cells were excited at 488 nm by the laser. Emission at wavelengths longer than 520 nm was then detected by a photomultiplier. After measurement of stable baseline fluorescence intensity, 10 μl of Ang II was added to the extracellular medium, and the fluorescence intensity was recorded. After 1 min, the same cells were stimulated by the addition of 10 μl of ionomycin (final concentration, 10 μM), and the relative fluorescence intensity was calculated. The fluorescence intensity, to estimate $[Ca^{2+}]_i$, was calculated from the difference between F_{max} and F_{min}. To estimate F_{min}, the cells were washed five times with Ca^{2+}-free K-H solution containing 3 mM EGTA, and the resting $[Ca^{2+}]_i$ was determined 2 min later. F_{max} was estimated from the intensity at 1 min after the addition of ionomycin in each experiment. Results are expressed as a percentage of the difference between F_{max} and F_{min}.

In Vitro PI3-K Assay. The immunoprecipitates obtained with the anti-p85 antibody (New England Biolabs) were assayed according to the method used by Whitman et al. (1988) with minor modifications. The protein G-Sepharose pellets were washed three times with assay buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$) and resuspended in 90 μl of assay buffer containing 20 μM [^{32}P]ATP. The reaction was initiated by adding 10 μl of phosphoinositide mixture. The lipid substrates were prepared by sonicating equal quantities of phosphatidylinositol and phosphatidylserine in assay buffer to yield a final concentration of 200 μg/ml. After a 10-min incubation at 37°C, the reaction was stopped by the addition of 200 μl of 1 M HCl/methanol (1:1), and the samples were extracted twice with 200 μl of chloroform. The lipids were recovered from the combined organic phases by evaporation, suspended with 10 μl of chloroform, and analyzed by thin layer chromatography on Silica Gel G plates (Kieselgel60 F254; Merck, Darmstadt, Germany).

Measurement of [^{3}H]Leucine Incorporation into the Cells. Subcultured cells in 35-mm dishes with 48 h of serum starvation were used. They were then stimulated for 24 h with 100 nM Ang II. The stimulated cells were pulsed with 1 μCi/ml [^{3}H]leucine during the last 8 h of culture. Cells were washed once with PBS and twice with ice-cold 5% trichloroacetic acid to remove the unincorporated [^{3}H]leucine, then solubilized in 300 μl of 1 N NaOH in 0.1% SDS and neutralized. Aliquots of samples were added to 10 ml of scintillation fluid and counted (Aloka 703, Tokyo, Japan).

Statistical Analysis. Values are reported as the mean ± S.D. from experiments done in triplicate. Statistical analysis was performed using the StatView (ver. 4.0; SAS Institute Inc., Cary, NC). Differences were analyzed with the use of an unpaired two-tailed Student's *t* test or Welch's *t* test as appropriate, and P values < 0.05 were considered significant.

Results

Time Courses and Concentration-Response Curves for the Activation of ERK 1/2, JNK, and p38 in RASMC Stimulated by Ang II. To evaluate the relative magnitude of MAP kinase activation by Ang II, growth-arrested RASMC were exposed to 100 nM Ang II. ERK1/2, JNK, and p38 activation in the cell lysate were determined as described under *Materials and Methods*. As shown in Fig. 1, A and B, ERK1/2 was activated most rapidly (peak at 5 min) and to the greatest extent (7.35-fold). JNK and p38 were activated within 5 min and peaked 10 min after the stimulation by Ang II in RASMC. JNK and p38 activations were similar (6.44- and 6.74-fold, respectively). Thereafter, the activities of ERK1/2 and p38 gradually declined. In contrast, JNK activation was sustained for 60 min. Figure 1, C and D, shows the concentration-response curves for the activation of ERK1/2, JNK, and p38 in RASMC stimulated by Ang II. The concentration dependence for ERK1/2 activation was determined by a 5-min incubation period, and for JNK and p38 activation, by 10 min incubation. Ang II-induced ERK1/2, JNK, and p38 activation increased in a concentration-dependent manner (from 100 pM to 1 μM). Ang II-stimulated ERK1/2 and JNK activations were maximal at 1 μM, whereas p38 activation peaked at 100 nM (Fig. 1, C and D).

Effect of Quercetin on Ang II-Induced ERK1/2, JNK, and p38 Activation. To clarify whether quercetin affects Ang II-induced MAP kinase activation, we examined the effect of various concentrations of quercetin on Ang II-induced ERK1/2, JNK, and p38 activation (Fig. 2). The cells were pretreated with quercetin for 30 min before the addition of Ang II (100 nM), for 5 min for ERK1/2 activation, and for 10 min for JNK and p38 activation. Ang II-induced JNK activation was inhibited by quercetin in a concentration-dependent manner (1–100 μM) with an IC_{50} value of ≈1 μM. In contrast, ERK1/2 and p38 activations were not influenced by quercetin (Fig. 2). These findings suggest that JNK, but not ERK1/2 and p38, is specifically sensitive to quercetin in RASMC.

Effect of Quercetin on Ang II-Induced Increase in $[Ca^{2+}]_i$ and Influence of PKC Depletion and PKC Inhibition on Ang II-Induced JNK Activation. It has been reported that Ang II increases intracellular Ca^{2+} and activates protein kinase C (PKC) in VSMC (Macrez-Lepretre et al., 1996). In addition, because it was reported that quercetin

affects intracellular Ca^{2+} mobilization and PKC activity in VSMC (Duarte et al., 1993), we examined the effect of quercetin on Ang II-induced increase in $[Ca^{2+}]_i$ and the influence of PKC depletion and PKC inhibition on Ang II-induced JNK activation in RASMC. As shown in Fig. 3A, Ang II caused a rapid and significant increase in $[Ca^{2+}]_i$, which peaked within 5 s and then decreased. Quercetin pretreatment for 30 min did not affect basal $[Ca^{2+}]_i$ as well as the Ang II-induced increase in $[Ca^{2+}]_i$ in RASMC. Figure 3B shows the influence of PKC depletion by PMA (1 μM) treatment for 24 h and PKC inhibition by pretreatment with staurosporine (1 μM) and calphostin C (100 nM) on Ang II-induced JNK activation and its inhibition by quercetin. Neither PKC depletion nor PKC inhibition affected Ang II-induced JNK activation or influenced quercetin inhibition of JNK activation by Ang II in RASMC. These findings suggest that inhibition by quercetin of Ang II-induced JNK activation is independent of intracellular Ca^{2+} and PKC in RASMC.

Ang II-Induced Shc Tyrosine Phosphorylation and Its Inhibition by Quercetin. Next, to clarify the intracellular mechanism(s) of inhibition by quercetin of Ang II-induced JNK activation other than through $[Ca^{2+}]_i$ and PKC, we focused on the role of the Shc adapter protein because Shc has been reported to be involved in JNK activation in some cells (Hashimoto et al., 1999). Shc is expressed as three alternatively spliced proteins of 66 kDa, 52 kDa, and 46 kDa that share an Src homology 2 domain and a phosphotyrosine binding domain (Migliaccio et al., 1999). As shown in Fig. 4A, p66 and p52Shc were rapidly tyrosine phosphorylated by Ang II within 2 min, peaked at 5 min, and then decreased. p46 Shc was also slightly phosphorylated, although its expression in RASMC was abundant (Fig. 4A, bottom). Moreover, quercetin inhibited Ang II-induced Shc tyrosine phosphorylation in a concentration-dependent manner (Fig. 4B). These findings are similar to those of quercetin inhibition of Ang II-induced JNK activation (Fig. 2) and suggest the possibility that Shc may be involved in Ang II-induced JNK activation in RASMC.

Ang II-Induced Shc·P85 Complex Formation and PI3-K/Akt Activation and Their Inhibition by Quercetin. Shc has been reported to transmit signals to downstream targets through its bindings to other molecules, such as Grb2, Src homology 2 domain-containing inositol 5′-phosphatase, and p85, a regulatory subunit of PI3-K (Lioubin et al., 1996; Thomas and Bradshaw, 1997; Dupont et al., 1998). Therefore, we investigated whether Ang II stimulates Shc·P85 association, because it has been reported that PI3-K is involved in JNK activation in various cells (Lopez-Ilasaca

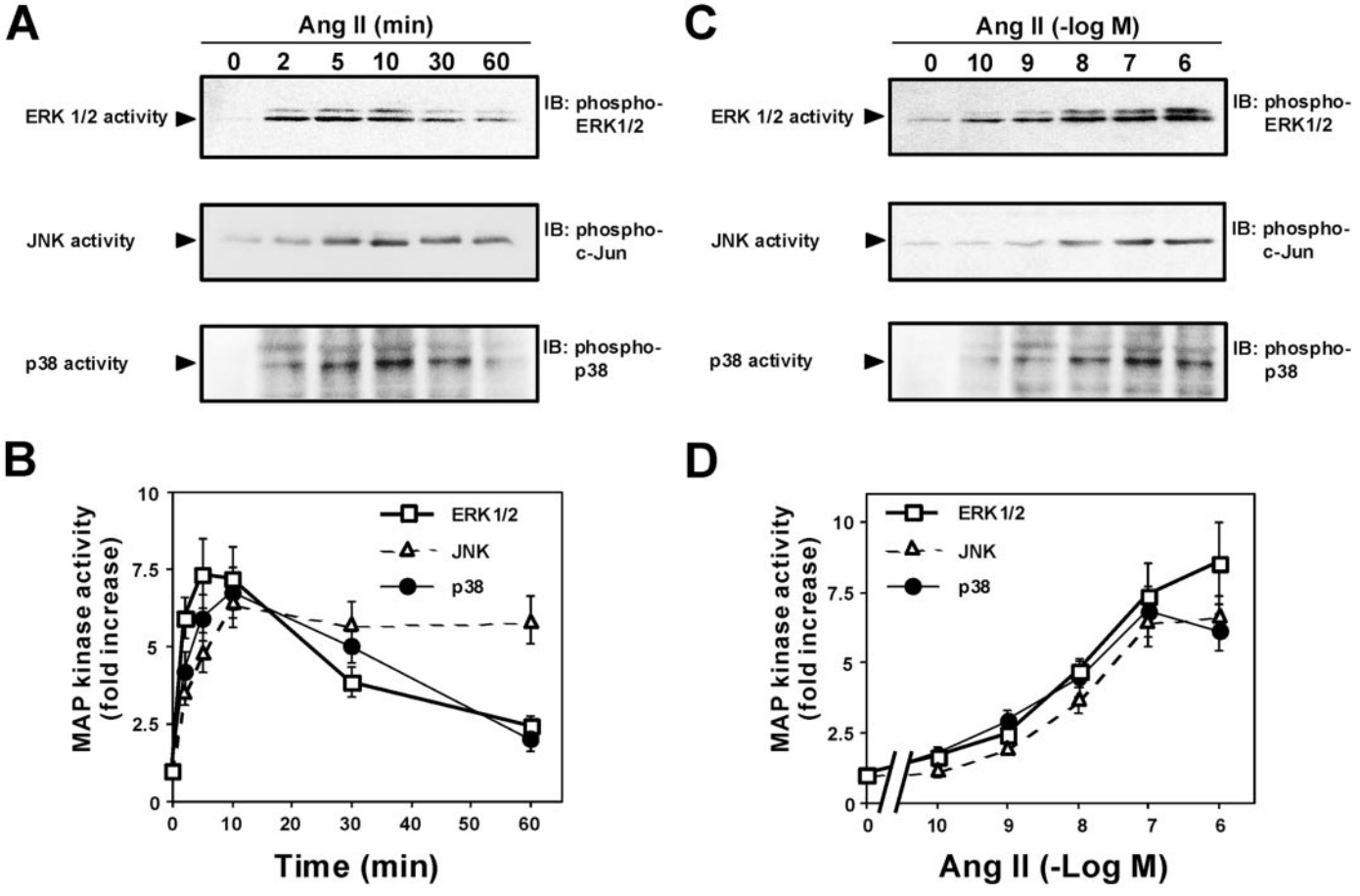

Fig. 1. Time courses (A, B) and concentration-response curves (C, D) of Ang II-induced ERK1/2, JNK, and p38 activation in RASMC. Cells were stimulated with 100 nM Ang II for the indicated periods of time (A) and were stimulated with the indicated concentrations of Ang II for 5 min for ERK1/2 and 10 min for JNK and p38 (C). Cells were harvested, lysed and used for subsequent analysis. The activities of ERK1/2, JNK, and p38 were measured as described under *Materials and Methods*. A and C, representative blots are shown. No significant differences in the amounts of ERK1/2, JNK, and p38 were observed in samples by Western blot analysis with anti-ERK1/2, JNK, and p38 antibodies (data not shown). B and D, densitometric analysis of ERK1/2, JNK, and p38 activation. Values were normalized by arbitrarily setting the densitometry of control cells (time = 0 and without Ang II) to 1.0 (values are mean ± S.D., n = 3).

et al., 1998; Inanami et al., 1999). As shown in Fig. 5A, Ang II caused a significant increase in the coimmunoprecipitation of p85 with Shc that was inhibitable by quercetin in a concentration-dependent manner. As a consequence, Ang II increased PI3-K activation and resultant Akt phosphorylation (Fig. 5B). Quercetin inhibited both Ang II-induced PI3-K activation and Akt phosphorylation in a concentration-dependent manner in RASMC. These findings are consistent with those shown in Fig. 4B, and suggest the signaling cascade of the Shc-p85-Akt pathway. Quercetin may affect this pathway that leads to JNK activation in RASMC.

LY294002, an Inhibitor of PI3-K and Quercetin Derivative, Inhibited Ang II-Induced Akt Phosphorylation and JNK Activation. To gain further insight into the significance of PI3-K/Akt pathway on Ang II-induced JNK activation, we used a PI3-K inhibitor, LY294002, that was originally developed from quercetin (Vlahos et al., 1994). As shown in Fig. 6A, LY294002 significantly inhibited Ang II-induced Akt phosphorylation in a concentration-dependent manner. Furthermore, LY294002, like quercetin, also inhibited Ang II-induced JNK activation in a fashion similar to its inhibition on Akt phosphorylation (Fig. 6B). These findings suggest the existence of a possible cross-talk between Akt and JNK signaling pathways in RASMC.

Quercetin and LY294002 Both Inhibited Ang II-Induced Increase in [^{3}H]Leucine Incorporation into RASMC. JNK activation by various stimuli has been implicated to be related to phenotypic modulations, such as proliferation, differentiation, and apoptosis (Kyriakis and Avruch, 1996; Higashita et al., 1997; Turchi et al., 2000). Ang

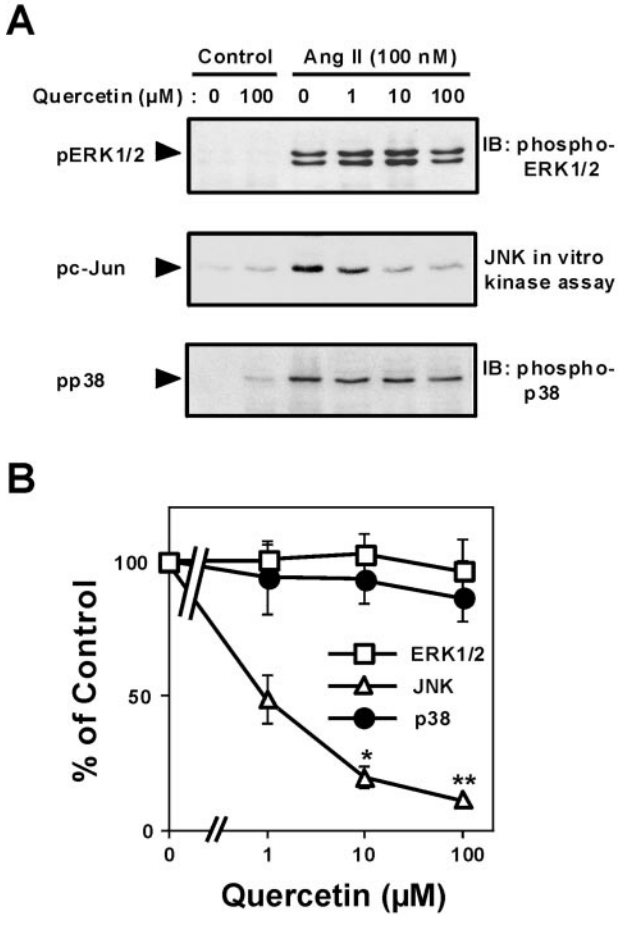

Fig. 2. Inhibition by quercetin of Ang II-induced JNK activation in a concentration-dependent manner, but not ERK1/2 and p38 activation in RASMC. Cells were pretreated with quercetin at the indicated concentrations for 30 min. Then the cells were stimulated with 100 nM Ang II for 5 min for ERK1/2 activity and 10 min for JNK and p38 activities. Cells were harvested, lysed, and used for subsequent analysis. The activities of ERK1/2, JNK, and p38 were measured as described under *Materials and Methods*. A, representative blots are shown. No significant differences in the amounts of ERK1/2, JNK, and p38 were observed in samples by Western blot analysis with anti-ERK1/2, JNK, and p38 antibodies (data not shown). B, densitometric analysis of the effect of quercetin on ERK1/2, JNK, and p38 activation. Values are expressed as the percentage of control, which was defined from each MAP kinase activity stimulated by 100 nM Ang II (values are the mean ± S.D., $n = 3$). The asterisks represent significant differences compared with Ang II stimulation ($^*p < 0.05$, $^{**}p < 0.01$).

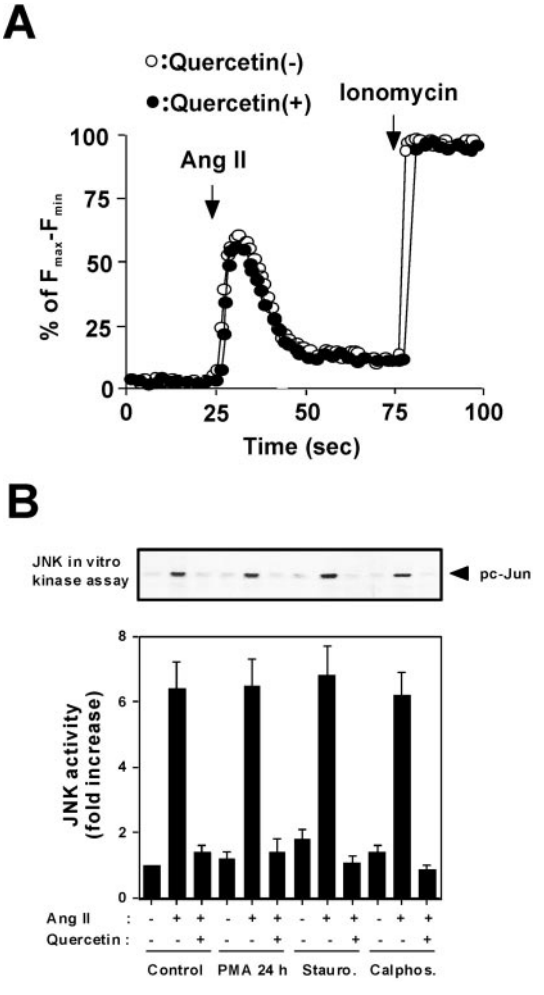

Fig. 3. Effect of quercetin on Ang II-induced increase in $[Ca^{2+}]_i$ (A) and influences of PKC depletion and PKC inhibition on Ang II-induced JNK activation and its inhibition by quercetin (B) in RASMC. A, cells were preincubated with (●) or without (○) 100 μM quercetin and then stimulated with 100 nM Ang II, followed by the addition of 10 μM ionomycin. Values are expressed as the mean of the percentage difference of F_{max} and F_{min} from three different experiments as described under *Materials and Methods*. Error bars are omitted for clarity. B, cells were preincubated with or without 1 μM PMA for 24 h to deplete PKC from the cells. Cells were also pretreated with or without 1 μM staurosporine and 100 nM calphostin C for 1 h to inhibit PKC in the cells. Quercetin (100 μM) was added to the medium 30 min before Ang II (100 nM) stimulation. JNK activity was measured as described under *Materials and Methods*. Top, representative autoradiogram. No significant differences in the amounts of JNK were observed in samples by Western blot analysis with JNK antibody (data not shown). Bottom, densitometric analysis of JNK activation. Values were normalized by arbitrarily setting the densitometry of control cells (without Ang II) to 1.0 (values are the mean ± S.D., $n = 3$).

II stimulation of VSMC has been shown to cause cellular hypertrophy (Griendling et al., 1994). Therefore, we investigated the effects of quercetin and LY294002 on Ang II-induced [^{3}H]leucine incorporation into the cells. Because long-time exposure to higher concentrations (100 μM) of quercetin and LY294002 themselves resulted in a significant loss of RASMC (data not shown), we applied 10 μM concentrations of the agents 30 min before Ang II stimulation. As shown in Fig. 7, Ang II treatment for 24 h caused a significant increase

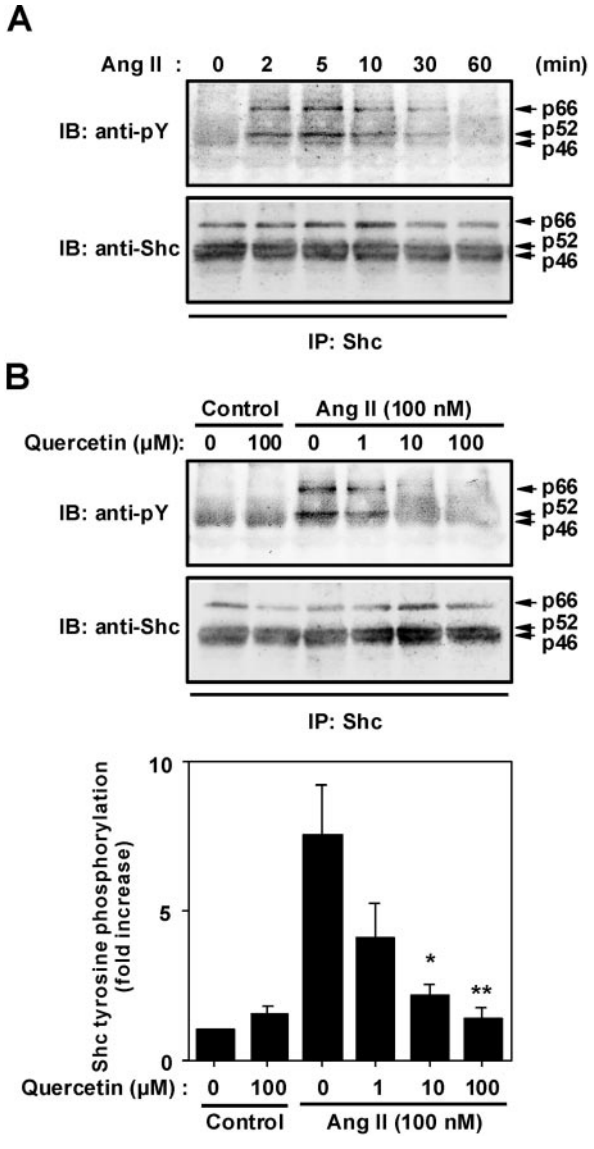

Fig. 4. Ang II-induced increase in Shc tyrosine phosphorylation and its inhibition by quercetin in RASMC. A, cells were stimulated for the indicated times with 100 nM Ang II. Cell lysates were incubated with anti-Shc antibody, and immunoprecipitates from each were analyzed by anti-phosphotyrosine (top) and anti-Shc (bottom) Western blotting. No differences in the amounts of Shc (p66, p52, and p46 isoforms) were observed in samples by Western blot analysis with anti-Shc antibody (bottom). B, cells were pretreated with quercetin at the indicated concentrations for 30 min, followed by stimulation with 100 nM Ang II for 5 min. Then the cells were treated as described above. Top and middle, representative autoradiograms with anti-phosphotyrosine and anti-Shc antibodies, respectively. No significant differences in the amounts of Shc (p66, p52, and p46 isoforms) were observed in samples by Western blot analysis with anti-Shc antibody (bottom). Bottom, densitometric analysis of Shc (sum of p66, p52, and p46 isoforms) tyrosine phosphorylation. Values were normalized by arbitrarily setting the densitometry of control cells (without Ang II) to 1.0 (values are the mean ± S.D., $n = 3$). The asterisks represent significant differences compared with Ang II stimulation (*$p < 0.05$, **$p < 0.01$).

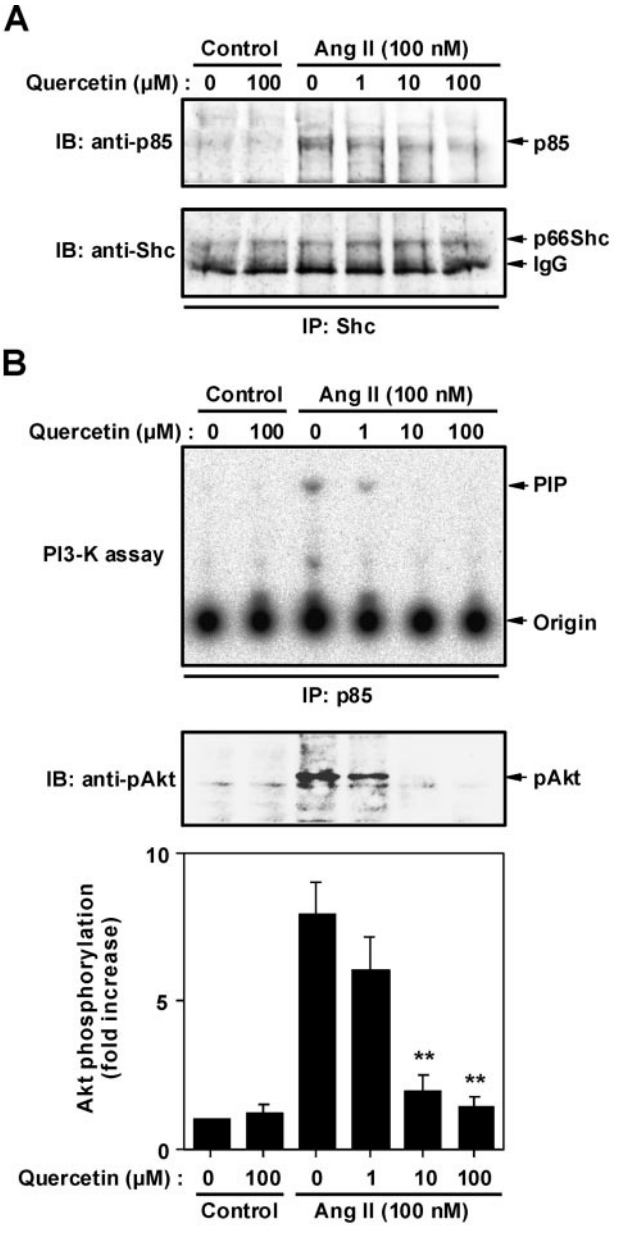

Fig. 5. Ang II-induced Shc-P85 complex formation (A) and PI3-K/Akt activation and their inhibition by quercetin (B) in RASMC. A, cells were pretreated with quercetin at the indicated concentrations for 30 min, followed by stimulation with 100 nM Ang II for 5 min. Cell lysates were incubated with the anti-Shc antibody, and immunoprecipitates from each were analyzed by anti-p85 (top) and anti-Shc (bottom) Western blotting. No significant differences in the amounts of p66 Shc were observed in samples by Western blot analysis with anti-Shc antibody (lower). In this set of experiments, p52 and p46 isoforms were hidden because of overlapping of IgG from antibodies. B, cells were treated as described above and incubated with anti-p85 antibody. The immunoprecipitates from each were assayed for PI3-K activity as described under *Materials and Methods* (top). The same samples were analyzed using anti-phosphospecific Akt antibody Western blotting (middle). No significant differences in the amounts of Akt were observed in samples by Western blot analysis with Akt antibody (data not shown). The lower panel shows the densitometric analysis of Akt activity evaluated by its phosphorylation. Values were normalized by arbitrarily setting the densitometry of control cells (without Ang II) to 1.0 (values are the mean ± S.D., $n = 3$). The asterisks represent significant differences compared with Ang II stimulation (**$p < 0.01$).

of greater than 2-fold in [^{3}H]leucine incorporation. Both quercetin and LY294002 almost abolished the Ang II-induced increase in [^{3}H]leucine incorporation into RASMC. These findings indicate that quercetin and LY294002 may inhibit RASMC hypertrophy and that this probably occurs through the inhibition of JNK activation by Ang II.

Discussion

Ang II has been shown to induce VSMC hypertrophy by acting through the G protein-coupled AT1 receptors (Griendling et al., 1994) and has been implicated in vascular proliferative diseases including hypertension, atherosclerosis, and myocardial infarction (Baker et al., 1992). The intracellular signal transduction pathways induced by Ang II have been investigated, and Ang II has been shown to cause mobilization of intracellular calcium via phospholipase C (Macrez-Lepretre et al., 1996), and to activate protein tyrosine kinase and PKC via the AT1 receptor (Sabri et al., 1998). During mitogenesis, accumulating evidence suggests that Ang II induces MAP kinase phosphorylation (Ushio-Fukai et al., 1998; Viedt et al., 2000) and subsequent activation of transcription factors such as activator protein-1 (Kim and Iwao, 2000). Consequently, this activates DNA synthesis, which leads to increased cellular proliferation in VSMC (Murrell et al., 1990). As shown in Fig. 1, Ang II rapidly and significantly activated three major MAP kinase family members, ERK1/2, JNK, and p38. These findings are consistent with those of Viedt et al. (2000), who reported rapid activation of these three MAP kinases by Ang II in VSMC. However, the time courses of each MAP kinase activation were different after Ang II stimulation as shown in Fig. 1A. One possible explanation is that there are different reactivities of the MAP kinase members to variant signaling mediators activated by Ang II. Some findings have shown that Ang II induced ERK1/2 activation through a tyrosine kinase-dependent and PKC-dependent pathway in VSMC (Tsuda et al., 1992). In contrast, JNK and p38 activation have been shown to be mediated in a tyrosine kinase-independent and PKC-independent manner in VSMC (Viedt et al., 2000). These different signaling pathways may affect the differences in the time courses of the activation of the three MAP kinase members by Ang II.

Quercetin has been shown to cause protective actions on the cardiovascular system, including an anti-inflammatory effect, antioxidative effect, and vasodilatory effect (Formica and Regelson, 1995). It has been reported that quercetin inhibited oxidant-induced glomerular mesangial cell apoptosis (Kitamura and Ishikawa, 1999). It has also been reported that quercetin showed an inhibitory effect on endothelial cell oxidant production (Holland et al., 2000) and platelet activation (Pignatelli et al., 2000). However, no evidence has been

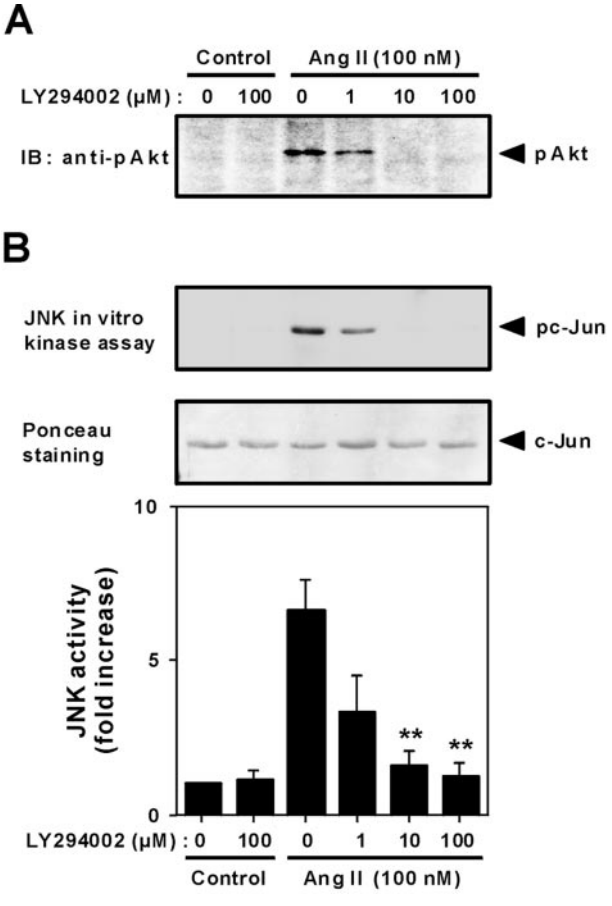

Fig. 6. Effects of LY294002 on Ang II-induced Akt activation and JNK activation in RASMC. A, cells were pretreated with LY294002 at the indicated concentrations for 30 min, followed by stimulation with 100 nM Ang II for 5 min. Cell lysates were analyzed using anti-phosphospecific Akt antibody Western blotting (middle), as in Fig. 5. No significant differences in the amounts of Akt were observed in samples by Western blot analysis with Akt antibody (data not shown). B, cells were pretreated with LY294002 at the indicated concentrations for 30 min, followed by stimulation with 100 nM Ang II for 10 min. JNK activity was measured as described under *Materials and Methods*. Top, representative autoradiogram. No significant differences in the amounts of c-Jun were observed in samples by ponceau de xylidine staining (middle). Bottom, densitometric analysis of JNK activation. Values were normalized by arbitrarily setting the densitometry of control cells (without Ang II) to 1.0 (values are the mean ± S.D., $n = 3$). The asterisks represent significant differences compared with Ang II stimulation (**$p < 0.01$).

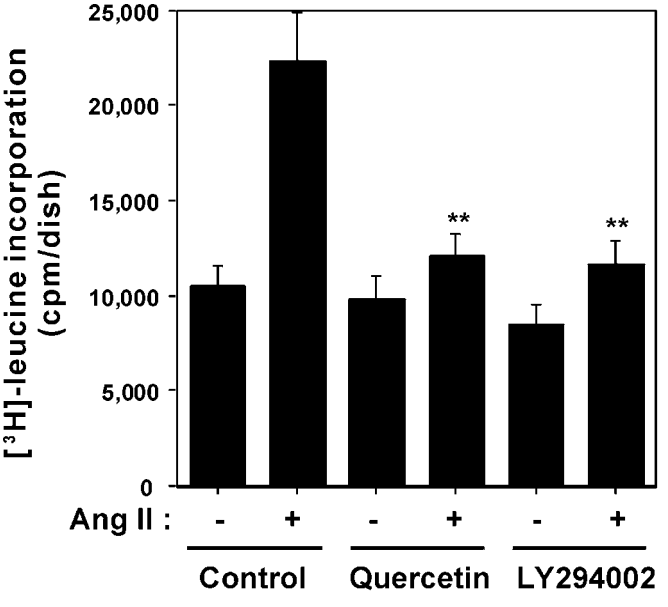

Fig. 7. Effects of quercetin and LY294002 on Ang II-induced increase in [^{3}H]leucine incorporation into RASMC. Growth-arrested cells were stimulated with or without Ang II (100 nM) for 24 h with pulsed addition of [^{3}H]leucine for the final 8 h as described under *Materials and Methods*. Then 10 μM quercetin and LY294002 were added to the medium 30 min before Ang II stimulation. Values are expressed as mean ± S.D. of cpm/dish from three different experiments. The asterisks represent significant differences compared with Ang II stimulation (**$p < 0.01$).

reported concerning the direct effect of quercetin on MAP kinase activity in VSMC. As shown in Fig. 2, we observed for the first time that quercetin specifically inhibited Ang II-induced JNK activation, but not ERK1/2 and p38 activation in RASMC. The inhibitory effect of quercetin on JNK activation may be attributable to its antioxidative effect because it has been reported that diphenyleneiodonium, a potent inhibitor of the flavin-containing NADH/NADPH oxidase enzyme, significantly attenuates JNK activation by Ang II in VSMC (Viedt et al., 2000). We also observed that *N*-acetyl-L-cysteine and ascorbic acid, both antioxidants, inhibited Ang II-induced JNK activation in RASMC (Kyaw et al., 2001). Because it has been reported that Ang II induced the generation of reactive oxygen species (ROS) via the NADH/NADPH oxidase enzyme in VSMC (Griendling et al., 1994; Ushio-Fukai et al., 1999), it may be reasonable to speculate that quercetin inhibited NADH/NADPH oxidase and the resultant JNK activation. It has also been reported that quercetin inhibited NADPH-dependent oxidation in endothelial cells (Holland et al., 2000) and hydrogen peroxide generation in platelets (Pignatelli et al., 2000). However, because we did not measure ROS directly within the cells, further studies are needed to define the effect of quercetin on ROS generation in VSMC.

Next, the intracellular signaling cascade from Ang II stimulation to JNK activation in RASMC was examined. Because it has been reported that quercetin inhibited intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization and PKC activation in VSMC (Duarte et al., 1993), we first examined the effect of quercetin on Ang II-induced change in $[Ca^{2+}]_i$ and the influence of PKC depletion and PKC inhibition on Ang II-induced JNK activation. As shown in Fig. 3A, Ang II caused a rapid and significant increase in $[Ca^{2+}]_i$ in RASMC, which was not inhibited by quercetin. Moreover, PKC depletion by 24-h PMA treatment and PKC inhibition by staurosporine and calphostin C did not affect Ang II-induced JNK activation or influence its inhibition by quercetin (Fig. 3B). These findings suggest that Ang II-induced JNK activation in RASMC is independent of intracellular Ca^{2+} and PKC. Consistent with the present findings, it has been reported that JNK and p38 activation have been shown to be mediated by a tyrosine kinase-independent and PKC-independent manner in VSMC (Viedt et al., 2000). However, it has also been reported that quercetin may influence these two major signaling molecules in some cells (Bastianetto et al., 2000; Cermak et al., 2000). It is difficult to explain the discrepancies at present; however, dependence on intracellular Ca^{2+} and PKC may be cell- and stimulus-specific.

We focused on the role of the Shc adapter protein because it was reported that Shc was involved in JNK activation in some cell types (Hashimoto et al., 1999), and Shc was suggested to be sensitive to oxidative stress (Migliaccio et al., 1999). The Shc adapter protein, composed of three isoforms of p66, p52, and p46, has been shown to possess binding ability to other proteins, such as Grb2, Src homology 2 domain-containing inositol 5′-phosphatase, and p85, via the Src homology 2 domain and phosphotyrosine binding domain and has a capability to transmit signals to downstream targets (Lioubin et al., 1996; Thomas and Bradshaw, 1997; Dupont et al., 1998). As shown in Fig. 4, Ang II caused a rapid tyrosine phosphorylation (peaked at 5 min) of the p52 and p66 subunits of Shc, and quercetin inhibited it in a concentration-dependent manner. These findings are consistent with those shown in Fig. 2 and suggest the possibility that Shc tyrosine phosphorylation may be involved in Ang II-induced JNK activation in RASMC. Because it was reported that stimulation of the G protein-coupled receptor induced Shc tyrosine phosphorylation and subsequent MAP kinase activation (Touhara et al., 1995), it is conceivable that Shc phosphorylation may relate to the transmission of signals to downstream molecules that lead to JNK activation. In fact, it was reported that *trans*-activation of EGF receptor by Ang II resulted in an increase in Shc tyrosine phosphorylation and its association with Grb2 in RASMC (Eguchi et al., 1999). However, it was reported that the Shc·Grb2 association and resultant Ras activation lead to downstream ERK activation (Eguchi et al., 1999), which is inconsistent with our notion that Shc phosphorylation may relate specifically to JNK activation. One possible explanation for this inconsistency is that Shc and Grb2 may have distinct roles in activation of ERK and JNK based on the findings that EGF-mediated JNK activation was abolished by loss of Shc, whereas it was not affected by loss of Grb2 (Hashimoto et al., 1999). Another possible explanation is the existence of other signaling pathways in which ERK is activated by hydrogen peroxide through Fyn and Ras, in addition to the Shc-Grb2-Sos pathway (Abe et al., 2000). Because much evidence suggests that Shc is tyrosine that is phosphorylated by the stimulation of receptor tyrosine kinases, such as the EGF or platelet-derived growth factor receptors, which have been shown to be *trans*-activated by Ang II, further studies are needed to define the upstream regulator of Shc, including receptor tyrosine kinases (Eguchi et al., 1999; Heeneman et al., 2000).

In addition to Shc tyrosine phosphorylation by Ang II, we also observed that Ang II caused an increase in the Shc·p85 association and resultant PI3-K/Akt activation; these were both inhibitable by quercetin (Fig. 5). These findings are consistent with those of previous studies in which Ang II activated the PI3-K/Akt pathway in VSMC (Takahashi et al., 1999; Ushio-Fukai et al., 1999). Because it was reported that PI3-K mediates JNK activation by G protein-coupled receptor stimulation (Lopez-Ilasaca et al., 1998) and by hydrogen peroxide (Inanami et al., 1999), it is reasonable to speculate that the PI3-K/Akt pathway may be involved in JNK activation by Ang II in RASMC. In addition, from the findings that the time course for activation of PI3-K/Akt were earlier (5 min) than that of JNK, it is conceivable that the PI3-K/Akt pathway may exist upstream of JNK. Additional evidence that LY294002, known as a PI3-K inhibitor, inhibited Ang II-induced Akt phosphorylation as well as JNK activation may strongly support the notion that PI3-K/Akt works as an upstream regulator of JNK (Fig. 6). LY294002 is also known as a quercetin derivative and originally developed from the chemical structure of quercetin (Vlahos et al., 1994). Thus, the inhibitory action of quercetin on Ang II-induced JNK activation may be attributed to its inhibitory effect on Shc and the PI3-K/Akt pathway in RASMC. However, inconsistent with our results, Akt-dependent inhibition of apoptosis induced by JNK activation in cardiac myoblasts was reported (Hong et al., 2001). Therefore, further studies such as the use of gene transfer of constitutive-active or dominant-negative mutants are important to clarify the interaction of PI3-K/Akt and JNK because we did not demonstrate whether the PI3-K/Akt pathway directly regulates JNK activity in this study. In addition, because quercetin inhibited tyrosine phosphory-

lation of Shc, which may exist upstream of PI3-K/Akt, a possibility arises that quercetin may inhibit cellular events upstream of Shc rather than directly inhibit PI3-K. We partly confirmed the possibility that quercetin did not show any inhibitory effects on Shc phosphorylation and p85 phosphorylation when administered 5 min after Ang II stimulation (data not shown).

JNK activation has been shown to lead to cellular hypertrophy (Higashita et al., 1997) or apoptosis (Kyriakis and Avruch, 1996), which may be cell- and stimulus-dependent. It has also been reported that Ang II-induced Akt up-regulation leads to VSMC hypertrophy (Ushio-Fukai et al., 1999). In the present study, we examined the effect of quercetin on Ang II-induced increase in [^{3}H]leucine incorporation into cells. Increase in [^{3}H]leucine incorporation has been implicated in the increase in protein synthesis and resultant cellular hypertrophy caused by various agonists, including Ang II (Huwiler et al., 1995). As shown in Fig. 7, Ang II caused an increase in [^{3}H]leucine incorporation that was almost abolished by both quercetin and LY294002. These findings suggest that quercetin may have an antihypertrophic effect on VSMC through the inhibition of PI3-K/Akt and JNK activation. Because accumulating evidence suggests that Ang II-induced JNK activation and/or PI-3K/Akt activation has been implicated in cellular hypertrophy in VSMC (Higashita et al., 1997; Ushio-Fukai et al., 1999), inhibition of both these kinases by quercetin may imply its usefulness for treating cardiovascular diseases in which VSMC growth may be involved.

In conclusion, we showed for the first time that quercetin specifically inhibited Ang II-induced JNK activation, but not ERK and p38 activation in RASMC. One possible target molecule for the inhibition by quercetin is the Shc adapter protein. It was suggested that the PI3-K/Akt pathway may be involved in Ang II-induced JNK activation in RASMC. Ang II-mediated resultant protein synthesis was inhibited by quercetin in RASMC. The findings of the present study may shed light on the pharmacological basis for the clinical application of bioflavonoids in cardiovascular diseases.

References

Abe J, Okuda M, Huang Q, Yoshizumi M and Berk BC (2000) Reactive oxygen species activate p90 ribosomal S6 kinase via Fyn and Ras. *J Biol Chem* **275:**1739–1748.

Baker KM, Booz GW and Dostal DE (1992) Cardiac actions of angiotensin II: role of an intracardiac renin-angiotensin system. *Annu Rev Physiol* **54:**227–241.

Bastianetto S, Zheng WH and Quirion R (2000) Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. *Br J Pharmacol* **131:**711–720.

Cermak R, Vujicic Z, Kuhn G and Wolffram S (2000) The secretory response of the rat colon to the flavonol quercetin is dependent on Ca^{2+}-calmodulin. *Exp Physiol* **85:**255–261.

Cobb MH and Goldsmith EJ (1995) How MAP kinases are regulated. *J Biol Chem* **270:**14843–14846.

Davis RJ (1993) The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem* **268:**14553–14556.

Duarte J, Perez Vizcaino F, Utrilla P, Jimenez J, Tamargo J and Zarzuelo A (1993) Vasodilatory effects of flavonoids in rat aortic smooth muscle. Structure-activity relationships. *Gen Pharmacol* **24:**857–862.

Dupont J, Derouet M, Simon J and Taouis M (1998) Effect of nutritional state on the formation of a complex involving insulin receptor IRS-1, the 52 kDa Src homology/collagen protein (Shc) isoform and phosphatidylinositol 3′-kinase activity. *Biochem J* **335:**293–300.

Eguchi S, Iwasaki H, Inagami T, Numaguchi K, Yamakawa T, Motley ED, Owada KM, Marumo F and Hirata Y (1999) Involvement of PYK2 in angiotensin II signaling of vascular smooth muscle cells. *Hypertension* **33:**201–206.

Formica JV and Regelson W (1995) Review of the biology of Quercetin and related bioflavonoids. *Food Chem Toxicol* **33:**1061–1080.

Griendling KK, Minieri CA, Ollerenshaw JD and Alexander RW (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* **74:**1141–1148.

Hashimoto A, Kurosaki M, Gotoh N, Shibuya M and Kurosaki T (1999) Shc regulates epidermal growth factor-induced activation of the JNK signaling pathway. *J Biol Chem* **274:**20139–20143.

Heeneman S, Haendeler J, Saito Y, Ishida M and Berk BC (2000) Angiotensin II induces transactivation of two different populations of the platelet-derived growth factor beta receptor. Key role for the p66 adaptor protein Shc. *J Biol Chem* **275:**15926–15932.

Higashita R, Li L, Van Putten V, Yamamura Y, Zarinetchi F, Heasley L and Nemenoff RA (1997) Galpha16 mimics vasoconstrictor action to induce smooth muscle alpha-actin in vascular smooth muscle cells through a Jun-NH_2-terminal kinase-dependent pathway. *J Biol Chem* **272:**25845–25850.

Holland JA, O'Donnell RW, Chang MM, Johnson DK and Ziegler LM (2000) Endothelial cell oxidant production: effect of NADPH oxidase inhibitors. *Endothelium* **7:**109–119.

Hollman PC and Katan MB (1999) Health effects and bioavailability of dietary flavonols. *Free Radic Res* **31(Suppl):**S75–S80.

Hong F, Kwon SJ, Jhun BS, Kim SS, Ha J, Kim SJ, Sohn NW, Kang C and Kang I (2001) Insulin-like growth factor-1 protects H9c2 cardiac myoblasts from oxidative stress-induced apoptosis via phosphatidylinositol 3-kinase and extracellular signal-regulated kinase pathways. *Life Sci* **68:**1095–1105.

Huwiler A, Stabel S, Fabbro D and Pfeilschifter J (1995) Platelet-derived growth factor and angiotensin II stimulate the mitogen-activated protein kinase cascade in renal mesangial cells: comparison of hypertrophic and hyperplastic agonists. *Biochem J* **305:**777–784.

Inanami O, Takahashi K, Yoshito A and Kuwabara M (1999) Hydrogen peroxide-induced activation of SAPK/JNK regulated by phosphatidylinositol 3-kinase in chinese hamster V79 cells. *Antioxid Redox Signal* **1:**113–121.

Kim S and Iwao H (2000) Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* **52:**11–34.

Kitamura M and Ishikawa Y (1999) Oxidant-induced apoptosis of glomerular cells: intracellular signaling and its intervention by bioflavinoid. *Kidney Int* **56:**1223–1229.

Kyaw M, Yoshizumi M, Tsuchiya K, Kirima K, and Tamaki T (2001) Antioxidants inhibit JNK and p38 MAPK activation, but not ERK1/2 activation by angiotensin II in rat aortic smooth muscle cells. *Hypertens Res* **24:**251–261.

Kyriakis JM and Avruch J (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J Biol Chem* **271:**24313–24316.

Lioubin MN, Algate PA, Tsai S, Carlberg K, Aebersold A and Rohrschneider LR (1996) p150Ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev* **10:**1084–1095.

Liu ZG, Hsu H, Goeddel DV and Karin M (1996) Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. *Cell* **87:**565–576.

Lopez-Ilasaca M, Gutkind JS and Wetzker R (1998) Phosphoinositide 3-kinase gamma is a mediator of Gbetagamma-dependent Jun kinase activation. *J Biol Chem* **273:**2505–2508.

Macrez-Lepretre N, Morel JL and Mironneau J (1996) Angiotensin II-mediated activation of L-type calcium channels involves phosphatidylinositol hydrolysis-independent activation of protein kinase C in rat portal vein myocytes. *J Pharmacol Exp Ther* **278:**468–475.

Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L and Pelicci PG (1999) The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature (Lond)* **402:**309–313.

Murrell GA, Francis MJ and Bromley L (1990) Modulation of fibroblast proliferation by oxygen free radicals. *Biochem J* **265:**659–665.

Nishida E and Gotoh Y (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem Sci* **18:**128–131.

Ohlstein EH, Douglas SA, Sung CP, Yue TL, Louden C, Arleth A, Poste G, Ruffolo RR Jr and Feuerstein GZ (1993) Carvedilol, a cardiovascular drug, prevents vascular smooth muscle cell proliferation, migration, and neointimal formation following vascular injury. *Proc Natl Acad Sci USA* **90:**6189–6193.

Pignatelli P, Pulcinelli FM, Celestini A, Lenti L, Ghiselli A, Gazzaniga PP and Violi F (2000) The flavonoids quercetin and catechin synergistically inhibit platelet function by antagonizing the intracellular production of hydrogen peroxide. *Am J Clin Nutr* **72:**1150–1155.

Renaud S and de Lorgeril M (1992) Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **339:**1523–1526.

Sabri A, Govindarajan G, Griffin TM, Byron KL, Samarel AM and Lucchesi PA (1998) Calcium- and protein kinase C-dependent activation of the tyrosine kinase PYK2 by angiotensin II in vascular smooth muscle. *Circ Res* **83:**841–851.

St. Leger AS, Cochrane AL and Moore F (1979) Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. *Lancet* **1:**1017–1020.

Takahashi T, Taniguchi T, Konishi H, Kikkawa U, Ishikawa Y and Yokoyama M (1999) Activation of Akt/protein kinase B after stimulation with angiotensin II in vascular smooth muscle cells. *Am J Physiol* **276:**H1927–H1934.

Thomas D and Bradshaw RA (1997) Differential utilization of ShcA tyrosine residues and functional domains in the transduction of epidermal growth factor-induced mitogen-activated protein kinase activation in 293T cells and nerve growth factor-induced neurite outgrowth in PC12 cells. Identification of a new Grb2. Sos1 binding site. *J Biol Chem* **272:**22293–22299.

Touhara K, Hawes BE, van Biesen T and Lefkowitz RJ (1995) G protein beta gamma subunits stimulate phosphorylation of Shc adapter protein. *Proc Natl Acad Sci USA* **92:**9284–9287.

Tsuda T, Kawahara Y, Ishida Y, Koide M, Shii K and Yokoyama M (1992) Angiotensin II stimulates two myelin basic protein/microtubule-associated protein 2 kinases in cultured vascular smooth muscle cells. *Circ Res* **71:**620–630.

Turchi L, Loubat A, Rochet N, Rossi B and Ponzio G (2000) Evidence for a direct correlation between c-Jun NH_2 terminal kinase 1 activation, cyclin D2 expression, and G(1)/S phase transition in the murine hybridoma 7TD1 cells. *Exp Cell Res* **261:**220–228.

Ushio-Fukai M, Alexander RW, Akers M and Griendling KK (1998) p38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. *J Biol Chem* **273:**15022–15029.

Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K and Griendling KK (1999) Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *J Biol Chem* **274:**22699–22704.

Viedt C, Soto U, Krieger-Brauer HI, Fei J, Elsing C, Kubler W and Kreuzer J (2000) Differential activation of mitogen-activated protein kinases in smooth muscle cells by angiotensin II: involvement of p22phox and reactive oxygen species. *Arterioscler Thromb Vasc Biol* **20:**940–948.

Vlahos CJ, Matter WF, Hui KY and Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* **269:**5241–5248.

Whitman M, Downes CP, Keeler M, Keller T and Cantley L (1988) Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature (Lond)* **332:**644–646.

Yoshizumi M, Abe J, Haendeler J, Huang Q and Berk BC (2000) Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. *J Biol Chem* **275:**11706–11712.

Yoshizumi M, Inui D, Okishima N, Houchi H, Tsuchiya K, Wakabayashi H, Kido H and Tamaki T (1998) Endothelin-1-(1–31), a novel vasoactive peptide, increases $[Ca^{2+}]_i$ in human coronary artery smooth muscle cells. *Eur J Pharmacol* **348:**305–309.

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