Involvement of PYK2 in Angiotensin II Signaling of Vascular Smooth Muscle Cells

Satoru Eguchi, Hiroaki Iwasaki, Tadashi Inagami, Kotaro Numaguchi, Tadashi Yamakawa, Evangeline D. Motley, Koji M. Owada, Fumiaki Marumo, Yukio Hirata

Abstract—PYK2, a recently identified Ca\(^{2+}\)-sensitive tyrosine kinase, has been implicated in extracellular signal-regulated kinase (ERK) activation via several G protein–coupled receptors. We have reported that angiotensin II (Ang II) induces Ca\(^{2+}\)-dependent transactivation of the epidermal growth factor receptor (EGFR) which serves as a scaffold for preactivated c-Src and downstream adaptors (Shc/Grb2), leading to ERK activation in cultured rat vascular smooth muscle cells (VSMC). Herein we demonstrate the involvement of PYK2 in this cascade. Ang II rapidly induced tyrosine phosphorylation of PYK2, whose effect was completely inhibited by an AT\(_1\) receptor antagonist and an intracellular Ca\(^{2+}\) chelator. A Ca\(^{2+}\) ionophore also induced PYK2 tyrosine phosphorylation to a level comparable with that by Ang II, whereas phorbol ester–induced phosphorylation was less than that by Ang II. Moreover, PYK2 formed a complex coprecipitable with catalytically active c-Src after Ang II stimulation. Although a selective EGFR kinase inhibitor completely abolished Ang II–induced recruitment of Grb2 to EGFR and markedly attenuated Ang II–induced ERK activation, it had no effect on Ang II–induced PYK2 tyrosine phosphorylation or its association with c-Src and Grb2. These data suggest that the AT\(_1\) receptor uses Ca\(^{2+}\)-dependent PYK2 to activate c-Src, thereby leading to EGFR transactivation, which preponderantly recruits Grb2 in rat VSMC. (Hypertension. 1999;33[part II]:201-206.)

Key Words: angiotensin II ▪ receptors, angiotensin ▪ proline-rich tyrosine kinase 2 ▪ c-Src ▪ epidermal growth factors ▪ muscle, smooth, vascular ▪ signal transduction

Angiotensin II (Ang II), a dominant hemodynamic effector of the renin-angiotensin system, has been shown to promote hypertrophy or hyperplasia, or both, of vascular smooth muscle cells (VSMC), cardiac myocytes\(^{1-3}\), and cardiac fibroblasts.\(^{5}\) Ang II also enhances migration and extracellular matrix production of VSMC.\(^{6}\) Therefore, it is now widely believed that Ang II plays a key role in cardiovascular remodeling associated with hypertension, atherosclerosis, restenosis after vascular injury, heart failure, and even diabetes. This notion is supported by results of numerous in vivo experiments, as well as recent clinical trials, demonstrating multiple beneficial effects of ACE inhibitors and angiotensin type 1 receptor (AT\(_1\),R) antagonists in these disease states.\(^{7,8}\)

Thus, much progress has recently made to elucidate the signal transduction mechanisms leading to the growth-promoting effect through a G protein–coupled receptor (GPCR), AT\(_1\),R. It provides an exciting aspect that AT\(_1\),R shares typical signaling events with growth factor receptor such as tyrosine kinase activation and subsequent phosphorylation of the specific substrates accompanied by selective protein/protein interaction, resulting in activation of extracellular signal-regulated kinases (ERKs).\(^{6,9}\) We recently reported that Ang II induces Ca\(^{2+}\)-dependent transactivation of the epidermal growth factor receptor (EGFR) that serves as a scaffold for preactivated c-Src kinase and downstream adapter proteins, Shc/Grb2, leading to p21\(^{ras}\)/ERK activation in cultured rat VSMC.\(^{10}\) However, the mechanism linking AT\(_1\),R to the receptor tyrosine kinase EGFR has not been clear.

Recently, a novel nonreceptor tyrosine kinase with a high homology to p125 focal adhesion kinase (FAK) was cloned by several groups and named proline-rich tyrosine kinase 2 (PYK2),\(^{11}\) cell adhesion kinase \(\beta\),\(^{12}\) related adhesion focal tyrosine kinase,\(^{13}\) and calcium-dependent tyrosine kinase.\(^{14}\) In PC12 cells, PYK2 mediates the recruitment of Grb2/Sos and subsequent p21\(^{ras}\)-dependent ERK activation in response to intracellular Ca\(^{2+}\) accumulation by a GPCR agonist, bradykinin, as well as membrane depolarization.\(^{11}\) Moreover, PYK2 seems to operate these process in concert with c-Src.\(^{15}\) Recently, Ang II has also been shown to activate PYK2 in liver epithelial cells.\(^{14}\) The common feature that both EGFR and PYK2 signaling by GPCRs require intracel-

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lular \(Ca^{2+}\) elevation and c-Src activation prompted us to examine the possible involvement of PYK2 in the growth-promoting signal by Ang II in VSMC. In the present study, we assessed the contribution of PYK2 to the tyrosine kinase cascade operated through AT1R that might exist upstream of the ERK activation in VSMC.

## Methods

### Materials

Ang II and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma. Recombinant human EGF was from Upstate Biotechnology. AG1478, A23187, and BAPTA-AM were from Calbiochem. An agarose-conjugated glutathione-S-transferase (GST)-Grb2 fusion protein and protein A/G-agarose were from Santa Cruz Biotechnology. CV11974 was a generous gift of Takeda Pharmaceutical Co. Anti-PYK2 polyclonal antibody (pAb) (06–559) and anti-phosphotyrosine monoclonal antibody (mAb) (4G10) were obtained from Upstate Biotechnology. Anti-PYK2 mAb (P47120) was from Amersham. Anti-Src pAb (SRC2) and anti-EGF receptor pAb (1005) were from Santa Cruz Biotechnology. Anti-Src mAb (clone 327) was from Calbiochem. The mAb directed to Tyr530-dephosphorylated c-Src (clone 28) was prepared as described previously and selectively recognizes the active form of c-Src. Horseradish peroxidase-conjugated second antibodies were from Amersham.

### Cell Culture

VSMC were prepared from the thoracic aorta of 12-week-old Sprague-Dawley rats (Charles River Breeding Laboratories) by the explant method and cultured in Dulbecco’s modified Eagle’s medium containing 10% FCS, penicillin, and streptomycin as previously described. Subconfluent VSMC from passages 3 through 15 were used in the experiments. The predominant expression of AT1R, but not of AT2R, was confirmed by the binding study. Subconfluent cells were made quiescent under serum-free condition for 3 days.

### Immunoprecipitation and Immunoblotting

Cells were lysed by adding ice-cold lysis buffer, pH 7.5, containing 50 mmol/L HEPES, 50 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 1 mmol/L Na3VO4, 100 mmol/L NaF, 30 mmol/L 2-(p-nitrophenyl)phosphate, 1 mmol/L PMSF, 10 mg/mL leupeptin, and 10 mg/mL aprotinin and centrifuged for 5 minutes at 14 000g. Supernatant was mixed with the antibodies for immunoprecipitation and rocked at 4°C for 2 to 16 hours. Immuno-precipitates were washed in lysis buffer, solubilized in Laemmli’s sample buffer with 2-mercaptoethanol, resolved by SDS-PAGE, and transferred to nitrocellulose membrane. After blocking with 5% milk, the membrane was treated with a primary antibody, followed by a secondary antibody conjugated with horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham) as described. For immunoblot analysis of Grb2-associable proteins, agarose-conjugated GST-Grb2 fusion protein was rocked with Triton X-100–treated cell lysate at 4°C for 2 to 16 hours and washed with lysis buffer. Bound proteins were solubilized, resolved by SDS-PAGE, and subjected to immunoblotting as described.

### Results

#### Ang II Activates PYK2 Through AT1R

To assess whether Ang II activates PYK2 in VSMC, the effect of Ang II on phosphotyrosine content of PYK2 was examined. Treatment of quiescent rat VSMC with Ang II (10^{-7} mol/L) markedly increased tyrosine-phosphorylated PYK2 as early as 2 minutes; neither phosphorylated band nor immunoprecipitated PYK2 was observed when normal rabbit IgG was used for the immunoprecipitation (Figure 1A). Pretreatment with 10^{-5} mol/L CV11974, a selective AT1R antagonist, completely blocked Ang II–induced tyrosine phosphorylation of PYK2 (Figure 1B). These data indicate that Ang II activates PYK2 via AT1R in rat VSMC.

#### Calcium-Dependent PYK2 Activation by Ang II

PYK2 activation through GPCRs involves intracellular Ca^{2+} elevation and/or protein kinase C (PKC) activation in PC12 cells. Stimulation of AT1R activates phospholipase C \(\beta\) to increase cytosolic free Ca^{2+} concentration ([\(Ca^{2+}\)]_i) and activates PKC in VSMC. However, both p21ras/ERK activation and EGFR transactivation via AT1R are mainly mediated by an increase in [\(Ca^{2+}\)]. To determine the Ca^{2+} dependence of PYK2 activation by Ang II in VSMC, the effect of an intracellular Ca^{2+} chelator (BAPTA-AM) was examined. Pretreatment with 10^{-5} mol/L BAPTA-AM, but not with its solvent DMSO (0.1%), completely inhibited the Ang II–induced PYK2 phosphorylation (Figure 2A). The Ca^{2+} ionophore A23187 (10^{-5} mol/L) also induced PYK2 tyrosine-phosphorylation comparable with that by 10^{-7} mol/L Ang II, whereas a PKC activator, PMA (10^{-6} mol/L), minimally induced PYK2 phosphorylation (Figure 2B). These data demonstrated that Ang II–induced PYK2 activation requires...
an increase in \([\text{Ca}^{2+}]_i\), rather than activation of PKC in rat VSMC.

**Association of PYK2 With c-Src by Ang II**

The autophosphorylation of PYK2 at Tyr402 with the conserved YAEI sequence provides a selective binding site for the SH2 domains of Src family tyrosine kinase for its activation, which is essential for the PYK2-mediated ERK activation by several GPCR agonists. We and others have recently demonstrated that c-Src is involved in the Ang II–induced ERK activation in rat VSMC. To determine whether c-Src plays a role in PYK2 signaling activated by Ang II, we performed immunoprecipitation (IP) with anti-PYK2 pAb, followed by immunoblotting (IB) with anti-phosphotyrosine (pTyr) mAb and anti-PYK2 mAb. Ang II (10\(^{-7}\) mol/L) initiated tyrosine phosphorylation of PYK2 as early as 1 minute, which was sustained up to 5 minutes, with concomitant transient (1 to 2 minutes) association of PYK2 with c-Src (clone 28) by repeated reprobing. Figure 3A shows that Ang II (10\(^{-7}\) mol/L) increased the association of PYK2 with active c-Src (clone 28). These data provide evidence for the involvement of c-Src in PYK2 signaling initiated by AT,R, presumably through an increase in \([\text{Ca}^{2+}]_i\), a new finding to our knowledge.

**Effect of EGFR Inhibition of PYK2 Signaling**

We have recently shown that c-Src exists upstream of EGFR transactivation, which plays an essential role in the AT,R-mediated Ca\(^{2+}\)-dependent ERK activation in rat VSMC. Thus, it could be hypothesized that PYK2 may contribute to the EGFR transactivation through c-Src. To elucidate the hierarchical order of PYK2, c-Src, and EGFR, the effect was studied of a selective EGFR kinase inhibitor, AG1478, on the PYK2 phosphorylation and its association with c-Src. Neither phosphorylation of PYK2 nor its association with c-Src by Ang II (10\(^{-7}\) mol/L) was inhibited by AG1478 at 2.5\(\times\)10\(^{-7}\) mol/L (Figure 4A), a concentration effective in inhibiting Ang II–induced ERK activation in rat VSMC. Furthermore, EGF (100 ng/mL) did not affect the phosphotyrosine content of PYK2 in rat VSMC (data not shown).
Ang II–induced association of PYK2 with c-Src and Grb2. A, VSMC were pretreated with 2.5 × 10^{-7} mol/L AG1478 or 0.1% dimethylsulfoxide (DMSO) for 30 minutes and then stimulated with Ang II (10^{-7} mol/L) for 2 minutes. Cell lysates were immunoprecipitated (IP) with anti-PYK2 pAb, followed by immunoblotting (IB) with anti-phosphotyrosine (pTyr) mAb, anti-PYK2 mAb, and anti-c-Src mAb (clone 28). B, VSMC were pretreated with 2.5 × 10^{-7} mol/L AG1478 or 0.1% dimethylsulfoxide (DMSO) for 30 minutes and then stimulated with Ang II (10^{-7} mol/L) for 2 minutes. After cell lysis, GST-Grb2 fusion protein was added. Proteins associated with the fusion protein were subjected to immunoblotting (IB) with anti-PYK2 mAb and anti-EGFR pAb.

The activated PYK2 has been shown to recruit Grb2 for the ERK activation in neuronal cells.\textsuperscript{11} To elucidate whether similar mechanism is operated in VSMC after stimulation with Ang II, the lysates of VSMC stimulated by Ang II (10^{-7} mol/L) with or without pretreatment of AG1478 (2.5 × 10^{-7} mol/L) for 30 minutes were coprecipitated with GST-Grb2-fusion protein, followed by immunoblotting with antibodies against EGFR or PYK2. Ang II increased the amounts of PYK2 coprecipitable with the fusion protein regardless of the presence of AG1478, whereas AG1478 completely inhibited Ang II–induced association of EGFR with the fusion protein (Figure 4B). Thus, activation of PYK2, as well as its association with c-Src and Grb2 in response to Ang II, occurs independent of EGFR kinase activity, suggesting that PYK2 may be located upstream of and/or in parallel with the EGFR in VSMC.

**Discussion**

A growing body of evidence indicates that the growth-promoting effect by Ang II is mediated by activation of several protein tyrosine kinases.\textsuperscript{6–9} Earlier studies from our laboratory indicated that a Ca\textsuperscript{2+}-dependent tyrosine kinase or kinases may transmit AT\textsubscript{1}R signal to the ERK cascade in rat VSMC.\textsuperscript{18} Subsequently, we have shown EGFR as such a tyrosine kinase.\textsuperscript{19} Here, we further identified and characterized another AT\textsubscript{1}R-responsive Ca\textsuperscript{2+}-dependent tyrosine kinase as PYK2 in rat VSMC.

PYK2 has been shown to be regulated by Ca\textsuperscript{2+} signal in PC12 cells\textsuperscript{11} and constitutes a major Ca\textsuperscript{2+}-dependent tyrosine kinase in Ang II–stimulated liver epithelial cells.\textsuperscript{14} The Ca\textsuperscript{2+}-dependency of the Ang II–induced PYK2 activation as demonstrated in this study is consistent with a recent report showing that Ang II– and platelet-derived growth factor–stimulated PYK2 activation was inhibitable with the intracellular Ca\textsuperscript{2+} chelator in rat VSMC,\textsuperscript{20} whereas the importance of PKC was also suggested. However, the present results appear to demonstrate that PYK2 phosphorylation by PMA is much weaker than those by Ang II and a Ca\textsuperscript{2+} ionophore, suggesting a preferential role of calcium to PKC in regulation of PYK2 in VSMC. Because PYK2, which lacks calmodulin-binding motif, cannot be activated by either Ca\textsuperscript{2+} or calmodulin in vitro,\textsuperscript{11} the mechanism by which Ca\textsuperscript{2+} signal activates PYK2 remains to be determined.

Src family tyrosine kinase has been implicated in the ERK activation by various agonists for GPCRs, including AT\textsubscript{1}R.\textsuperscript{21–23} Recently, it has been reported that both G\textsubscript{i} and G\textsubscript{q} agonists, such as bradykinin and lysophosphatidic acid, respectively, induced association of PYK2 with c-Src through binding of autophosphorylated Tyr402 of PYK2 to the SH2 domain of c-Src, thereby leading to c-Src activation.\textsuperscript{15} The activated c-Src could further phosphorylate PYK2 at Tyr881 followed by the LNV sequence and an adaptor protein Shc, thereby recruiting the Grb2/Sos complex. These events are believed to be essential for the ERK activation by GPCR agonists in PC12 cells.\textsuperscript{15} The calcium-dependent PYK2/c-Src activation has also been shown to bridge both G\textsubscript{i} and G\textsubscript{q}-coupled receptors to the ERK activation in HEK 293 cells.\textsuperscript{24} In rat VSMC, we have recently shown that Ang II increased transient association of active c-Src with Shc that is contingent on Shc phosphorylation.\textsuperscript{10} In the present study, we further demonstrated that PYK2 formed a complex with an active c-Src and Grb2 on Ang II stimulation. Therefore, it is reasonable to speculate that PYK2 may contribute to the Ang II–induced ERK activation in concert with Src family tyrosine kinase and adaptors (Shc and Grb2) in cells where AT\textsubscript{1}R promotes cell growth, such as in VSMC.

In addition to PYK2 and c-Src, combination of multiple tyrosine kinases appears to be involved in the ERK activation by GPCR agonists depending on cell type. For example, the G\textsubscript{i}-coupled ERK activation requires Csk, Lyn, and Syk, whereas the G\textsubscript{q}-coupled activation requires Btk and Syk in avian lymphoma cells.\textsuperscript{25} We and others have recently shown that c-Src acts upstream of EGFR transactivation to feed into the ERK cascade through G\textsubscript{i}-coupled AT\textsubscript{1}R in rat VSMC\textsuperscript{10} and G\textsubscript{i}-coupled lysophosphatidic acid and α\textsubscript{2A}-adrenergic receptors in COS-7 cells,\textsuperscript{26} respectively. Interestingly, not only PYK2 and c-Src,\textsuperscript{15} but also EGFR,\textsuperscript{27} appear to be essential for the Ca\textsuperscript{2+}-dependent ERK activation by GPCR agonists in PC12 cells. Thus, the Ang II–induced Ca\textsuperscript{2+}-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
 & DMSO & AG1478 \\
\hline
Ang II (100 nM) & - & + \\
\hline
IB: pTyr & - & + \\
\hline
IP: PYK2 & - & + \\
\hline
Ang II (100 nM) & - & + \\
\hline
IB: EGFR & - & + \\
\hline
IB: PYK2 & - & + \\
\hline
\end{tabular}
\caption{Effects of AG1478 on Ang II–induced association of PYK2 with c-Src and Grb2.}
\end{table}
Figure 5. Possible involvement of PYK2 in signal transduction of VSMC operated through AT_R. Ang II induces ERK cascade (Ras, Raf, MEK, ERK) through adaptors (Shc and Grb2) and Sos recruited through EGFR that is transphosphorylated by c-Src. The c-Src activation is mainly mediated through PYK2, which senses intracellular Ca\textsuperscript{2+} elevation by G\textsubscript{i} phospholipase C–coupled AT\textsubscript{1}R. PYK2 may also contribute to the direct recruitment of Grb2/Sos complex for ERK activation, although possibly constituting a minor component.

dependent PYK2 activation accompanied by its interaction with c-Src as demonstrated in this study and the Ang II–induced association of c-Src with EGFR as demonstrated in our previous study\textsuperscript{10} strongly suggest that PYK2 function is mainly located upstream of the AT\textsubscript{1}R-mediated EGFR transactivation. This is consistent with the present observation that neither PYK2 phosphorylation nor its association with active c-Src requires EGFR kinase activity after Ang II stimulation.

Alternatively, PYK2 could function in parallel with EGFR to feed into the ERK cascade because Ang II–induced association of Grb2 with PYK2 occurred even when the association of Grb2 with EGFR was completely blocked by AG1478. In liver epithelial cells, Ca\textsuperscript{2+}– and PKC-dependent PYK2 activation by Ang II was reported,\textsuperscript{14} whereas the Ang II–induced EGFR transactivation appeared to be driven only when cellular PKC was depleted.\textsuperscript{28} However, we have recently shown that AG1478 markedly inhibited the Ang II–induced ERK activation.\textsuperscript{10} Taken together, we submit that the recruitment of Grb2 by PYK2 contributes little, if any, to the ERK activation through AT\textsubscript{1}R and that the ERK activation by Ang II appears to be preferentially mediated by the recruitment of Grb2 to the EGFR in VSMC. A possible involvement of PYK2 in signal transduction of Ang II–induced ERK activation is illustrated in Figure 5.

PYK2 may account for other signaling pathways than the ERK cascade by AT\textsubscript{1}R in VSMC. PYK2 is involved in c-Jun amino-terminal kinase (JNK) activation induced by tumor necrosis factor–α, ultraviolet irradiation, and osmotic shock.\textsuperscript{29} It has been shown that PYK2 activation is correlated with JNK activation\textsuperscript{14} and p70 ribosomal S6 kinase activation, but not ERK activation.\textsuperscript{30} In Ang II–stimulated rat liver epithelial cells, in addition, PYK2 has a “focal adhesion-targeting domain” homologous to that of FAK.\textsuperscript{13} In fact, PYK2 has been shown to be tyrosine-phosphorylated after β\textsubscript{1}-integrin stimulation\textsuperscript{11} and to be associated with a cytoskeletal protein, paxillin.\textsuperscript{32} In this regard, it has recently been reported that paxillin is tyrosine-phosphorylated by and associates with PYK2 in Ang II–stimulated rat liver epithelial cells.\textsuperscript{33} Because these tyrosine kinases (PYK2, c-Src, EGFR) may phosphorylate each other as well as respective specific substrates and recruit additional signaling molecules, several signaling events branching at the level of these kinases will account for diverse functions of the AT\textsubscript{1}R in a tissue- and cell type–specific manner.

In conclusion, we have demonstrated that Ang II induces a Ca\textsuperscript{2+}–dependent PYK2 activation and its interaction with c-Src and Grb2 in rat VSMC. Further elucidation of cross-talk between AT\textsubscript{1}R and protein tyrosine kinases, as well as their downstream signals, should unravel the exact role of Ang II in the mechanism of vascular remodeling under pathological states, such as in hypertension, atherosclerosis, and restenosis after angioplasty.

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