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THE ANGIOTENSIN II AT_1 RECEPTOR IS TYROSINE AND SERINE PHOSPHORYLATED AND CAN SERVE AS A SUBSTRATE FOR THE SRC FAMILY OF TYROSINE KINASES

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SUMMARY - Angiotensin II AT_1 receptor signal transduction has recently been shown to function through the phospholipase C isozyme, PLC-γ. Since PLC-γ is known to interact with phosphotyrosine containing proteins through SH_2 domains, we examined the phosphorylation state of the AT_1 receptor. Immunoprecipitation of the [^{32}P] labeled AT_1 receptor from rat aortic smooth muscle cells followed by alkali hydrolysis demonstrated the presence of tyrosine phosphorylation. Phosphoamino acid analysis of the excised bands demonstrated the presence of phosphoserine and phosphotyrosine residues. A fusion protein comprising the intracellular tail of the AT_1 receptor was used to screen for candidate kinases, and the src kinase family displayed high activity. In summary, this study shows that the AT_1 receptor is serine and tyrosine phosphorylated in vivo and suggests that a soluble kinase related to the src family may be responsible for the tyrosine phosphorylation.

Tyrosine phosphorylation of proteins is widely recognized as a critical event in intracellular signal transduction (1). Many growth factor receptors such as PDGF and EGF are receptor tyrosine kinases, and ligand binding to these receptors activates an intracellular cascade of protein phosphorylations (2). The Ang II AT_1 receptor falls into the G-protein class of cell surface receptors and does not contain an intrinsic kinase activity (3). However, like PDGF and EGF, Ang II has been shown to act as a mitogen for several cell types (4). Recently it has been shown in VSMC that Ang II signal transduction through the AT_1 receptor occurs via tyrosine phosphorylation of PLC-γ (5). This method of activation is an intracellular pathway common to the PDGF and EGF receptor signalling pathways (2). Because PLC-γ interacts with other proteins through SH_2 domain binding to phosphotyrosine containing sequences (6), we examined the phosphotyrosine state of the AT_1 receptor. Since the receptor does not have an intrinsic

Abbreviations: VSMC - rat aortic vascular smooth muscle cells; PLC - phospholipase C; SH_2 - src homology 2; DMEM - Dulbecco's modified eagle medium; GST - glutathione-S-transferase; PDGF - platelet derived growth factor; EGF - epidermal growth factor; PKC - protein kinase C; PKA - cAMP dependent protein kinase.

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kinase activity, we also examined the ability of the intracellular tail of the AT_1 receptor to serve as a substrate for several classes of protein kinases. The src-family which is known to function in many different signal transduction pathways through a diverse group of cell surface proteins showed high activity against the AT_1 intracellular tail (7). These studies show that the AT_1 receptor is phosphorylated on tyrosine and serine and that the receptor tail serves as an excellent substrate for src kinases.

MATERIALS AND METHODS

Production of anti-AT_1 antibody - A polyclonal rabbit anti-AT_1 antibody, designated Ang2.15 was raised and affinity purifed as described previously (8).

Cell culture - VSMC were grown in DMEM supplimented with 10% fetal bovine serum at 37°C in a 5% CO_2 humidified atmosphere, and utilized between passage 13 and passage 20. Medium was changed at 2-3 day intervals and cells were routinely subcultured 1:10 by trypsin/EDTA dissociation at approximately seven day intervals. All cell culture reagents were purchased from Gibco\BRL.

In vivo ^{32}P labeling of phosphoproteins - VSMC were grown to near confluence on 100 mm culture plates (Corning). Cells were growth arrested in serum free DMEM for 48 hours prior to Ang II stimulation. Cells were phosphate depleted for 4 hr. in phosphate-free DMEM then labeled for 4 hr. in 3 ml phosphate-free DMEM containing 2 mCi ^{32}P-orthophosphate, 9120 Ci/mmol (DuPont/NEN). Immunoprecipitation of Ang II receptor - ^{32}P-labeled VSMC were preincubated with 100 nM sodium orthovanadate for 30 min. then stimulated with 100 nM Ang II. Cells were washed three times in 5 ml ice cold PBS and placed in 3 ml lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2.5 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotonin) on ice for 30 min. Cell lysates were centrifuged at 6000g for 20 min. to pellet any insoluble material. Supernatants were precleared against Immunoprecipitin (Gibco) for 1 hr on ice. Immunoprecipitin was pelleted by centrifugation at 3000g for 10 min. Supernatants (3 ml) were removed to fresh tubes, and incubated overnight with 7 μl (1.4 mg/ml) affinity purified Ang2.15 anti-AT_1 receptor antibody. Antibody complexes were collected by the addition of 100 μl 10% Protein A-agarose (Gibco) which was allowed to incubate at 4°C for 4 hrs on a rocking platform. Beads were collected at 3000g for 10 min at 4°C and washed x 4 in 1 ml lysis buffer. Beads were resuspended in 200 ul SDS-gel loading buffer (50 mM Tris, pH 6.8, 100 mM DTT, 2% SDS, 0.05% bromophenol blue, 10% glycerol, 5% 2-mercaptoethanol) and heated to 95°C for 5 min. Samples run on a 10% SDS-polyacrylamide gel by the method of Laemmli in a Protean II apparatus (Bio-Rad) (9). Proteins were transfered to an Immobilon-P membrane (Millipore) in a Trans-blot cell (Bio-Rad) for 5 hrs. at 60 V, at 4°C by the method of Burnette (10).

Base hydrolysis of immobilized proteins - Following transfer, the Immobilon-P membrane was washed in 50 mM Tris, pH 7.4, 150 mM NaCl for 10 min, then placed in 1.0 N KOH for 2 hr. at 55°C (11). The filter was neutralized for 1 min. in wash buffer, 5 min. in 1.0M Tris, pH 7.0, and twice for 5 min in distilled water. The filter was dried prior to autoradiography.

Cloning of AT_{1A} tail into a glutathione-S-transferase fusion protein vector - A 166 bp fragment of the Ca18b AT_1 receptor cDNA was PCR amplified and cloned into the pGEX-KG vector via XbaI/HindIII restriction sites (4,12) and verified by DNA sequence analysis. The vector was transformed into to $CaCl_2$ competent MC1061 E. coli as described by Miller (13). This allowed the production of a 32kDa fusion protein composed of glutathione-S-transferase linked to the carboxyl terminal 54 amino acids of the Ang II AT_{1A} receptor. The fusion protein is referred to as AT1A306-359. The portion of the AT_1 receptor present in this fusion protein corresponds

to the intracellular tail of the Ang II receptor. The modified vector is referred to as pGEX-14/10.

Production of fusion protein- The fusion protein AT1A306-359 was harvested by the method of Smith and Johnson (14).

In vitro kinase assay- Raytide (Oncogene Science), angiotensin II (Sigma) and AT1A306-359 fusion protein were resuspended in kinase assay buffer (50 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.015% Triton X-100) to a concentration of 1.5 mg/ml. Src, lyn, lck, and fyn kinases (Upstate Biotechnology Incorporated) were each resuspended in kinase dilution buffer (kinase assay buffer containing 0.1 mg/ml BSA and 0.2% β-mercaptoethanol) to a concentration of 0.3 units/ul. For each reaction, 10 μl of substrate was combined with 10 μl enzyme on ice. The reaction was started by the addition of 10 ul ATP mix (0.3 mM ATP, 30 mM $MgCl_2$, and 200 μCi [γ-^{32}P] ATP per ml in kinase assay buffer) and brief vortexing. The reaction was incubated at 30°C over a time course from 0 to 30 min. The reaction was stopped by the addition of 120 μl 10% phosphoric acid followed by vortexing. 120 μl of each reaction was blotted onto 3 cm x 3 cm squares of P81 paper (Whatman). The P81 papers were washed x 4 for 10 min in 1 L cold 0.5% phosphoric acid, followed by a final wash in acetone. Papers were allowed to dry and then quantitated by liquid scintillation counting in a Beckman LS 6000SC.

Phosphoamino acid analysis - Two-dimensional phosphoamino acid analysis was performed as previously described (15).

RESULTS

Immunoprecipitation of [^{32}P]-labeled AT_1 receptor from VSMC yielded two major bands with approximate molecular masses (Mr) of 68 kDa and 49 kDa on SDS-PAGE as shown in Figure 1. These sizes are in agreement with previously reported Mr for the AT_1 receptor, and most likely represent the fully and partially glycosylated forms of the receptor (68 and 49kDa respectively) (8,17). Each immunoprecipitate was split so that two identical sets of samples could be run on each SDS-PAGE. Following transfer of proteins to Immobilon-P, the

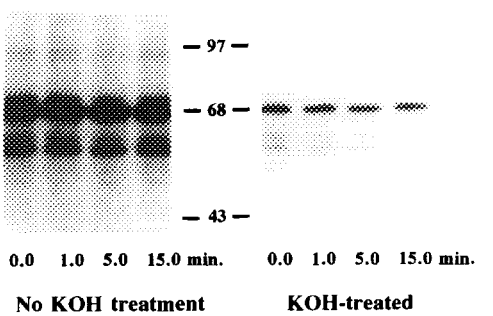

Figure 1. Immunoprecipitation of [^{32}P] labeled AT_1 receptor from VSMC. VSMC were incubated with [^{32}P] orthophosphate and stimulated with Ang II (100 nM) over the time course indicated. AT_1 receptor was immunoprecipitated with anti-AT_1 antibody, run on a 10% SDS-PAGE gel and transferred to Immobilon-P. The bands at 68 kDa and 49 kDa represent different glycosylation states of the AT_1 receptor. The right half of the membrane was treated with KOH as indicated in the materials and methods. [^{32}P] label remaining after alkali hydrolysis indicates the presence of phosphotyrosine residues. Molecular weight markers in kDa are shown in the middle of the figure. This figure is representative of three separate experiments.

membrane was cut in half and one set of samples was subjected to base hydrolysis. This procedure allows for the selective phosphate hydrolysis of phosphoserine and phosphothreonine, while leaving phosphotyrosine intact, and has previously been used to demonstrate the presence of tyrosine phosphorylation of proteins (11,16). The right half of Figure 1 shows the immunoprecipitate following base hydrolysis of the proteins. Both the 68 and 49 kDa bands retain [^{32}P] labeling indicating the presence of tyrosine phosphorylation. There does not appear to be any change in total phosphate level or tyrosine phosphate level following Ang II stimulation over the fifteen minute time course studied. Additionally, the majority of phosphate present on the receptor represents non-tyrosine phosphorylation, as it is alkali labile. To further investigate the AT_1 receptor phosphorylation, phosphoamino acid analysis was performed on the 68 kDa bands after excision from the Immobilon-P membrane. As shown in Figure 2 the

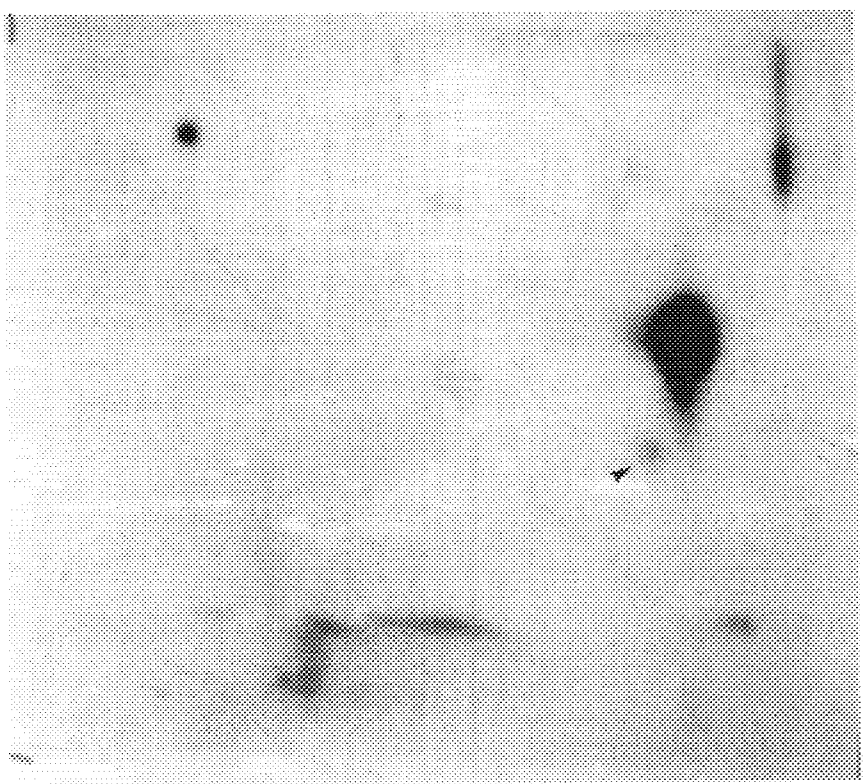

Figure 2. Phosphoamino acid analysis of immunoprecipitated AT_1 receptor from VSMC. [^{32}P] labeled AT_1 receptor bands were excised from Immobilon-P and subjected to hydrolysis in 6N HCl at 110°C for 1 hr. Two-dimensional electrophoresis of amino acids was performed. Position of phosphoamino acids was determined relative to internal control standards as indicated. The large dark spot represents phosphoserine. The arrowhead indicates the phosphotyrosine spot. This figure is representative of four separate experiments.

majority of the protein phosphorylation present is in the form of phosphoserine, but phosphotyrosine is also demonstrated.

We next examined the ability of the cytosolic carboxyl terminal tail of the AT_{1A} receptor to serve as a substrate for several protein kinases. The AT1A306-359 fusion protein was prepared and consisted of a glutathione-S-transferase (GST) protein followed by the C-terminal 54 amino acids of the AT_{1A} receptor. The ability of this fusion protein to serve as a substrate for src-like kinases was compared to Raytide and angiotensin II, two commonly used substrates which are both accepted as control substrates for src-kinase assays (18,19). All kinase assays were run with a substrate concentration of 0.5 mg/ml which was found to give optimal activity (Data not shown). Figures 3A-D show the levels of [^{32}P] incorporation by the src, lck, lyn and fyn kinases respectively. GST not containing the AT_{1A} intracellular tail did not show [^{32}P]

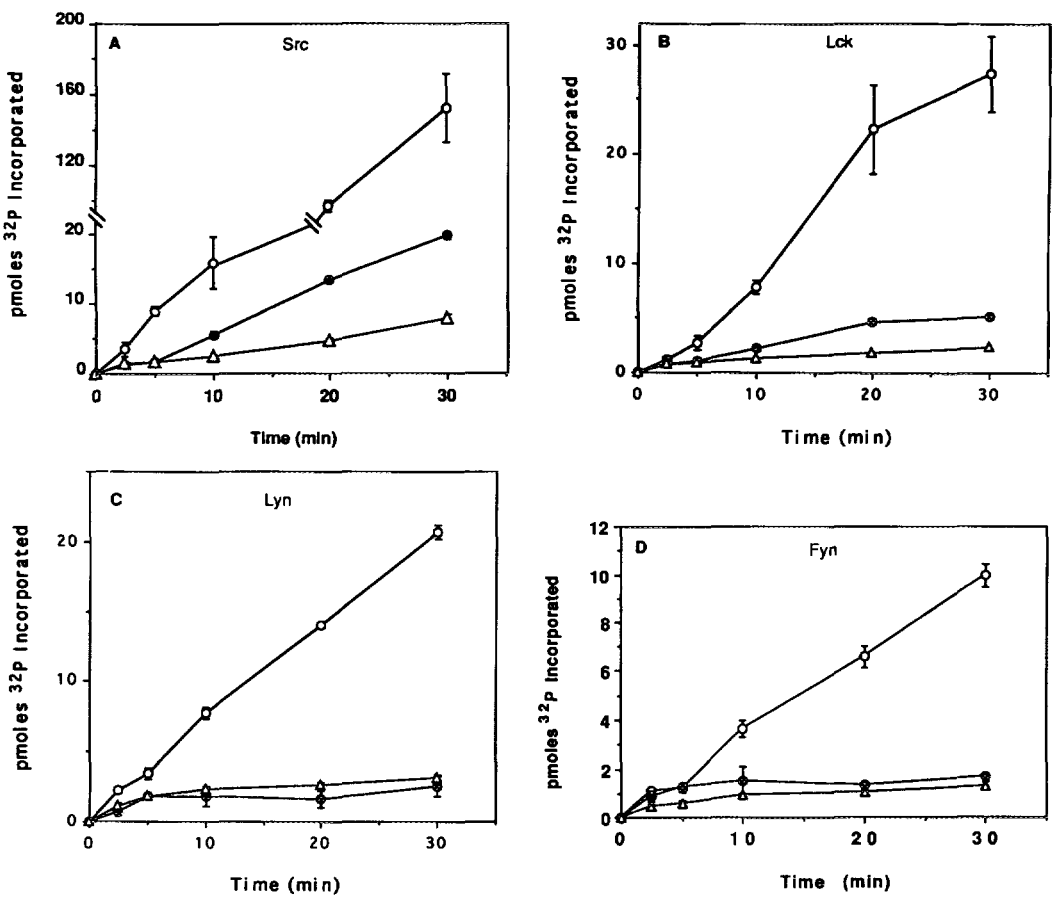

Figure 3.A-D- In vitro kinase assays. Raytide (○), AT_1 receptor tail fusion protein (●), and Ang II (△) were assayed as substrates for src (A), lck (B), lyn (C), and fyn (D) tyrosine kinases. Experimental details are given in the materials and methods section. Each point represents the mean ± SE for three separate experiments.

incorporation above background levels (Data not shown). This is in agreement with Valius et al. who also found that GST did not serve as a substrate for PDGF receptor kinase (19). On a per milligram basis, the AT_1 receptor tail present in the fusion protein was equal to or better than ang II as a substrate for all four of the kinases assayed, but was not as good a substrate as Raytide. There is a large discrepancy in Mr between ang II, Raytide and the AT1A306-359 fusion protein, and this results in a much lower molar concentration of the fusion protein in the kinase assays. Table 1 shows the [^{32}P] incorporation standardized per mole of protein. The fusion protein containing the AT_1 receptor tail showed the greatest rate of phosphate incorporation for all of the kinases studied except src which showed highest activity against Raytide. For lck, lyn and fyn kinases the rate of [^{32}P] incorporation into the AT1A306-359 fusion protein was approximately twofold than that of Raytide. Ang II was the least effective substrate for all four kinases. It should be noted that the AT1A306-359 fusion protein has three potential tyrosine phosphorylation sites in the tail and the rate is calculated as if all three sites serve as kinase substrates. In the event that only one or two of the sites serve as substrates, the rate of incorporation would be higher.

A series of serine/threonine kinases were also examined using AT1A306-359 fusion protein as a substrate. Protein kinase A, protein kinase C, and MAP kinase were all examined. In each case the kinases showed good activity against positive control substrates but no activity toward AT1A306-359 as a substrate (Data not shown).

DISCUSSION

The major findings of this study are that the AT_1 receptor is tyrosine and serine phosphorylated in vivo, and that in vitro the carboxyl terminal tail of the AT_{1A} receptor is an

Table 1 Initial rates of protein tyrosine kinases of the Src family expressed as μmoles of [^{32}P] incorp./min/mol substrate

	protein tyrosine kinases			
Substrates	src	lck	lyn	fyn
AT1A306-359	218	143	253	178
Raytide	255	77	97	63
Angiotensin II	24	14	27	9

Values indicated are the mean of three separate experiments.

excellent substrate for the src family of protein tyrosine kinases. These findings are significant because we have recently demonstrated that Ang II signal transduction in VSMC occurs through a PLC-γ mediated pathway (5). PLC-γ is known to associate with receptors through specific interaction of an SH_2 domain in PLC-γ with phosphotyrosine residues located in the receptor (20, 21). Thus tyrosine phosphorylation of the AT_1 receptor may be central to its association with PLC-γ and Ang II signal transduction.

The AT_1 receptor does not contain an intrinsic kinase activity as do the receptor tyrosine kinases. However, it has recently been shown that soluble tyrosine kinases can associate with cell surface proteins and facilitate cell surface initiated signal transduction much like receptor tyrosine kinases (7). To assess the possibility that a src-like soluble tyrosine kinase may phosphorylate the AT_1 receptor, we used a fusion protein containing the intracellular C-terminal tail of the AT_1 receptor to determine its suitability as a substrate for the src-family of protein tyrosine kinases. Figures 3A-D illustrate that the fusion protein was found to be better than ang II but not as good as Raytide as a substrate for the src family of protein kinases on an equal mass basis. When compared on a molar basis the AT1A306-359 fusion protein was found to be equal to or better than Raytide as a substrate for the src family tyrosine kinases studied. This suggests that the tail might be a natural substrate for a src-like kinase in vivo.

The serine\threonine kinases studied did not show any activity toward the AT1A306-359 fusion protein. This is a bit surprising, as three consensus PKC sites and a PKA site are predicted in the AT_{1A} receptor tail (3). One possible explanation is that the GST present in the AT1A306-359 fusion protein is inhibitory to these kinases by either steric or allosteric effects. Additionally, only the carboxyl terminal tail of the receptor is present in the AT1A306-359 fusion protein, while the immunoprecipitation experiments represent the entire receptor molecule. As a result, the serine phosphorylation seen by phosphoamino acid analysis may represent phosphorylation of the receptor on sites not present in the AT1A306-359 fusion protein.

The recent implication of PLC-γ1 in the Ang II signalling pathway suggests a functional basis for AT_1 receptor phosphorylation, namely that a src-like kinases tyrosine phosphorylates the AT_1 receptor and allows binding of PLC-γ through SH_2 domains. The data presented in this paper illustrate two novel concepts regarding Ang II mediated signal transduction. First, immunoprecipitation studies and phosphoamino acid analysis with [^{32}P] labeled VSMC indicate that the AT_1 receptor is tyrosine phosphorylated in vivo. Second, a survey of several members of the src kinase family demonstrates that the intracellular tail of the AT_1 serves as an excellent substrate for src-like tyrosine kinases in vitro. These findings are critical to an understanding of the mechanism of the early signalling events in the AT_1 signal transduction pathway.

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