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Effect of Azelnidipine on Angiotensin II-Mediated Growth-Promoting Signaling in Vascular Smooth Muscle Cells

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ABSTRACT

The detailed mechanism of the effects of extracellular Ca^{2+} entry blockade on angiotensin II (Ang II) type 1 (AT1) receptor-mediated growth-promoting signals in vascular smooth muscle cells (VSMCs) is not fully understood. Ang II stimulation caused biphasic activation of growth-promoting signals, reaching a peak at 5 to 10 min followed by a decrease and a second peak at around 2 to 4 h. Addition of PD98059 (2′-amino-3′-methoxyflavone), a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor, or AG490 [α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide], a Janus-activated kinase 2 (Jak2) inhibitor, even 4 h after Ang II treatment inhibited [^{3}H]thymidine incorporation. The calcium channel blocker azelnidipine attenuated the later peaks of extracellular signal-regulated kinase (ERK), tyrosine kinase 2, Jak2 activation, and phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3. Interestingly, azelnidipine increased rather than decreased the later ERK peaks in cells treated with small interfering RNA against mitogen-activated protein kinase phosphatase-1. Ang II-mediated [^{3}H]thymidine incorporation was inhibited dose dependently by azelnidipine and also by azelnidipine, plus olmesartan, whereas olmesartan or azelnidipine alone at such lower doses did not affect [^{3}H]thymidine incorporation. These data provide new insight into the manner in which calcium channels exert an essential action in the AT1 receptor-mediated growth-promoting actions in VSMCs.

Angiotensin II (Ang II) has direct effects on endothelial and vascular smooth muscle cells (VSMCs) and plays a key role in the initiation and amplification of pathobiological events that lead to vascular disease (Dzau, 2001). These major vascular actions of Ang II have been reported to be mediated by the type 1 Ang II (AT1) receptor, and AT1 receptor blockers (ARBs) have been widely used as antihypertensive drugs with the expectation of a vascular protective effect (de Gasparo et al., 2000). Ca^{2+} entry into vascular cells has been reported to be necessary for Ang II-induced DNA synthesis (Saward and Zahradka, 1997), and AT1 receptor stimulation has been reported to stimulate L-type Ca^{2+} channels and induce influx of extracellular Ca^{2+} through calcium channels, resulting in a sustained elevation of intracellular calcium (Brock et al., 1985; Berridge, 1993; Macrez et al., 1997). It has been reported that DNA synthesis induced by both Ang II and growth factors such as platelet-derived growth factor in VSMCs is significantly blunted by calcium channel blockers (CCBs) and that CCBs inhibit neointimal formation in the injured artery (Ko et al., 1992; Dol et al., 1995; Hirata et al., 2000).

Nifedipine has been shown to block the Ang II-induced increase in intracellular calcium nearly completely in freshly prepared VSMCs (Iversen and Arendshorst, 1998), and amlodipine has been demonstrated to retard or even prevent the progression of atherosclerosis and has antiproliferative effect on VSMCs from spontaneously hypertensive rats (Stepien et al., 1998). In addition, other L-type voltage-gated calcium channel blockers, such as nitrendipine, nisoldipine, nimodipine, and isradipine, have been shown to blunt Ang II-induced DNA synthesis in VSMCs (Ko et al., 1993). These results suggest the possibility that combination therapy with an

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ABBREVIATIONS: Ang II, angiotensin II; VSMC, vascular smooth muscle cell; AT1, type I angiotensin II receptor; ARB, AT1 receptor blocker; CCB, calcium channel blocker; Pyk2, proline-rich nonreceptor tyrosine kinase 2; ERK, extracellular signal-regulated kinase; Tyk2, tyrosine kinase 2; Jak, Janus-activated kinases; STAT, signal transducer and activator of transcription; PD98059, 2′-amino-3′-methoxyflavone; AG490, α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; siRNA, small interfering RNA; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; Mek/MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MKP-1, mitogen-activated protein kinase phosphatase-1.

ARB and CCB could more effectively prevent vascular damage than monotherapy. We recently reported that in polyethylene-cuff-induced vascular injury of the femoral artery in mice, proliferation of VSMCs and neointimal formation, associated with activation of extracellular signal-regulated kinase (ERK) and tyrosine-phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3, were significantly inhibited by coadministration of lower doses of both an ARB, olmesartan, and azelnidipine (Jinno et al., 2004). Evidence has suggested that the intracellular signaling mechanisms by which the AT1 receptor exerts hypertrophic and/or hyperplastic effects on targets such as VSMCs are closely associated with receptor and nonreceptor tyrosine kinases and that some AT1 receptor-mediated signaling requires Ca^{2+}-sensitive tyrosine kinases (Eguchi and Inagami, 2000). However, the effect of Ca^{2+} entry blockade on AT1 receptor mediated growth-promoting signaling and its detailed mechanism remains to be elucidated. In this study, we focused on the AT1 receptor-mediated Pky2/c-Src/ERK pathway and Jak/STAT pathway and explored the possibility that azelnidipine may exaggerate the inhibitory effect of an ARB, olmesartan, on AT1 receptor-mediated VSMC proliferation and its related signaling.

Materials and Methods

Cell Culture and Treatment. VSMCs were prepared from thoracic aorta adult of Sprague-Dawley rats (Clea Japan Inc., Tokyo, Japan) as described previously (Li et al., 1999; Cui et al., 2002). VSMCs were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and supplemented with antibiotics. AT1 receptor or AT2 receptor expression was examined by radioligand binding assay as reported previously (Li et al., 1999). Olmesartan (donated by Sankyo Pharmaceutical Co., Tokyo, Japan), a specific AT1 receptor blocker, and/or azelnidipine (donated by Sankyo Pharmaceutical Co., Tokyo, Japan) were administered with Ang II. PD98059 (Cell Signaling Technology Inc., Beverly, MA), a mitogen-activated protein kinase/ERK kinase inhibitor, or AG490 (Calbiochem, San Diego, CA), a Jak kinase inhibitor, was added to VSMCs and incubated with Ang II. For small interfering RNA assay, VSMCs were transiently transfected with lamin A/C siRNA as a control or MKP-1-specific siRNA, a cocktail of three siRNA designed by B-Bridge (Sunnyvale, CA), by LipofectAMINE Plus (Invitrogen, Carlsbad, CA). Thirty-six hours after transfection, cells were treated with or without Ang II and/or azelnidipine.

[^{3}H]Thymidine Incorporation. DNA synthesis was assayed by measurement of [^{3}H]thymidine incorporation (Cui et al., 2002). VSMCs were serum-starved for 48 h to induce a quiescent state. Subconfluent and quiescent cells cultured in 24-well plates were stimulated with various reagents for 12 h and pulsed with 1 μCi/ml [*methyl*-^{3}H]thymidine (specific activity, 60 Ci/mmol) (PerkinElmer Life and Analytical Sciences, Boston, MA) for an additional 24 h. The cells were washed twice with ice-cold phosphate-buffered saline and subsequently incubated with ice-cold 5% trichloroacetic acid for 20 min at 4°C. The cells were washed twice with ice-cold 5% trichloroacetic acid and then with ice-cold phosphate-buffered saline and lysed with 0.5 N NaOH. The radioactivity of the cell lysate was determined using a liquid scintillation counter.

Immunoprecipitation and Western Blot Analysis. Subconfluent VSMCs were kept in a serum-free condition for 48 h and then treated as indicated in the figure legends. Total proteins were prepared from the cultured VSMCs, and Western blot was performed as described previously (Cui et al., 2002). Immunoprecipitation was performed using anti-Pky2 and anti-Jak2 (Upstate Biotechnology, Waltham, MA), anti-Tyk2, and anti-epidermal growth factor receptor antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoblotting was performed using anti-phospho-tyrosine (4G10), anti-Pyk2, anti-c-Src, and anti-Jak2 (Upstate Biotechnology), anti-phospho-c-Src, anti-STAT1, anti-phospho-tyrosine-STAT1, anti-STAT3, anti-phospho-tyrosine-STAT3, anti-ERK, and anti-phospho-ERK (Cell Signaling Technology, Inc.), anti-phospho-serine-STAT3 (New England Biolabs, Beverly, MA), anti-Tky2, anti-epidermal growth factor receptor, and anti-MKP-1 (Santa Cruz Biotechnology, Inc.), and anti-phospho-serine-STAT1 and anti-α smooth muscle actin antibodies (clone 1A4; Sigma-Aldrich, St. Louis, MO). The cell lysate (20 μg) was run on 10% SDS-polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred onto nitrocellulose membrane (Hybond ECL; Amersham Biosciences Inc., Piscataway, NJ). Blots were incubated with specific antibodies as indicated. The bands were visualized with enhanced chemiluminescence system (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Densitometric analysis was performed using NIH Image software.

Statistical Analysis. Values are expressed as mean ± S.E.M. in the text and figures. The data were analyzed using analysis of variance followed by Newman-Keuls test for multiple comparisons. Values of $p < 0.05$ were considered to be statistically significant.

Results

Effect of Azelnidipine on Ang II-Induced DNA Synthesis of VSMCs. To determine the role of extracellular Ca^{2+} influx in AT1 receptor-mediated proliferation of adult rat aortic VSMCs, we examined the effects of azelnidipine. Radioligand binding assay showed that rat aortic VSMCs used in our study exclusively expressed the AT1 receptor (10.02 ± 0.85 fmol/10^6 cells, mean ± S.E.M., $n = 4$) and no detectable level of the AT2 receptor. We observed that Ang II significantly increased [^{3}H]thymidine incorporation in VSMCs (Fig. 1A), and this Ang II (10^{-7} M)-mediated [^{3}H]thymidine incorporation was inhibited dose dependently by the addition of olmesartan or azelnidipine, resulting in the basal level of [^{3}H]thymidine incorporation by treatment with azelnidipine (1 to 5×10^{-6} M) or olmesartan (1×10^{-6} to 10^{-5} M) alone (Fig. 1, B and C). Moreover, Ang II (10^{-7} M)-mediated [^{3}H]thymidine incorporation was inhibited by lower doses of azelnidipine (5×10^{-7} M) and olmesartan (10^{-10} M) together, whereas olmesartan or azelnidipine alone at these doses did not affect [^{3}H]thymidine incorporation in VSMCs (Fig. 1D). These results suggest an involvement of extracellular Ca^{2+} influx via the L-type calcium channel in Ang II-stimulated VSMC proliferation.

Effect of Azelnidipine on AT1 Receptor-Mediated Growth-Promoting Signaling in VSMCs. To examine the underlying signaling mechanism of the inhibitory effect of azelnidipine on AT1 receptor-mediated VSMC proliferation, we focused on the Pyk2/c-Src/ERK cascade and Jak/STAT cascade. We observed that Ang II (10^{-7} M) treatment activated Pyk2, c-Src, Jak2, Tyk2, and ERK determined by their phosphorylation, reaching a peak at 5 to 10 min, followed by a decrease in their activities and then reactivation, showing a second peak at 2 to 4 h after Ang II stimulation (Fig. 2, A and B). Similar results were observed for Ang II-induced tyrosine- and serine-phosphorylation of STAT1 and STAT3 (Fig. 2, A and B). These effects of Ang II were inhibited by the addition of olmesartan (10^{-5} M), but not by an AT2 receptor-specific blocker, PD123319 (data not shown). Addition of azelnidipine (5×10^{-6} M) markedly inhibited Ang II-stimulated tyrosine phosphorylation of Pyk2 and c-Src (Fig. 2,

C and D). Azelnidipine partially suppressed the initial Ang II-mediated ERK activation and serine-phosphorylation of STAT1 and STAT3 and did not inhibit Ang II-mediated initial activation of Jak2, Tyk2, and tyrosine phosphorylation of STAT1 and STAT3 (Fig. 2, C and D). In contrast, azelnidipine attenuated the later peaks of Ang II-mediated ERK activation, tyrosine- and serine-phosphorylation of STAT1 and STAT3, and activation of Jak2 and Tyk2 (Fig. 2, C and D). The protein levels of Pyk2, c-Src, ERK, Jak2, Tk2, STAT1, and STAT3 did not change throughout the experimental period.

Effect of Azelnidipine on Ang II-Induced MKP-1 Expression in VSMCs. The late phase activation of ERK, STAT1, and STAT3 by Ang II was markedly attenuated by azelnidipine. MKP-1 is a protein phosphatase that can dephosphorylate multiple mitogen-activated protein (MAP) kinases (Keyse, 1995) and has been reported to be activated by Ang II stimulation (Sandberg et al., 2004), which might contribute to inhibition of overstimulation of ERK (Viedt et al., 2000). In addition, Venema et al. (1998) have demonstrated that MKP-1 induces STAT1 tyrosine dephosphorylation in VSMCs. Consistent with a previous report, we observed that Ang II stimulation increased MKP-1 expression, reaching a peak at around 60 min, followed by a decrease in MKP-1 expression (Fig. 3). We found that the addition of azelnidipine increased Ang II-induced MKP-1 expression and retarded its decrease. To determine whether MKP-1 induction is required for azelnidipine-mediated ERK inactivation, we examined ERK phosphorylation under conditions where small interfering RNA specifically blocked MKP-1 induction. VSMCs were transiently transfected with lamin A/C siRNA as a control or MKP-1-specific siRNA. Thirty-six hours after transfection, cells were treated with or without Ang II and/or azelnidipine. MKP-1 protein levels were evaluated by immunoblot. As shown in Fig. 4A, MKP-1 expression increased in Ang II-treated control siRNA-expressing VSMCs but was blocked in MKP-1-siRNA-expressing VSMCs. MKP-1-siRNA-expressing cells retained ERK phosphorylation after Ang II and azelnidipine treatment (Fig. 4B). Interestingly, the reduction of the late phase ERK activation was significantly blocked in MKP-1-siRNA-expressing VSMCs.

Role of AT1 Receptor-Mediated Late Phase of Activation of ERK and STAT in VSMC Proliferation. To address the roles of the AT1 receptor-mediated distinct phases of activation of ERK and STAT in VSMC proliferation induced by Ang II, we examined the effect of PD98059, an Mek inhibitor, and the effect of AG490, a Jak2 inhibitor. Simultaneous addition of PD98059 and AG490, or addition of PD98059 or AG490 1 h after Ang II stimulation, abolished Ang II-mediated [^{3}H]thymidine incorporation in VSMCs (Fig. 5). Addition of PD98059 and AG490 2 to 4 h after Ang II stimulation also significantly inhibited Ang II-mediated [^{3}H]thymidine incorporation in VSMCs, whereas addition of PD98059 and AG490 8 h after Ang II stimulation did not affect Ang II-mediated [^{3}H]thymidine incorporation in VSMCs (Fig. 5).

Effect of Combination of Lower Doses of Azelnidipine and Olmesartan on AT1 Receptor-Mediated Growth-Promoting Signals in VSMCs. We showed that a combination of lower doses of azelnidipine and olmesartan decreased AT1 receptor-mediated DNA synthesis in VSMCs (Fig. 1D). To explore the signaling mechanism of this combined

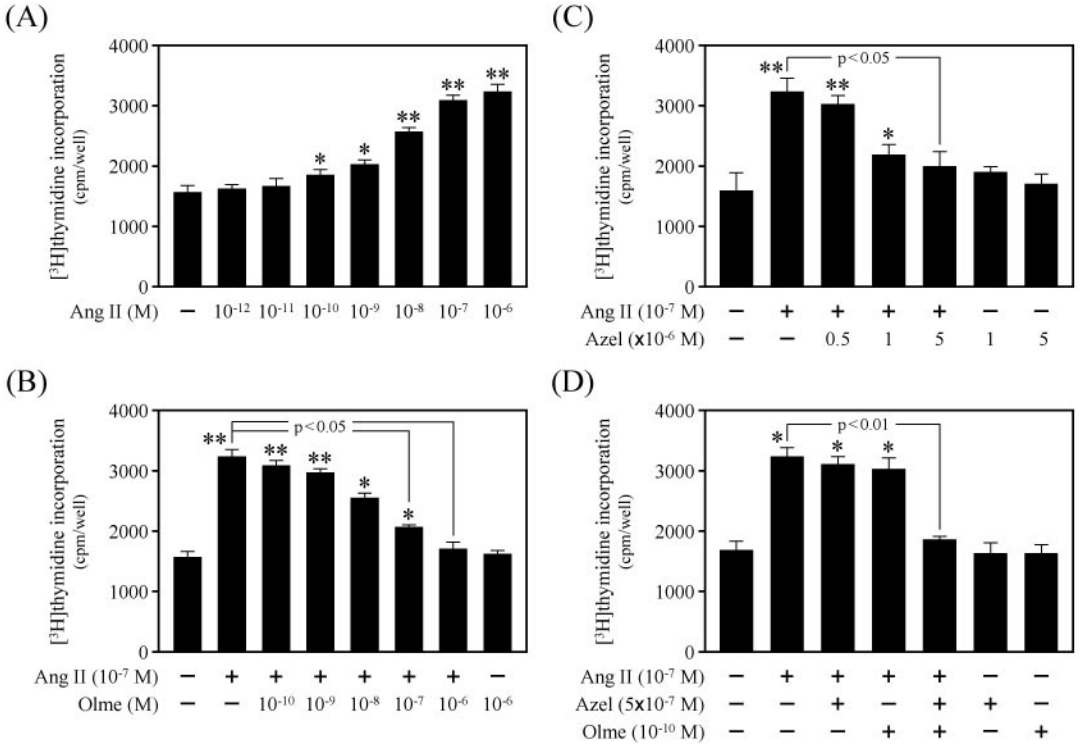

Fig. 1. Effect of angiotensin II (A) on DNA synthesis in VSMCs. Effect of azelnidipine (B), olmesartan (C), and azelnidipine plus olmesartan (D) on Ang II-induced DNA synthesis in VSMCs. Subconfluent, quiescent VSMCs were treated with Ang II (10^{-7} M), azelnidipine (Azel), and/or olmesartan (Olme) as indicated for 36 h. DNA synthesis was assayed by measuring [^{3}H]thymidine incorporation as described under *Materials and Methods*. Similar results were obtained in four different VSMC cultures. Values are expressed as mean ± S.E.M. ($n = 4$). *, $p < 0.05$; **, $p < 0.01$ versus control.

effect, we examined the effects of low doses of azelnidipine (5×10^{-7} M) and olmesartan (10^{-10} M), which did not affect Ang II-mediated growth-promoting signals in VSMCs (Fig. 6). We observed that coadministration of azelnidipine and olmesartan at these doses decreased Ang II-activated rapid tyrosine phosphorylation of Pyk2, c-Src, Jak2, Tyk2, and ERK activity, and partially suppressed Ang II-activated rapid serine phosphorylation of STAT1 and STAT3, whereas the combination of these two drugs had no significant effect on Ang II-activated rapid tyrosine phosphorylation of STAT1 and STAT3. In contrast, a combination of azelnidipine and olmesartan significantly

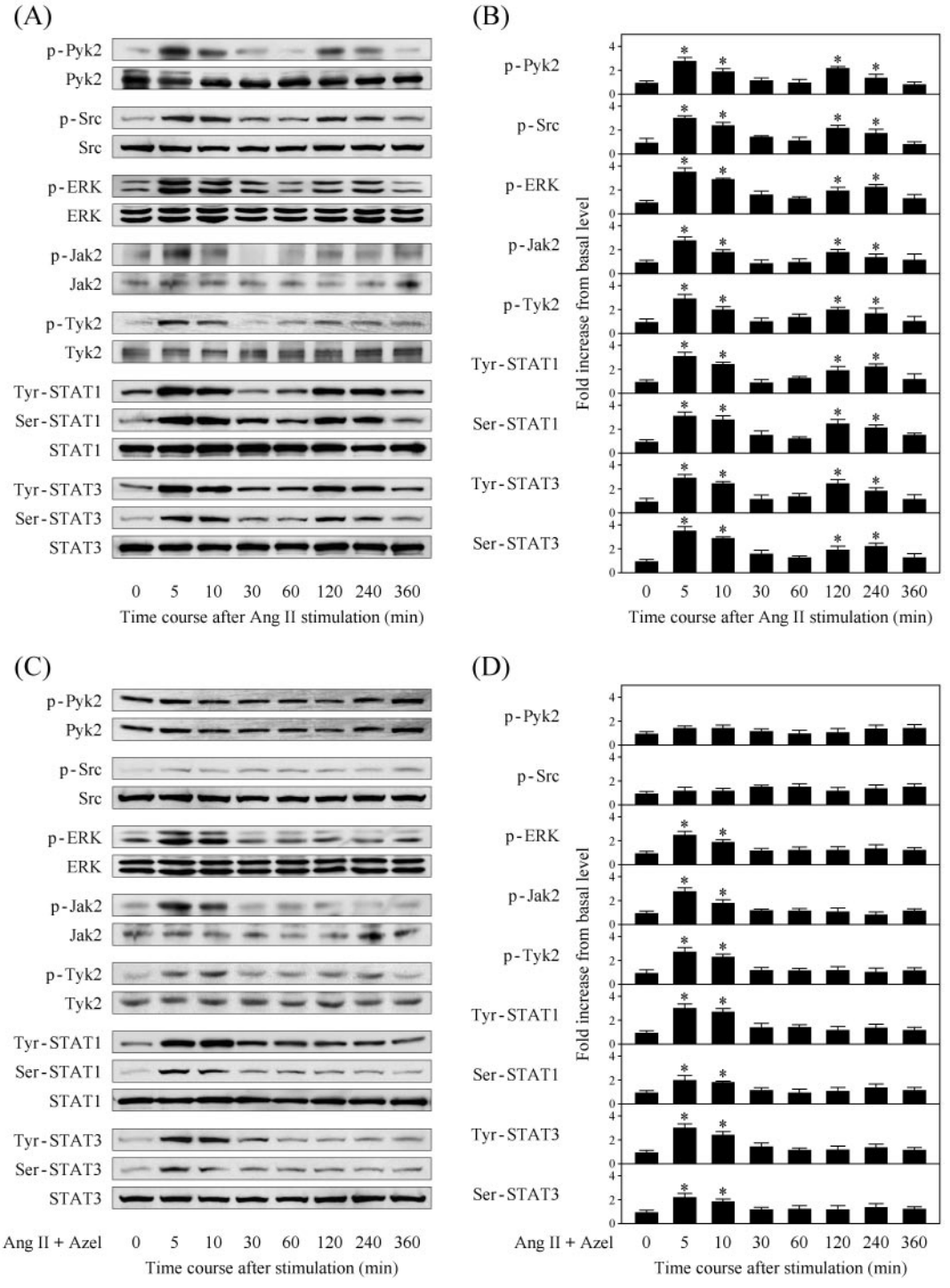

Fig. 2. Effect of Ang II on activation of Pyk2, c-Src, ERK, Jak2, Tyk2, and tyrosine- and serine-phosphorylation of STAT1 and STAT3 in VSMCs (A and B). Effect of azelnidipine on Ang II-induced phosphorylation of Pyk2, c-Src, ERK, Jak2, Tyk2, and tyrosine- and serine-phosphorylation of STAT1 and STAT3 in VSMCs (C and D). Subconfluent and quiescent VSMCs were treated with Ang II (10^{-7} M) and/or azelnidipine (Azel; 5×10^{-6} M) as indicated. Immunoprecipitation was performed using anti-Pyk2, anti-Jak2, and anti-Tyk2 antibodies. Immunoblotting was performed using anti-phosphorylated-tyrosine (4G10), anti-Pyk2, anti-c-Src, anti-phospho-c-Src, anti-ERK, anti-phospho-ERK, anti-Jak2, anti-Tyk2, anti-STAT1, anti-phospho-tyrosine-STAT1, anti-phospho-serine-STAT1, anti-STAT3, anti-phospho-tyrosine-STAT3, and anti-phospho-serine-STAT3 antibodies. Figures show representative data from three separate experiments. Values are expressed as mean ± S.E.M. ($n = 3$). *, $p < 0.05$ versus time 0.

inhibited Ang II-mediated tyrosine- and serine-phosphorylation of STAT1 and STAT3 as well as Pyk2, c-Src, Jak2, Tyk2, and ERK activation in the last stage of Ang II-induced growth-promoting signals.

Discussion

The Pky2/c-Src/ERK pathway and the Jak/STAT pathway, which are activated by stimulation of the AT1 receptor and

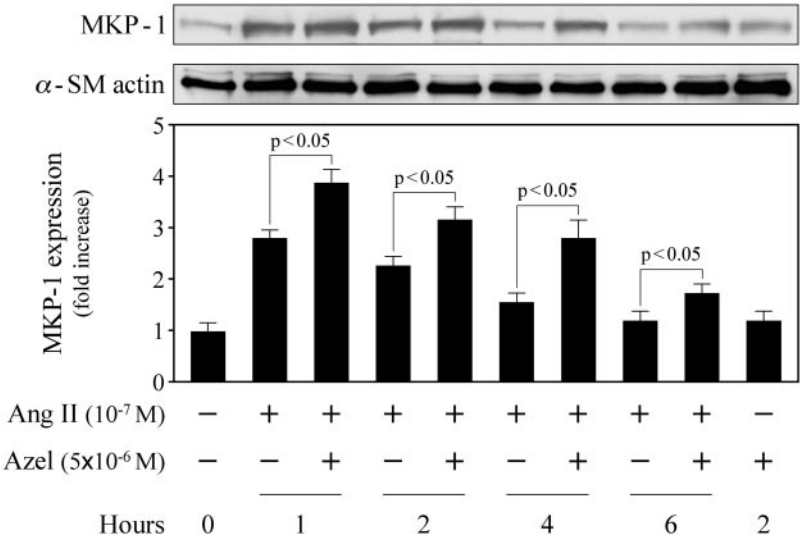

Fig. 3. Effect of azelnidipine on Ang II-induced MKP-1 expression. Subconfluent and quiescent VSMCs were treated with Ang II (10^{-7} M) and azelnidipine (Azel; 5×10^{-6} M) for different times. Immunoblotting was performed using anti-MKP-1 and anti-α-SM actin antibodies. Figures show representative data from three separate experiments. Values are expressed as mean ± S.E.M. ($n = 3$).

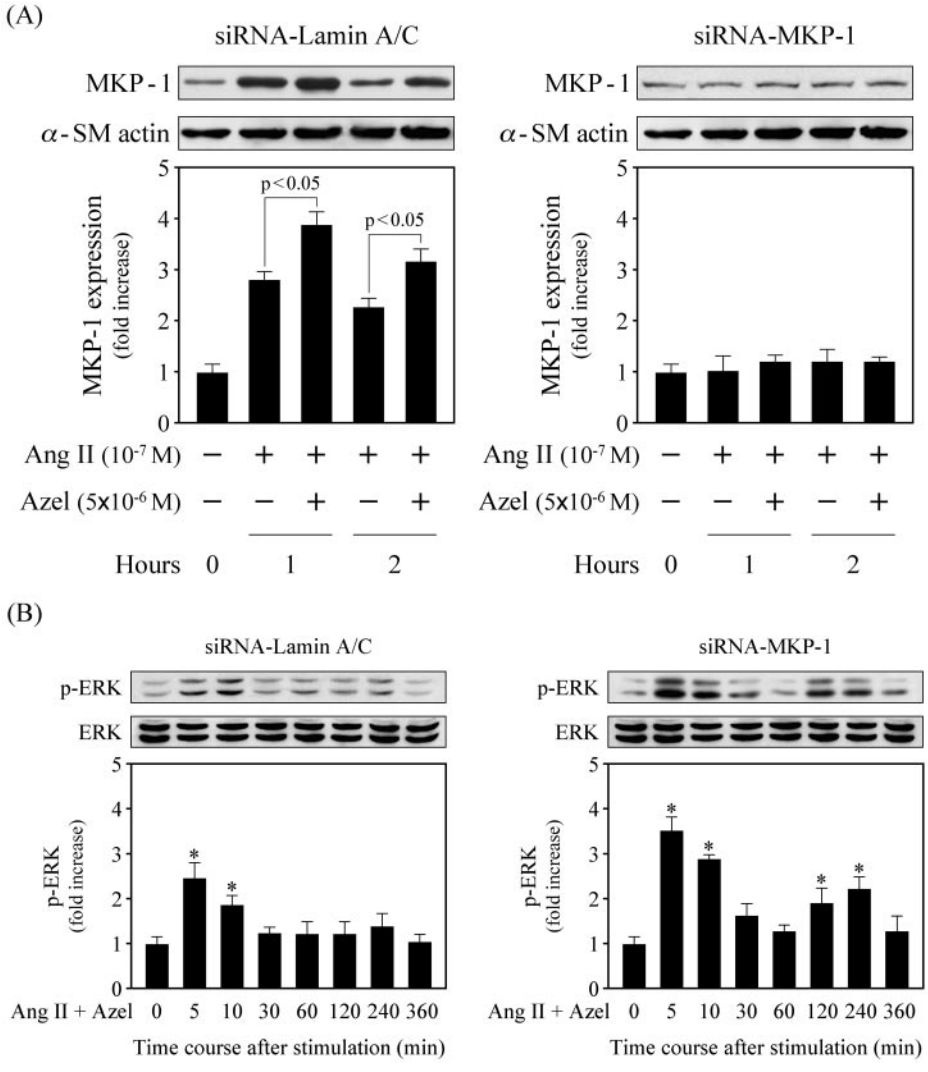

Fig. 4. A, effect of siRNA-MKP-1 on Ang II and azelnidipine-induced MKP-1 expression. Subconfluent and quiescent VSMCs were treated with Ang II (10^{-7} M) and azelnidipine (Azel; 5×10^{-6} M) for different times. Immunoblotting was performed using anti-MKP-1 and anti-α-SM actin antibodies. Figures show representative data from three separate experiments. Values are expressed as mean ± SEM ($n = 3$). B, effect of siRNA-MKP-1 on Ang II and azelnidipine-induced ERK phosphorylation. Subconfluent and quiescent VSMCs were treated with Ang II (10^{-7} M) and azelnidipine (Azel; 5×10^{-6} M) for different times. Immunoblotting was performed using anti-ERK and anti-phospho-ERK antibodies. Figures show representative data from three separate experiments. Values are expressed as mean ± SEM ($n = 3$).

various growth factors, are critical for cell proliferation, differentiation, and hypertrophy (Force and Bonventre, 1998). In our experiment, we observed that Ang II (10^{-7} M) treatment activated Pyk2, c-Src, Jak2, Tyk2, ERK, and STATs, reaching a peak at 5 to 10 min, and then reactivated Pyk2, c-Src, Jak2, Tyk2, ERK, and STATs, showing a second peak at around 2 to 4 h after Ang II stimulation in VSMCs. Moreover, we demonstrated that addition of PD98059 or AG490 even 4 h after Ang II treatment effectively inhibited Ang II-mediated [^{3}H]thymidine incorporation in VSMCs, suggesting that AT1 receptor-mediated activation of ERK or STAT within the first hour after Ang II stimulation was not sufficient to induce VSMC proliferation, and the later phase of AT1 receptor-mediated activation of ERK and STAT seems to be required for the full induction of VSMC proliferation. We insist again both the early and late phase activation of ERK and STAT are essential for Ang II-stimulated VSMC proliferation. Consistent with our observation, it has been shown that growth factor-induced late phase activation of ERK is coupled with cellular responses of proliferation (Cook and McCormick, 1996; Weber et al., 1997; Nelson et al., 1998). Moreover, the possibility that temporal activation of ERK and STATs might mediate distinct cellular events has been reported previously (Pang et al., 1995; Wu and Bradshaw, 1996; Kodama et al., 1997; Pelletier et al., 2003).

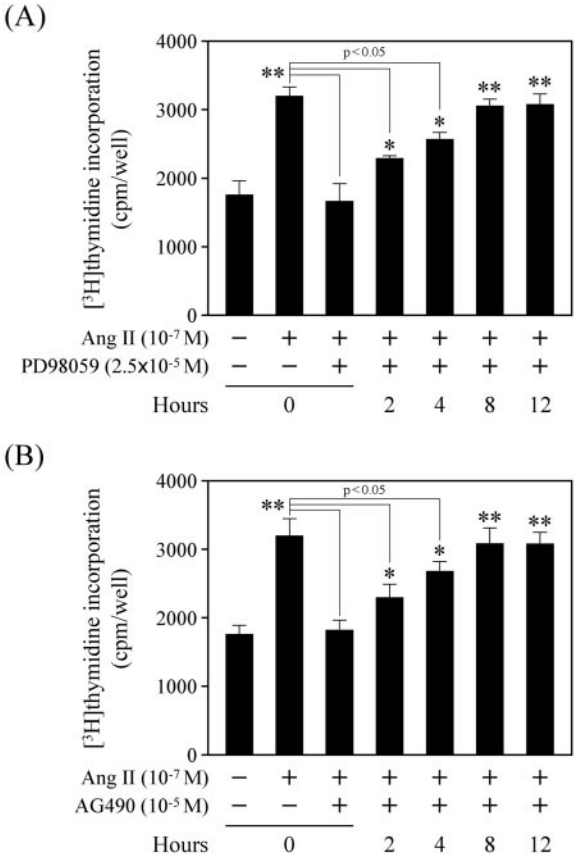

Fig. 5. Effect of MEK inhibitor PD98059 (A) and Jak2 kinase inhibitor AG490 (B) on Ang II-mediated DNA synthesis of VSMCs. Subconfluent, quiescent VSMCs were treated with Ang II (10^{-7} M) for 36 h, with or without PD98059 (2.5×10^{-5} M) or AG490 (10^{-5} M) as indicated. DNA synthesis was assayed by measuring [^{3}H]thymidine incorporation as described under *Materials and Methods*. Similar results were obtained in four different cultured cell lines. Values are expressed as mean ± S.E.M. ($n = 4$). *, $p < 0.05$; **, $p < 0.01$ versus control.

The detailed functional roles of AT1 receptor-mediated biphasic activation of the Pky2/c-Src/ERK pathway and the Jak/STAT pathway remain to be elucidated.

Activation of ERK may also result in increased production of serum response factor, and this may act in concert with the activation of STATs, thereby resulting in an increase of *c-fos* transcription, which is a critical determinant of VSMC proliferation and is regulated by the net interaction with different transcriptional factors. We demonstrated previously that in response to AT1 receptor stimulation, tyrosine- and serine-phosphorylated STAT1 and STAT3 accumulated in the nuclei of VSMCs and became a component of the nuclear *sis*-inducing factor complex, resulting in enhancement of *c-fos* promoter activity (Horiuchi et al., 1999). In the present study, we also observed that AT1 receptor stimulation by Ang II increased tyrosine- and serine-phosphorylated STAT1 and STAT3. These results suggest that both tyrosine- and serine-phosphorylated STAT1 and STAT3 may contribute to the proliferation of VSMCs.

Calcium has also been shown to regulate gene expression via multiple signaling pathways by activating calcium-sensitive kinases such as MAP kinases. It has been reported that AT1 receptor stimulation increases intracellular calcium (Ca^{2+}) via influx of extracellular Ca^{2+} by opening cell membrane calcium channels or release from the intracellular Ca^{2+} pool (Eguchi and Inagami, 2000). We previously reported (Jinno et al., 2004) that ERK activation and tyrosine-phosphorylation of STAT1 and STAT 3 were partially but not totally inhibited in cuff-induced vascular injury of AT_{1a} receptor-null mice, indicating that Ca^{2+} influx via the L-type calcium channel, which results in phosphorylation of growth-promoting signals in VSMCs, may be not only in parallel but also in series to activation of AT1 receptors. The MAP kinases ERK1 and ERK2 have been reported to be activated by an increase in Ca^{2+} influx, which activates upstream kinases. For example, in PC12 cells, membrane depolarization leading to calcium influx through the L-type calcium channels activates the dual specificity MAPK kinase, MEK1, which phosphorylates and activates MAPK (Rosen et al., 1994). Moreover, calcium influx leads to activation of the small guanine nucleotide-binding protein, Ras, which is also required for signaling to MAPK (Rosen and Greenberg, 1996). In the present study, we demonstrated that addition of azelnidipine (5×10^{-5} M) markedly inhibited biphasic Ang II-stimulated Pyk2 and c-Src activation and partially suppressed the initial Ang II-mediated ERK activation and serine-phosphorylation of STAT1 and STAT3, whereas azelnidipine did not inhibit initial activation of Jak2 and Tyk2 and tyrosine-phosphorylation of STAT1 and STAT3 and markedly attenuated the later peaks of ERK, Jak2, and Tyk2 activation and phosphorylation of STATs. Ang II-mediated [^{3}H]thymidine incorporation in VSMCs was also inhibited dose dependently by the addition of azelnidipine. These results suggest that azelnidipine-mediated inactivation the early and late phase of signaling kinases may contribute to its inhibitory effect on the proliferation of VSMCs via inhibition of Ca^{2+} influx.

To elucidate the mechanism by which azelnidipine inhibited the late phase activation of ERK, we focused on MKP-1. Consistent with a previous report (Sandberg et al., 2004), we observed that Ang II stimulation increased MKP-1 expression, reaching a peak at around 60 min, followed by a

decrease in MKP-1 expression. Moreover, we found that the addition of azelnidipine increased Ang II-regulated MKP-1 expression and retarded its decease. On the other hand, siRNA-MKP-1 studies clearly showed that the reduction of the late-phase ERK activation was significantly blocked by MKP-1 knockdown, indicating that MKP-1 expression has contributed to the late phase of ERK phosphorylation. Therefore, it would be possible that blocking of Ca^{2+} entry in VSMCs by azelnidipine increases AT1 receptor-mediated MKP-1 expression, thereby inhibiting Ang II-activated late phase activation of ERK and contributing to the inhibition of Ang II-mediated VSMC proliferation. These studies suggest an additional role of calcium influx in controlling MKP-1 expression and ERK and that studying the detailed mechanism of effect of azelnidipine on MCP-1 expression could contribute to further understanding of the mechanism of VSMC proliferation.

Combined antihypertensive therapy with a CCB and ARB seems to have crucial roles in achieving targeted blood pressure reductions (Kuriyama et al., 2002), and we could

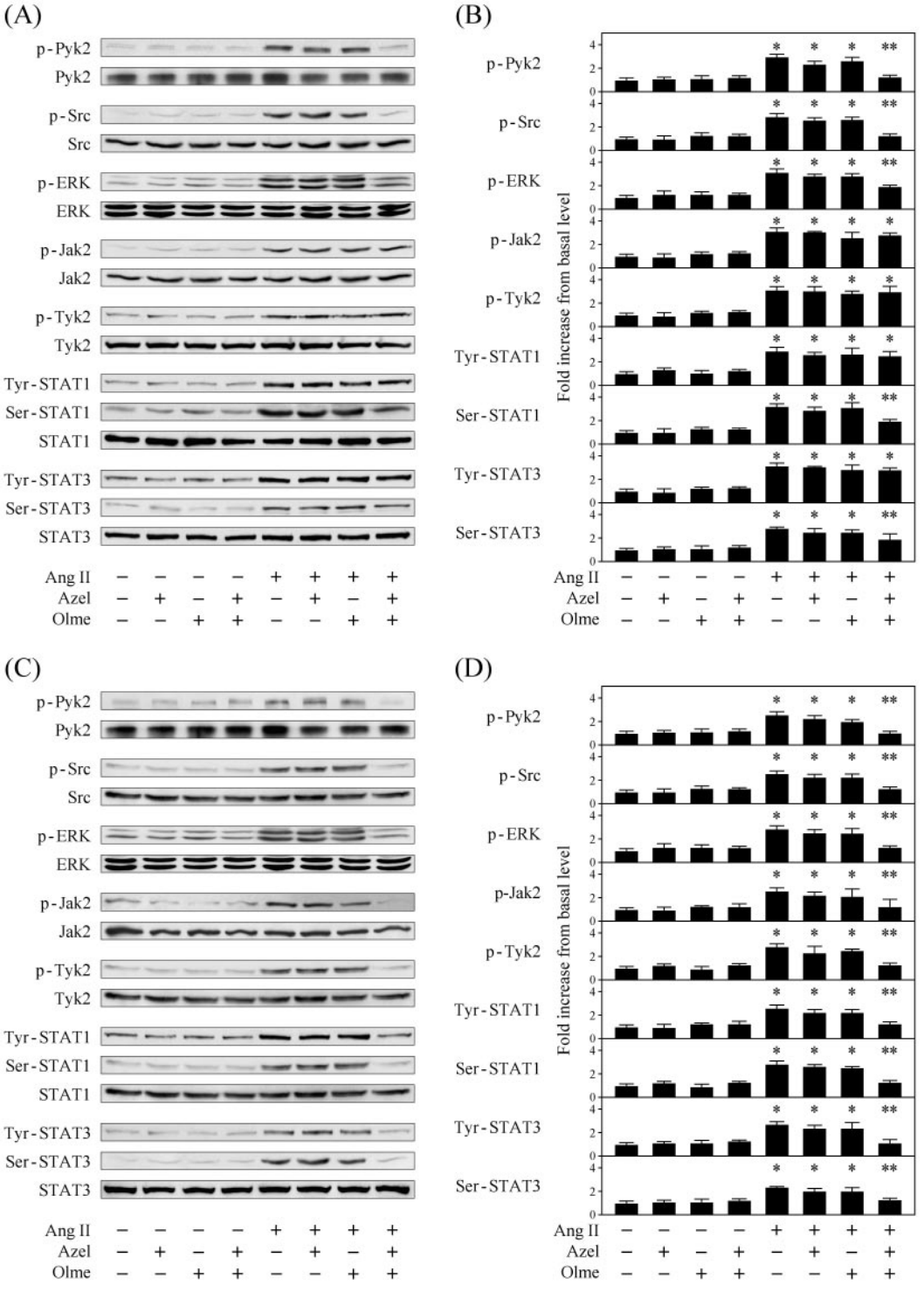

Fig. 6. A and B, effect of combination of azelnidipine (Azel) and olmesartan (Olme) on Ang II-mediated rapid phase phosphorylation of Pyk2, c-Src, ERK, and tyrosine- and serine-phosphorylation of STAT1 and STAT3 in VSMCs. C and D, effect of combination of azelnidipine plus olmesartan on Ang II-mediated late phase of phosphorylation of Pyk2, c-Src, ERK, Jak2, Tyk2, and tyrosine- and serine-phosphorylation of STAT1 and STAT3 in VSMCs. Immunoprecipitation and immunoblotting were performed as in Fig. 2. Figures show representative data from three separate experiments. Values are expressed as mean ± S.E.M. ($n = 3$). *, $p < 0.05$ versus control; **, $p < 0.05$ versus Ang II (+).

anticipate that this combined therapy could contribute to more effective cardiovascular protection and fewer side effects than monotherapy. We demonstrated in this study that Ang II-mediated [^{3}H]thymidine incorporation in VSMCs was inhibited dose-dependently by the addition of azelnidipine (5×10^{-7} M) with olmesartan (10^{-10} M), whereas olmesartan or azelnidipine alone at these doses did not affect [^{3}H]thymidine incorporation in VSMCs. Furthermore, the combination of lower doses of azelnidipine and olmesartan also inhibited the AT1 receptor-mediated early-phase and late-phase phosphorylation of growth-promoting signaling factors Pky2, c-Src, ERK, and STAT. Consistent with these observations, we recently reported that azelnidipine also enhanced the vascular protective effects of olmesartan in polyethylene-cuff-induced vascular injury of the femoral artery in mice, with the inhibition of proliferation of VSMCs and neointimal formation associated with decreased activation of ERK and tyrosine-phosphorylation of STAT1 and STAT3 (Jinno et al., 2004).

Together, these results suggest that Ang II (10^{-7} M) stimulation exerted biphasic activation of Pyk2, c-Src, Jak2, Tyk2, and ERK and tyrosine- and serine-phosphorylation of STAT1 and STAT3, both of which seem to be necessary for the full induction of VSMC proliferation. The L-type calcium channel plays an essential role in AT1 receptor-mediated growth-promoting actions in VSMCs, and MKP-1 is at least one of the critical phosphatases that mediate the antiproliferative actions of the CCB azelnidipine. A CCB had a synergistic inhibition of Ang II-induced DNA synthesis with an ARB, accompanied by marked inhibition of Pyk2, c-Src, Jak kinases, ERK, and STAT activity. Dominant negative-ERK gene transfer significantly suppressed VSMC proliferation in both the intima and the media after balloon injury (Izumi et al., 2001), indicating that ERK pathway has a pivotal role of atherosclerosis formation. Thus, our findings provide novel insights into the pathogenesis of VSMC proliferation and vascular atherosclerosis and might initiate rational and new therapeutic concepts. Our findings also suggest that a combination of CCBs and ARBs might be a useful and effective therapy for the treatment of cardiovascular diseases associated with hypertension.

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