

Angiotensin II-mediated vascular smooth muscle cell growth signaling

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Abstract

The mechanism by which Ang II stimulates the growth of vascular smooth muscle cells was investigated by measuring the phosphorylation of mitogen-activated protein kinases ERK 1 and ERK 2. Ca^{2+} ionophore was found to have effects practically analogous to Ang II. We found that the signaling pathway involves the activation of epidermal growth factor receptor (EGFR) kinase, activation of the adaptor proteins Shc and Grb2, and the small G-protein Ras. Although the mechanism of AT_1 - (or Ca^{2+})-induced activation of EGFR is not yet clear, we have found that calcium-dependent protein kinase $CAK\beta$ /PYK2 and c-Src are involved in this process. These studies indicate a transactivation mechanism that utilizes EGFR as a bridge between a Gq-coupled receptor and activation of phosphotyrosine generation.

Key words

- Angiotensin II
- Type 1 receptor
- Epidermal growth factor receptor
- Vascular smooth muscle cells

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Introduction

Heptahedral receptors have been considered to be coupled to various heterotrimeric G-proteins and transmit their signals through the classical 3',5'-cyclic adenosine mononucleotide (cAMP) by Gs or Gi, or by elevation of cytoplasmic Ca^{2+} or stimulation of protein kinase C (PKC) through the activation of $Gq_{/11}$. On the other hand, signals regulating cellular growth have been considered to go through growth factor or cytokine receptors, which have a single transmembrane domain, like receptors for epidermal growth factor (EGFR) and platelet-derived growth factor (PDGFR). However, some of the heptahedral (heterotrimeric G-protein-coupled) receptors, such as the type 1 angiotensin II (Ang II) receptor, AT_1 , have been recognized to be one of the most potent stimulators of hypertrophic remodeling of

the vascular and cardiac ventricular wall (1-5). The mechanism for such a growth stimulation by Ang II, other than the well-known vascular contraction, regulation of renal tubular electrolyte handling, aldosterone release and facilitation of adrenergic release, has not been clear.

However, in recent years observations of Ang II-stimulated activation of some of the mitogen-activated protein kinase (MAPK) such as extracellular signal-regulated kinase (ERK 1/2) have been reported, even though the pathway leading to ERK activation is, in many cases, mediated by protein tyrosine kinases and is thought to require the growth factor, or cytokine receptor signaling mechanisms since it seems to require tyrosine phosphorylation (6,7).

As Ang II receptor subtypes were defined as subtype-specific non-peptidic receptors (8), the growth stimulation by Ang II was

identified to reside in the type 1 receptor AT₁. Following the cloning and structure determination of AT₁, by us (9) and Murphy et al. (10), studies by Morrero et al. (11-13) and Dostal et al. (14) suggested possible direct binding of phospholipase C- γ (PLC- γ) and Janus kinase (JAK) to a phosphotyrosyl residue in the carboxyterminal segment of AT₁, possibly Tyr³¹⁹ or Tyr³¹⁹-Ile-Pro-Pro-³²², and subsequent activation of these enzymes.

However, we entertained a different hypothesis since we found that the C-terminus truncation of rat AT₁ at residue Lys³¹⁸ or eliminating the Tyr-Ile-Pro-Pro did not eliminate the activation of ERK. Another rationale for the hypothesis of the direct activation of the tyrosine kinase system by Tyr³¹⁹ was due to the abundant presence of PLC- γ and failure to observe PLC- β in certain vascular smooth muscle cell (VSMC) lines which was consistently not the case in explanted VSMC we had used for a long time.

Elevation of Ca²⁺ mimics Ang II in MAPK/ERK activation

We hypothesized that the PLC- β -mediated increase in Ca²⁺ or PKC may be the mechanism of Ang II-mediated MAPK/ERK activation and demonstrated that calcium ionophore A23187 increased MAPK. Furthermore, the intracellular calcium chelator

BAPTA-AM or calcium release inhibitor TMB8 abolished the MAPK activation by Ang II, as shown in Figure 1. Interestingly, similar patterns of regulation were also seen in the activation of the small G-protein Ras which is located upstream of ERK. Thus, we were able to show that the activation of ERK by intracellular calcium proceeds through the Ras-dependent pathway (15). The precise mechanism of activation of Ras or MAPK by Ca²⁺ is not known. The possibility of PKC-mediated signaling was eliminated for VSMC pretreated with a PKC inhibitor GF109203X or by PKC depletion by overnight exposure to phorbol ester. This is a unique and important feature of VSMC in contrast to cardiomyocytes in which a PKC-mediated mechanism seems to be involved (16).

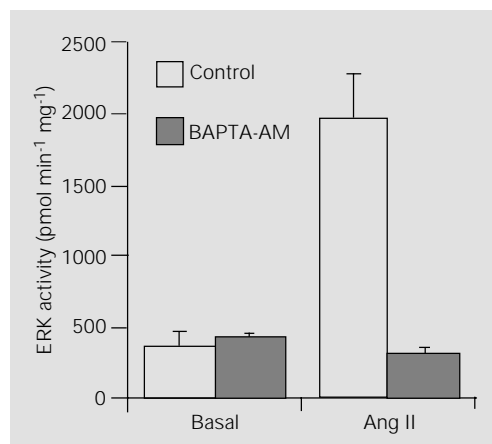
Involvement of EGFR in the activation of Ras and MAPK

However, the role of Ca²⁺ (but not PKC) in the activation of Ras and MAPK was not clear. To clarify the mechanism we examined steps upstream of Ras, which were most likely growth factor receptors.

As shown in Figure 2, activated EGFR transmits its signal by tyrosine phosphorylation of several tyrosine residues by EGFR kinase, subsequently forming a complex with Shc and then Grb2, or directly with Grb2. Grb2, in turn, activates the GDP-GTP exchange protein mSOS for Ras. This pathway was revealed by experiments using a fusion protein formed between glutathione-S-transferase (GST) and Grb2 for affinity precipitation of complex of Grb2 with Shc, EGFR and/or Sos.

By this technique we have demonstrated that activation of EGFR, as reflected by a complex formation with Grb2-GST, is induced by elevated cytoplasmic Ca²⁺, and the activated EGFR leads to the activation of MAPK/ERK (17). Interestingly, the transactivation of EGFR occurs by an intracellular

Figure 1 - Effects of the intracellular Ca²⁺ chelator BAPTA-AM on Ang II-induced extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) activation. Cultured rat VSMC were pretreated with BAPTA-AM (10 μ M) for 30 min, and then stimulated with Ang II (100 nM) for 5 min. The ERK/MAPK activity toward a specific substrate was determined.



mechanism rather than by EGF, which may have been produced and released from the Ang II-activated VSMC because the conditioned medium of Ang II-activated VSMC was not capable of activating EGFR in VSMC (17). This is a new concept of EGFR activation by an intracellular mechanism through a Ca^{2+} -dependent system.

If EGFR mediates Ang II-dependent activation of ERK, it is tempting to examine if a similar but distinct PDGFR could function as a similar mediator. EGFR kinase and PDGFR kinase specific inhibitors tyrphostin AG1478 and AG1295, respectively, became available (18). The expected inhibition of the activation of ERK by Ang II was observed only with the EGFR kinase inhibitor AG1478 (75% reduction), whereas the PDGFR kinase inhibitor AG1295 showed no significant effect (17). At present the mechanism for the selective activation of EGFR by Ang II but not PDGFR is not clear. However, it is interesting to note that the quantity of PDGFR in VSMC is much greater than that of EGFR. For understanding the mechanism of the specific activation of EGFR, a major void in our knowledge exists for the mechanism between increased cytoplasmic Ca^{2+} and EGFR activation. However, it is important to recognize that G-protein-coupled receptors are capable of transactivating a receptor tyrosine kinase system in many cells (19-21). This new concept is now supported by a similar mechanism mediated by a variety of heptahedral receptors.

Calcium-activated tyrosine kinase

Recently, several investigators reported a new Ca^{2+} -activated tyrosine kinase in the cytoplasmic fraction, which was named $CAK\beta$ (22)/ $PYK2$ (23)/ $RAFTK$ (24)/ $CADTK$ (25). Although the structure of this kinase indicates that it belongs to the family of focal adhesion kinase (FAK), this Ca^{2+} -activated proline-rich tyrosine kinase was

different from FAK.

The $CAK\beta/PYK2$ type soluble tyrosine kinase is a good candidate to mediate the signal of an elevated cytoplasmic Ca^{2+} to EGFR and/or ERK 1/2. We and others were able to show that such a mechanism is activated by Ang II or Ca^{2+} in VSMC (26,27), liver epithelial cells (25), cardiac fibroblasts (28), and pheochromocytoma cells (23). The cytoplasmic tyrosine kinase was activated by Ang II through AT_1 or the Ca^{2+} ionophore A23187, but not by PKC (26).

Interestingly, a member of an oncogene and a soluble protein tyrosine kinase, c-Src, is also activated in this process of Ang II (AT_1)-stimulated transactivation of EGFR, as detected by the monoclonal antibody P28 specific for the active form of c-Src (17). The activation of $PYK2$ and c-Src precedes the transactivation of EGFR, since the inhibition of the EGFR kinase by tyrphostin AG1478 does not affect the activation of $PYK2$ or c-Src (26). Active c-Src and $PYK2$ also form a coprecipitable complex in agreement with the observation of Dikic et al. (29) in a neuronal cell system. This system is very complex and we are not yet able to describe it as a linear vertical flow of signaling events. This mechanism involving at least three kinases seems to be much more complex than we have imagined. It is possible that this area may also involve mechanisms and systems

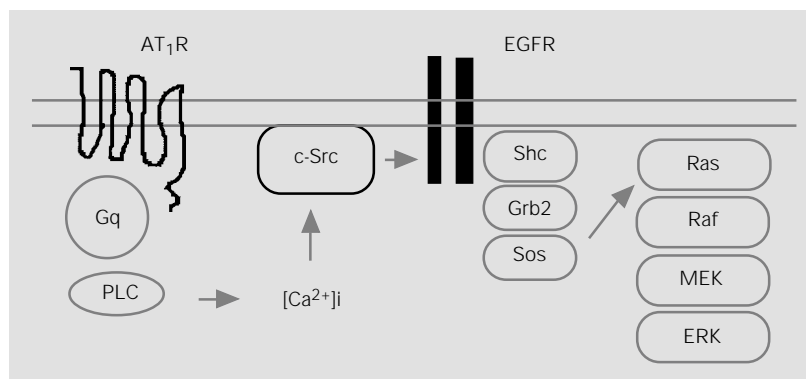


Figure 2 - In VSMC, Ca^{2+} -dependent transactivation of epidermal growth factor receptor (EGFR) mediates Ang II-induced Ras/ERK MAPK activation. AT_1R , Type 1 angiotensin II receptor; PLC, phospholipase C; ERK, extracellular signal-regulated kinase; MEK, MAPK kinase.

that may be stimulated by reactive oxygen species.

Roles of activated ERKs in VSMC

ERKs are a focal point for diverse cell growth and proliferating responses. Activation of ERK induces the transcription of the *fos* gene. Indeed, we were able to directly demonstrate c-Fos expression in a rapid response to Ang II-induced EGFR transactivation and subsequent ERK activation. Ang II-

induced protein synthesis is also mediated by the EGFR and ERK (30).

Dual signals for S6 kinase activation

In the hypertrophic responses of VSMC, the net effects of multiple effectors such as Ang II, insulin, etc., are syntheses of two discrete S6 kinases (p90 and p70 S6 kinases), which regulate the function of ribosomes. p90^{S6k} is subject to direct activation by ERK, but the upstream of p70^{S6k} still remains unclear.

Quite recently, we found that regulation of p70^{S6k} is complex, since it is activated by Ang II through two separate but related signaling pathways (31), as discussed below. We have shown that Ras-dependent ERK activation results in the activation of p70^{S6k}. A dominant negative mutant of Ras was found to inhibit not only ERK 1/2, but also p70^{S6k} (Figure 3). Inhibition of MEK (MAPK kinase) by PD98059 (the ERK pathway on the left side below Ras in Figure 4) was sufficient to block p70^{S6k}, whereas phosphatidyl inositol-3-kinase (PI3K) inhibitor blocked the p70^{S6k} activation without affecting the ERK. This observation suggested the presence of a second pathway. Indeed, we observed that Ang II activates Akt/protein kinase B (PKB). Akt/PKB was known to be activated by insulin through the activation of PI3K (32). However, we have found that Ang II activates wortmannin/LY294002-sensitive PI3K and its downstream components Akt/PKB and p70^{S6k} by phosphorylating the Ser⁴¹¹ residue of the latter, shown on the right side of Figure 4.

Activation of this pathway by insulin does not activate ERK. Of particular interest is that the PI3K-activating insulin effect shares a common signaling pathway with Ang II. The role of Ras is also intriguing in that it activates two bifurcating pathways. Although, Akt/PKB is a multifunctional activator of various signaling pathways, its activation of p70^{S6k} results

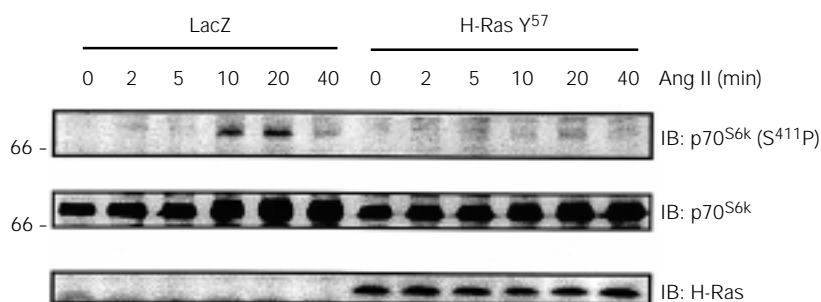


Figure 3 - Effect of dominant-negative H-Ras transfection of angiotensin II (Ang II)-induced Ser⁴¹¹ phosphorylation (S⁴¹¹P) of p70^{S6k}. VSMC were transfected with AdRas Y⁵⁷⁷ or control AdLacZ as indicated before stimulation. Cells were stimulated with Ang II (100 nM) for indicated durations. Cell lysates were analyzed by immunoblotting (IB) with anti-[phospho-Ser⁴¹¹]-p70^{S6k}, anti-p70^{S6k}, and anti-H-Ras antibodies as indicated by repeated reprobing.

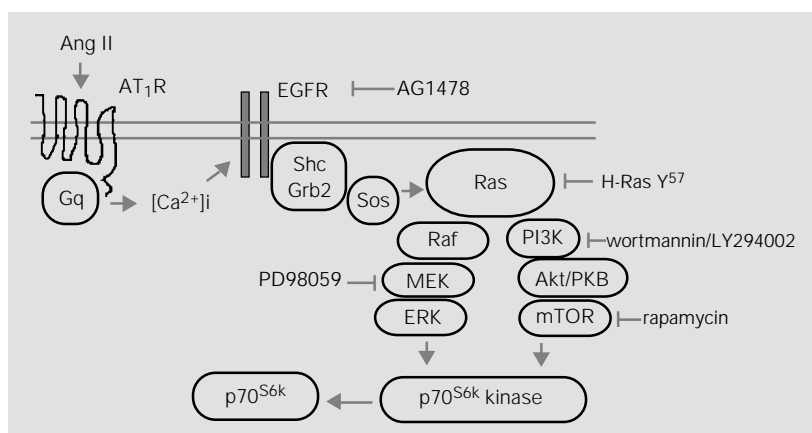


Figure 4 - Hypothetical pathways involved in the phosphorylation of p70^{S6k} by angiotensin II (Ang II). According to this model, activation of the putative S6 kinase (S6k) by Ang II requires two separate pathways which bifurcate at the point of Ras activation through transactivated epidermal growth factor receptor (EGFR). AT₁R, Type 1 Ang II receptor; ERK, extracellular signal-regulated kinase; PKB, protein kinase B; PI3K, phosphatidylinositol-3-kinase; MEK, MAPK kinase.

in resynthesis of the pathways leading to the hypertrophy of vascular smooth muscle cells (Figure 4).

Conclusion

We tried to summarize recent findings from our laboratory and others focusing on the hypertrophic effects of Ang II mediated by p70^{S6k} in quiescent cultured vascular smooth muscle cells. Although there are some large gaps in our knowledge, we have provided unequivocal evidence of transactivation of EGFR by a Gq₁₁-coupled receptor in a unique fashion since PDGFR is not activated.

Some of the components of pivotal importance in this system are Src family tyrosine kinase, EGFR, Ras-ERK, or Ras-PI3K and Akt, ending at p70^{S6k}. CAK β /PYK2 may

be a potential candidate for bridging Ca²⁺ to EGFR.

These studies are of particular relevance to vascular remodeling since this system seems to provide possible common sites of actions of two major components of vascular degenerative change, insulin and Ang II. Furthermore, it is important to note that the Ang-II-to-ERK pathway is mediated by reactive oxygen species (Frank GD, Eguchi S, Yamakawa T, Tanaka S, Inagami T and Motley ED, unpublished results).

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