Angiotensin II has been demonstrated to act as a growth factor in rat cardiac fibroblasts. However, the signaling events that lead to fibroblast cell growth in response to angiotensin II remain to be elucidated. This study was designed to determine whether angiotensin II stimulated tyrosine phosphorylation of proteins in cardiac fibroblasts. Immunoblot analysis demonstrated rapid tyrosine phosphorylation of distinct substrates of 125, 95, 46–60, and 44 kDa in response to 10 nM angiotensin II. Tyrosine phosphorylation was maximal at 5 min and persisted for at least 180 min. Additional tyrosine-phosphorylated proteins of 185, 145, and 85 kDa were detected in response to 10 ng/ml platelet-derived growth factor BB. A cluster of 75–80-kDa proteins were phosphorylated in response to angiotensin II, phorbol ester, and platelet-derived growth factor. Angiotensin II-induced tyrosine phosphorylation was unaffected by phorbol ester-sensitive protein kinase C down-regulation and could be partially blocked by pertussis toxin pretreatment. Angiotensin II stimulation resulted in increased cytosolic tyrosine kinase activity which was recovered by immunoprecipitation. Immunoblot analysis demonstrated tyrosine phosphorylation of p44MAPK and, in addition, we demonstrated for the first time tyrosine phosphorylation of p125FAK, p46SHP, and p66SHC in response to angiotensin II. The finding that angiotensin II and platelet-derived growth factor stimulated tyrosine phosphorylation of p46SHP and p66SHP suggested that this protein may serve as a common tyrosine kinase substrate in the mitogenic signaling cascade induced by G-protein-coupled receptors and growth factors and is consistent with the hypothesis that angiotensin II-induced tyrosine phosphorylation is involved in mitogenic signaling pathways in neonatal rat cardiac fibroblasts.

Angiotensin II (AII) has been shown to act as a cellular growth factor and to be involved in the regulation of growth in different tissues in response to a variety of physiological and pathological processes, including vascular cell growth in response to injury (1, 2). In addition, AII has been implicated in the rapid developmental growth of the left ventricle during the neonatal period and the remodeling of the heart following chronic hypertension and myocardial infarction (3–5). In particular, the inhibition of DNA synthesis and collagen deposition in the myocardial interstitium postinfarction (in rats), by the angiotensin converting enzyme inhibitor captopril, suggests that blockade of the renin-angiotensin system prevents interstitial and perivascular fibrosis by inhibiting fibroblast proliferation (6). The observation that vasodilators, in contrast to angiotensin converting enzyme inhibitors, are ineffective in preventing interstitial remodeling suggests that AII promotes fibroblast proliferation in the heart independently of changes in afterload (7). In addition, AII has been shown to promote hypertrophy of cardiomyocytes and hyperplasia of rat cardiac fibroblasts in cell culture (8–14).

Angiotensin II, acting via the AT1 receptor, has been reported to initiate early biochemical cellular events similar to peptide growth factors, i.e. rapid production of diacylglycerol and inositol 1,4,5-trisphosphate by phospholipase C-mediated hydrolysis of inositol phospholipids and activation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and ribosomal S-6 kinase (15–19). It has also been demonstrated that AII induces the expression of growth-associated early (jun, fos) and intermediate response (myc) proto-oncogenes (20–23). These data suggest that AII induces proximal and distal signaling events that ultimately lead to cardiac cellular growth. Phosphorylation of tyrosine residues are early and critical events in transmembrane signaling pathways necessary for the induction of cell growth (24–26). Recent studies in rat liver epithelial, renal mesangial, and vascular smooth muscle cells have demonstrated that AII stimulated tyrosine phosphorylation of several different substrates (19, 27, 28). However, tyrosine kinase substrates phosphorylated in response to G-protein-coupled receptors such as bombesin, vasopressin, and endothelin which exhibit mitogenic effects like AII, were unrelated to the targets which were observed upon stimulation of growth factor receptors with intrinsic tyrosine kinase activity (29). For example, in vascular smooth muscle cells, phosphatidylinositol-3-kinase, phosphatase C-γ, and GTPase activating protein were not tyrosine-phosphorylated in response to AII (19).

Recently, p125 focal adhesion kinase (p125FAK) has been identified as a dominant tyrosine kinase substrate in Swiss 3T3 cells stimulated with bombesin, vasopressin, or endothelin (30). In addition, FAK has been postulated to contribute to oncogenic transformation by v-src, v-fes, or v-erb. As a component of focal adhesion plaques, FAK is co-localized with cytoskeletal proteins such as vinculin, talin, tensin, paxillin, zyxin, and actin and the integrin family of heterotrimeric cell surface receptors, which mediate cellular adhesion to the extracellular matrix (31–34). Thus, FAK has been implicated to be involved in processes regulating cell motility and adhesion, integrity of the...
actin filament network, as well as intracellular signaling events.

We have previously reported that primary cultures of neonatal rat cardiac fibroblasts possess abundant AT(1)AII receptors that are coupled to a proliferative growth response. In these cells, the peptide receptor agonist [Sar1]AII enhanced the rates of incorporation of precursors into protein, DNA, and RNA and increased total protein, RNA, and cell number (35). Thus, this system is useful to elucidate the signal transduction pathway(s) involved in AII-mediated cell proliferation. We present data demonstrating that AII stimulated tyrosine kinase activation and identify several tyrosine kinase substrates involved in the mitogenic signal transduction cascade.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture**— Cultures of ventricular fibroblasts were prepared from hearts of 0-3-day-old Sprague-Dawley rat pups and grown as previously described (35). Two or three days following dispersion, cells were passaged and seeded at a density of 10^5/cm^2 on 100-mm plates. The cells were grown for 72 h and serum-starved for an additional 24-48 h. At the time of experiments, cultures were subconfluent (35).

**Immunoprecipitation and Immunoblot Analysis**— Cultures of cardiac fibroblasts were grown until subconfluent on 100-mm dishes, then incubated in the presence or absence of angiotensin II (United States Biochemical Corp.), platelet-derived growth factor (PDGF-BB, Upstate Biotechnology Inc.), or phorbol 12,13-dibutyrate (PDBu, Sigma) for various periods of time, as indicated. Incubations were terminated by aspirating the media, the plates were placed on ice, washed twice with cold Hank’s buffered salt solution, and scraped into homogenization buffer (20 mM Tris, pH 7.4, 2 mM EGTA, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 μM phenylmethylsulfonyl fluoride, and 100 mM Kallikrein inactivating units/ml of aprotinin) and centrifuged at 4°C for 5 min at 15,000 rpm in a Eppendorf Microfuge. Cell lysates were adjusted for different protein concentrations (Bradford Assay) and electrophoresed on an 8% SDS-polyacrylamide gel (36). Proteins were transferred to nitrocellulose, and blots were incubated overnight in Tris-buffered saline, TBST (20 mM Tris base, 137 mM NaCl, pH 7.6, 5% dry milk (w/v), and 1% Tween (w/v)). Immunoblots were probed with Anti-P-Tyr antibody (1:1000) overnight (Upstate Biotechnology Inc.). Blots were washed in TBS (20 mM Tris base, 137 mM NaCl, pH 7.6, and 0.1% Tween (v/v)) and immunoreactivity was determined using the enhanced chemiluminescence reaction (ECL, American). For immunoprecipitation, cells were lysed in homogenization buffer with the addition of 1% Triton X-100 (v/v) or P-Tyr lysis buffer (50 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and leupeptin). Equal protein aliquots were used for immunoprecipitations (250 μg of total protein) using anti-p125φs, anti-SHC antibody recognizing p46φs (Upstate Biotechnology Inc.), anti-MAPK antibody (Upstate Biotechnology Inc.), and protein A-G Agarose (Santa Cruz Biotechnology) or anti-P-Tyr antibody coupled to protein agarose (Santa Cruz Biotechnology) at 4°C. Immune complexes were boiled in SDS-sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Bands were quantified by densitometric analysis of film exposed to the ECL blots (Molecular Dynamics Personal Densitometer) using ImageQuant, Version 3.22 software (Molecular Dynamics). Densitometric values were expressed as percent or fold increase of control.

**In Vitro Phosphotyrosine Kinase Assays**—Immunocomplexes were washed five times with anti-P-Tyr lysis buffer and then with 50 mM HEPES, pH 7.4, and 150 mM NaCl. The complexes were resuspended in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MnCl, 0.5 μM ATP, and 100 μCi of γ[32P]ATP (3000 Ci/mmol, Dupont NEN), and the protein kinase reaction was performed for 15 min at 37°C. The reaction was terminated by addition of SDS-sample buffer. Samples were boiled at 95°C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (8%), and labeled phosphoproteins were visualized by autoradiography (19).

**RESULTS**

Angiotensin II, Phorbol Ester, and PDGF Stimulate Protein Tyrosine Phosphorylation in Neonatal Rat Cardiac Fibroblasts—Serum-starved, subconfluent cultures of neonatal rat cardiac fibroblasts were exposed to AII (10 nM), PDGF-BB (10 ng/ml), or PDBu (100 nM). Cell lysates were prepared, and proteins were separated and subjected to immunoblot analysis with monoclonal anti-P-Tyr antibody. Fig. 1A shows that AII stimulation resulted in tyrosine phosphorylation of several prominent bands with an apparent molecular mass in the range of 125 kDa and a cluster of proteins of 75-80 kDa. Lesser phosphorylation of 46-, 56-, and 44-kDa proteins was noted. Fig. 1. Time course of AII (A), PDBu (B), and PDGF-BB (C) induced tyrosine phosphorylation in neonatal rat cardiac fibroblasts. Serum-starved cultures of neonatal rat cardiac fibroblasts were exposed to 10 nM AII, 100 nM PDBu, or 10 ng/ml PDGF-BB for the indicated times. Cell lysates were separated using 8% SDS-gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted with anti-P-Tyr antibody. Enhanced chemiluminescence was used in secondary detection. Shown are representative blots of three experiments using cells from different dispersions.
Angiotensin-induced Tyrosine Kinase Activity

Fig. 2. Tyrosine phosphorylation in response to AII is reduced by pertussis toxin pretreatment. Cells were pretreated for 4–6 h with 100 ng/ml pertussis toxin (lanes 2 and 4) and exposed for 5 min to vehicle (lanes 1 and 2) or 10 nM AII (lanes 3 and 4). Lysates were prepared and analyzed by anti-P-Tyr immunoblotting.

Fig. 3. All-induced protein tyrosine phosphorylation is independent of phorbol ester-sensitive PKC. Cells were PKC-depleted by exposure to 500 nM PDBu for 48 h (indicated as +; lanes 2, 4, and 6), vehicle (lanes 1 and 2), 10 nM AII (lanes 3 and 4), or 100 nM PDBu (lanes 5 and 6) for 5 min. Cells were lysed and protein size-fractionated by SDS-PAGE and probed with anti-P-Tyr antibody.

Tyrosine Phosphorylation Induced by Angiotensin II Is G Protein-dependent—It has been reported that G,-dependent pathways are involved in the mitogenic responses to thrombin and lysosphosphatidic acid (37). Experiments were performed in which cells were pretreated for 4–6 h with 100 ng/ml pertussis toxin (Fig. 2). Subsequent stimulation of the cells with AII greatly reduced tyrosine phosphorylation of the 125- and 75–80-kDa substrates (lanes 4, pertussis toxin + AII) as compared with non-pertussis toxin-treated cells (lanes 3, AII). Pertussis toxin pretreatment did not affect basal tyrosine phosphorylation (lane 1, control; lane 2, pertussis toxin).

Angiotensin II-induced Tyrosine Phosphorylation Is Independent of PKC—In certain cells, PKC activation has been shown to be involved in the activation of p21ras and c-raf, thus modulating cell growth (38). We have previously shown that AII stimulates PKC in neonatal rat cardiac fibroblasts. Prolonged exposure of the cells to phorbol 12-myristate 13-acetate, however, had no effect on AII-induced [3H]thymidine incorporation, suggesting that phorbol ester-sensitive PKC isoforms were not sufficient to induce cell growth.2 Fig. 1B shows that exposure of the cells to 100 nM PDBu induced tyrosine phosphorylation of substrates (75–80 kDa, 125 kDa) similar to those observed in response to AII. In order to determine the involvement of phorbol ester-sensitive PKC isoforms, tyrosine phosphorylation in response to AII was determined in PKC-depleted cells (Fig. 3). Cultures were exposed to 500 nM PDBu for 48 h to down-regulate PKC, a period of time which has been reported to result in the loss of cellular PKC activity (38). Prolonged exposure to PDBu moderately increased basal tyrosine phosphorylation (Fig. 3) and markedly reduced tyrosine phosphorylation in response to 100 nM PDBu. However, AII-induced tyrosine phosphorylation of the 75–80- and 125-kDa bands was not affected by PDBu pretreatment, suggesting that AII effects were independent of a phorbol ester-sensitive PKC. PDBu-stimulated tyrosine phosphorylation was unaffected by PDBu pretreatment (data not shown).

Angiotensin II Induces Tyrosine Kinase Activity—It has been demonstrated that activation of receptor and cytoplasmic tyrosine kinases resulted in autophosphorylation of the kinase on tyrosine residues (26, 29, 39). This observation was used to further characterize the cellular tyrosine kinase activity in response to AII. Cellular lysates were prepared, the tyrosine kinase(s) were immunoprecipitated with anti-P-Tyr antibody, and in vitro autophosphorylation assays were performed (Fig. 4A). Alternatively, immunocomplexes were subsequently immunoblotted with anti-P-Tyr antibody (Fig. 4B). Stimulation with 10 nM AII for 5 min (lane 2) resulted in increased recovery of P-Tyr kinase activity toward substrates of 52, 56 (6-fold), 60, 125 (6.6-fold), and faint bands of 75–80 and 154 kDa, when compared to vehicle-treated cells (lane 1). Exposure of the cells to 10 ng/ml PDGF-BB for 5 min increased tyrosine kinase activity toward additional proteins of 185, 145, 125, 85, 60, 56, 54, 52, and 40 kDa (lane 3). The data suggest that AII effects on tyrosine phosphorylation are mediated in part by activation of a tyrosine kinase (Figs. 1A and 4A). The addition of 50 mM phoshotyrosine to the cell lysates prior to the antibody reduced the recovery of tyrosine-phosphorylated substrates (data not shown). However, the autophosphorylation appears in part to be on residues other than tyrosine, because the pattern in Fig. 4B does not coincide with Fig. 4A. These data suggest that inhibition of a tyrosine phosphatase may be involved in enhancing the tyrosine phosphorylation seen in Fig. 4B. Alternatively, co-precipitation of serine/threonine kinases (associated with tyrosine-phosphorylated proteins) or a tyrosine phosphatase may account for the different pattern. These data are consistent with a previous report in vascular smooth muscle stimulated with AII, in which it was demonstrated that the extent of tyrosine phosphorylation of the 75–80-kDa proteins in the autophosphorylation assay was barely detectable (19).

2 K. M. Baker, unpublished data.
Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases which are activated by phosphorylation on tyrosine and threonine residues in response to growth factors, insulin, phorbol ester, and G-protein-coupled receptors like thrombin, endothelin, and AII. Activated MAPKs have been reported to phosphorylate substrates which include the transcription factors c-fos, c-jun, c-myc, and p62 transcripts, the epidermal growth factor receptor, and S6 and c-raf kinase (40). Recently, it has been shown using dominant negative mutants and antisense constructs of p44 MAPK that MAPK activation is required for the mitogenic effect of p44MAPK in response to AII (130% increase over control) and PDGF (148% increase over control).

Angiotensin II-stimulated Tyrosine Phosphorylation of p44MAPK—Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases which are activated by phosphorylation on tyrosine and threonine residues in response to growth factors, insulin, phorbol ester, and G-protein-coupled receptors like thrombin, endothelin, and AII. Activated MAPKs have been reported to phosphorylate substrates which include the transcription factors c-fos, c-jun, c-myc, and p62 transcripts, the epidermal growth factor receptor, and S6 and c-raf kinase (40). Recently, it has been shown using dominant negative mutants and antisense constructs of p44 MAPK that MAPK activation is required for the mitogenic effect of p44MAPK in response to AII (130% increase over control) and PDGF (148% increase over control).

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Angiotensin II-stimulated Tyrosine Phosphorylation of p46SHC and p56SHC—The mammalian shc gene encodes two proteins of 46 and 56 kDa that induce malignant transformation when overexpressed in mouse fibroblasts (42). In cells stimulated with epidermal growth factor or insulin, SHC has been demonstrated to form a complex with GRB2 via phosphorylated SH2 domains (42, 43). This protein complex has been shown to bind to the nucleotide exchange factor SOS, which subsequently activates p21ras. Thus, SHC phosphorylation is likely to be an important tyrosine kinase substrate involved in the regulation of p21ras (44). In order to determine if exposure to AII resulted in tyrosine phosphorylation of SHC, cells were exposed to vehicle, 10 nm AII, or 10 ng/ml PDGF. Cytosolic extracts were prepared and immunoprecipitated with anti-SHC antibody, which recognized p46SHC and p56SHC, and the blots were probed with anti-P-Tyr antibody. Fig. 7A demonstrates rapid tyrosine phosphorylation of p46SHC (400%) and p56SHC (1000%) in response to AII (1 min) and PDGF (5 min). A similar result was obtained when cytosolic extracts were immunoprecipitated with anti-P-Tyr antibody and blotted with anti-SHC antibody (Fig. 7B). Tyrosine phosphorylation of SHC was easily detected following a 30-s exposure to AII. The difference in the extent of tyrosine phosphorylation of p44MAPK, p125FAK, and p46SHC may be due to variable amounts of protein immunoprecipitated with antibodies of varying affinity, the tyrosine phosphorylation may have occurred with different stoichiometries, or there may exist a variable abundance of these proteins in the cytosol. All of the AII-stimulated tyrosine phosphorylation events could be blocked or attenuated by EXP3174, demonstrating mediation of the responses, primarily via the AT1 receptor.
Angiotensin-induced Tyrosine Kinase Activity

**Fig. 6. Expression and tyrosine phosphorylation of p125tyk2.** Lysates (50–60 μg of protein/lane) of neonatal rat cardiac fibroblasts, were immunoblotted using polyclonal anti-p125tyk2 antibody (A). Lysates of vehicle (lane 1) or 10 nM AII for 5 min (lane 2) treated cells were immunoprecipitated using anti-125tyk2 antibody. Immunocomplexes were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose and probed with anti-P-Tyr antibody (B). ECL was used in secondary detection.

**DISCUSSION**

Interstitial fibroblast proliferation and collagen accumulation following myocardial infarction is associated with compensatory remodeling of the hypertrophying myocardium (3, 4). It has been postulated that the process of structural remodeling leads to diastolic and systolic dysfunction and ultimately heart failure (45). Several in vivo studies have implicated AII as the principal factor involved in the remodeling process, mediated through the modulatory affects of the peptides on cardiac fibroblast growth. We have recently demonstrated that AII stimulated hyperplastic growth of cultured neonatal rat cardiac fibroblasts (35). Since the signaling pathway(s) by which AII mediates growth in these cells is (are) unknown, the present study was designed to identify intracellular events that may be necessary for AII-induced cell proliferation. Several lines of evidence suggest that activation of intrinsic receptor or soluble tyrosine kinases play a crucial role in the signaling cascade regulating cell growth. Tyrosine phosphorylation and dephosphorylation of signaling proteins which have SH2 domains regulated by tyrosine kinases and phosphatases are considered early and critical events that lead to cellular proliferation and/or differentiation (29). In particular, ligand binding of growth factors like PDGF or EGF induce receptor dimerization, stimulate tyrosine kinase activation and autophosphorylation of the receptor on tyrosine residues (24, 25), which results in association of cytoplasmic signaling proteins like phospholipase Cγ, phosphatidylinositol-3'-kinase, p21ras GTPase activating protein, tyrosine kinases of the src family, and adapter proteins like GRB2 (29, 46). Similar to vascular smooth muscle cells, AII stimulation of neonatal rat cardiac fibroblasts induced tyrosine phosphorylation of several substrates which persisted for at least 180 min (Fig. 1A). This response could be blocked by pretreatment of the cells with EXP3174, a nonpeptide, noncompetitive AT1 receptor antagonist, demonstrating that AII effects were mediated by the AT1 receptor.

PKC activation has been shown to modulate the activity of p21ras in T cells, and PKC activity is required to fully activate c-src in SF9 cells and NIH3T3 fibroblasts, which suggested that PKC might be involved in growth regulation (98, 47, 48). Many cell types exhibit a mitogenic response to phorbol ester-induced PKC activation (49). Rat cardiac fibroblasts, exposed to PDBu, exhibit tyrosine phosphorylation of substrates identical with those following AII stimulation. However, the AII effects on cardiac fibroblasts appeared to be independent of phorbol ester-sensitive PKC isoforms, in that down-regulation of PKC had no effect on MI-induced tyrosine phosphorylation. These data do not exclude the involvement of a phorbol ester-insensitive PKC isoform, such as ζ. The observation that the pattern of tyrosine phosphorylation in response to PDBu was similar to that of AII, and, additionally, that PKC activation failed to induce [3H]thymidine uptake, implies that additional signaling proteins might be tyrosine-phosphorylated which have not been detected in our experiments or more likely that additional pathways are required to mediate the mitogenic effect of AII in these cells.

The AT1 receptor belongs to a family of receptors with seven transmembrane-spanning domains which are coupled to G pro-
teins (50). These receptors do not have intrinsic tyrosine kinase activity and therefore must activate soluble membrane-associated tyrosine kinases, although the mechanism of coupling has not been elucidated. Recently, Gi subunits have been reported to be involved in mitogenic responses to thrombin and lysophosphatidic acid (51). In addition, when activated, the 8β subunit dissociates from α, GTP and may activate second messengers, i.e. specific isofoms of adenyl cyclase and phospholipase C (53, 54). It has been demonstrated that pp60Src phosphorylated the α subunit of several G-proteins in vitro (55). In Rat 1a cells transfected with GTPase-inhibited α, a constitutively activated MAPK was observed (37). The effects of AII on tyrosine phosphorylation are pertussis toxin-sensitive, and the formation of a protein-protein complex with GRB2 and the nucleotide exchange factor SOS (42-44). These protein interactions have been implicated in growth factor-induced mitogenic signal transduction, have been identified to be phosphorylated on tyrosine residues in response to AII. In addition, p42 and p56Lck were tyrosine-phosphorylated in response to both AII and PDGF. This finding suggested that SHC phosphorylation may serve as a converging target in the growth factor and AII-stimulated signaling cascade, which ultimately may result in activation of p21ras. Further studies will be directed toward identifying the mechanisms by which all stimulates tyrosine kinases and the role of these phosphorylation events in the mitogenic signaling cascade in neonatal rat cardiac fibroblasts.

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REFERENCES

Angiotensin-induced Tyrosine Kinase Activity