Angiotensin II-induced Stimulation of p21-activated Kinase and c-Jun NH$_2$-terminal Kinase is Mediated by Rac1 and Nck

Udo Schmitz, M.D., Kerstin Thömmes, Ph.D., Imke Beier, Wolfgang Wagner, Agapios Sachinidis, Ph.D., Rainer Düsing, M.D. and Hans Vetter, M.D.

Medizinische Universitäts-Poliklinik
Wilhelmstr. 35-37, 53111 Bonn
Germany

Running title: PAK and JNK activation by AngII is dependent on Rac1 and Nck

Correspondence:  Udo Schmitz, M.D.
Medizinische Universitäts-Poliklinik
Wilhelmstr. 35-37
53111 Bonn
Germany
Tel.: +49-228-287-2263
Fax: + 49-228-287-2658
e-mail: uschmitz@uni-bonn.de

Copyright 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
PAK and JNK activation by AngII is dependent on Rac1 and Nck

SUMMARY

p21-activated kinase (PAK)\(^1\) has been shown to be an upstream mediator of JNK in angiotensin II (AngII) signaling. Little is known regarding further signaling molecules involved in activation of PAK and JNK by AngII. Rho-family GTPases Rac and Cdc42 have been shown to enhance PAK activity by binding to p21-binding domain of PAK (PAK-PBD). In vascular smooth muscle cells (VSMC) AngII stimulated Rac1 binding to GST-PAK-PBD fusion protein. Pretreatment of VSMC by Genistein inhibited AngII-induced Rac1 activation, whereas src inhibitor PP1 had no effect. Inhibition of PKC by PDBU pretreatment also decreased AngII-mediated activation of Rac1. The adaptor molecule Nck has been previously shown to mediate PAK activation by facilitating translocation of PAK to the plasma membrane. In VSMC AngII stimulated translocation of Nck and PAK to the membrane fraction. Overexpression of dominant negative Nck in CHO cells, stably expressing the AngII type I receptor (CHO-AT1), inhibited both PAK and JNK activation by AngII, whereas it did not affect ERK1/2. Finally, dominant negative Nck inhibited AngII-induced DNA synthesis in CHO-AT1 cells. Our data provide evidence for Rac1 and Nck as upstream mediators of PAK and JNK in AngII signaling and implicate JNK in AngII-induced growth responses.

INTRODUCTION

Stimulation of the renin-angiotensin system has been previously shown to significantly contribute to cardiovascular pathology such as arterial hypertension, left ventricular hypertrophy and restenosis. Angiotensin II (AngII) exerts its pathological effects by promoting hypertrophic and/or hyperplastic growth responses in vascular smooth muscle cells (VSMC) (1), cardiomyocytes (2) and cardiac fibroblasts (3). Recently, the MAP kinase family of serin threonin kinases has become a focus of interest in cardiovascular research. In many cell systems MAP kinases are known to regulate hypertrophic and/or hyperplastic growth by
PAK and JNK activation by AngII is dependent on Rac1 and Nck various stimuli. In VSMC, AngII has been shown to stimulate ERK1/2 (4), JNK (5) and p38 (6, 7). A positive contribution of ERK1/2 to AngII induced protein and DNA synthesis in VSMC was first demonstrated by Servant et al. (8) and Wilkie et al. (9), and has been subsequently confirmed by Marrero et al. (10). Ushio-Fukai et al. (7) recently defined p38 as a critical component in AngII-mediated protein and DNA synthesis in VSMC. Furthermore, they showed that inhibition of both ERK1/2 and p38 had an additive effect on AngII-induced growth in VSMC (7). The above mentioned studies used PD98059 and SB203580 as specific inhibitors of ERK1/2 and p38, respectively. However, compounds that specifically inhibit JNK activation by AngII have not been available up to now. Therefore, no data are available considering the contribution of JNK to AngII-mediated growth responses.

The signal transduction events that lead to stimulation of ERK1/2 by AngII have been elucidated in recent years (11) and have been shown to include stimulation of the small G-protein Ras (12, 13). The small G-proteins Rac and Cdc42 are known to be important upstream mediators of JNK activation since constitutively active mutants of these small GTPases enhance JNK activity, whereas dominant negative mutants block activation of JNK (14, 15). A putative downstream component of Rac and Cdc42 in the signaling pathway leading to JNK activation is the serine-threonine kinase PAK (for p21-activated kinase) (16), which becomes activated upon binding to GTP-bound Rac or Cdc42. We have previously shown, that PAK is stimulated by AngII in VSMC in a tyrosine kinase and PKC dependent manner (5). Furthermore, our data implicated PAK as an upstream mediator of JNK in VSMC (5).

PAK consists of an N-terminal regulatory domain, comprising the p21-binding domain (PBD), an adjacent auto-inhibitory domain (ID), 4 putative SH3 domain binding PXXP motifs, and a C-terminal catalytic domain (17, 18). Rho family GTPases Cdc42 and Rac have been demonstrated to bind to PAK solely in their active forms, i.e. the GTP-bound state, via...
PAK and JNK activation by AngII is dependent on Rac1 and Nck interaction with the p21-binding domain (PBD) (16). PAK has been shown to be activated by tyrosine kinase receptors (19, 20), cytokines (21) and G protein coupled receptors (5, 22). In tyrosine kinase receptor signaling the adaptor molecule Nck has been identified as a critical component mediating translocation of PAK to the plasma membrane (19, 23). Nck consists of 3 SH3 and 1 SH2 domain (24). PAK binds to the second SH3 domain of Nck (19, 20). Nck is known to bind stimulated tyrosine kinase receptors such as PDGF-receptor (25), EGF-receptor (26) and Eph receptor (23, 27, 28) by its SH2 domain. Recently, signal transduction pathways leading to AngII-induced activation of ERK1/2 in VSMC have been shown to include transactivation of the EGF receptor (29, 30). By analogy to Grb2 and AngII-induced ERK1/2 activation, we surmised that the adaptor molecule Nck might play a role in AngII-mediated JNK activation by mediating translocation of PAK to the plasma membrane.

In the present study we present evidence that AngII stimulates Rac1 in VSMC. Furthermore, we demonstrate that AngII promotes translocation of Nck and PAK to the plasma membrane in VSMC and that AngII-stimulated PAK activity is greatly enhanced in membrane fractions compared to the cytosol. Overexpression of dominant-negative Nck mutants, in CHO cells stably expressing the AT1 receptor (CHO-AT1 cells), led to inhibition of PAK and JNK activation by AngII and significantly inhibited AngII-induced DNA synthesis. In addition, overexpression of a PAK mutant (PAK-A13) that cannot bind Nck could not be stimulated by AngII in CHO-AT1 cells.

EXPERIMENTAL PROCEDURES

Materials - Antibodies were purchased from the following vendors: Upstate Biotechnology (rabbit polyclonal antibodies: Nck (06-288), monoclonal anti-Myc-tag (clone 9E10, 05-419), Transduction Laboratories (monoclonal Nck (N15920), Rac1 (R56220), Cdc42 (C70820), Santa Cruz Biotechnology (rabbit polyclonal PAK1 (sc-881), EGFR (sc-03), PDGFR-α (sc-338), PDGFR-β (sc-432), p38 (C-20) (sc-535-G), JNK-1 (C-17) (sc-474-G), monoclonal PY-
PAK and JNK activation by AngII is dependent on Rac1 and Nck

99 (sc-7020), New England BioLabs (monoclonal phospho-p44/42 MAP Kinase
(Thr202/Tyr204) E10). Cell Signaling Technology (polyclonal phospho-c-Jun (Ser63) II. c-
Jun (79) and ATF-2 (1-96) substrate were purchased from Santa Cruz Biotechnology, Inc..
Genistein, PP1, Phorbol 12,13-Dibutyrate (PDBu) and GF109203X (GFx) were purchased
from Biomol Research Laboratories, Inc and AG 1296, AG 1478 from Calbiochem. MBP was
purchased from Sigma. Plasmids containing wild-type human PAK1 (pCMV6M-PAK1) and
kinase dead PAK-K299R (pCMV6M-PAK1-K299R) were kindly provided by Gary Bokoch
(20). The PAK-A13 mutant (pCMV6M-PAK-A13), that lacks Nck binding, was kindly
provided by Jonathan Chernoff (31). Plasmids containing wild-type Nck (pEBB-Nck) and
mutant Nck pEBB-Nck-W38,143,229K with inactivating mutations of all 3 Nck-SH3
domains (denoted Nck-KSH3all in this paper) and pEBB-Nck-R308K, with an inactivating
mutation in the Nck-SH2 domain (denoted Nck-K308 in this paper) were kindly provided by
Bruce Mayer and have been previously described (32).

Cell Culture and transfection - VSMC were isolated from 200-250 g male Wistar-Kyoto rats
and maintained in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10%
bovine calf serum, as previously described (33). Passage 8 to 15 VSMC at 80% confluence
were growth arrested by incubation in 0.4 % calf serum for 24 hr prior to use. Chinese
hamster ovary (CHO) cells stably transected with angiotensin II type 1 receptor (AT1R) were
kindly obtained from Dr. Kenneth Baker and maintained in F-12 media supplemented with
20mmol/L Hepes, 0.2 mg/ml G418, and 10% fetal calf serum. Transient transfection of
CHO-AT1 cells using PAK1 and Nck plasmids was performed using LipofectAMINE (Life
Technologies, MD). Expression of transfected proteins was checked in each experiment by
Western blotting with anti-Myc-tag antibody 9E10 for detection of PAK1, or monoclonal Nck
for detection of Nck.
PAK and JNK activation by AngII is dependent on Rac1 and Nck

**Immunoprecipitation, in-vitro binding studies and Western-blot analysis** - Growth arrested VSMC or CHO-AT1 cells were either left untreated or stimulated by 100 nM - 1 µM AngII for the indicated times. Cells were lysed with lysis buffer containing 20 mM HEPES pH=7.5, 150 mM NaCl, 1 % Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 µg/ml leupeptin and 1 mM PMSF. The indicated antibodies or GST-fusion proteins were added to equal amounts of protein per sample and incubated for 12 hr at 4 °C. Antibody complexes were collected by addition of protein A-agarose for 3 hr. GST-fusion proteins were collected by addition of glutathione Sepharose 4B (Pharmacia) for 3 hr. Precipitates were washed 5 times in cell lysis buffer, resuspended in SDS-sample buffer and boiled for 10 min. After centrifugation for 10 min at 10,000 g the supernatants were size fractionated by SDS-PAGE, transferred to nitrocellulose membranes and probed with the indicated antibodies. Secondary antibodies were coupled to HRP and Western-blot detection was done by enhanced chemiluminescence (ECL Amersham). Equal loading of the immunoprecipitated protein of interest was ascertained in every experiment by Western-blotting.

**Preparation of cytosolic and membrane fractions** - VSMC were incubated in hypotonic buffer containing 10 mM HEPES pH= 7.4, 100 mM NaCl, 10 % glycerol and 1mM EDTA for 1 hr. Cells were scraped off the dishes and lysates were precleared by centrifugation at 10.000 x g at 4 °C for 10 min. Cell lysates were then homogenized with a Teflon Wheaton Homogenizer by 30 dounces and centrifuged in an ultra-centrifuge at 100.000 x g for 30 min. Supernatants were denoted cytosolic fraction. Pelleted proteins were solubilized by addition of lysis buffer containing 2% N-octyl β-D glucoside and centrifuged a second time in an ultra-centrifuge at 100.000 x g for 30 min. The supernatant of the second ultra-centrifugation was denoted membrane fraction. For determination of PAK activity from membrane and cytosolic fractions hypotonic buffer of cytosolic fraction was adjusted in order to obtain the same buffer conditions as was used for the membrane fraction.
PAK and JNK activation by AngII is dependent on Rac1 and Nck

**PAK-immunocomplex MBP-in-gel kinase assay** - Growth arrested VSMC were stimulated and cells were lysed with lysis buffer containing 10 mmol/L HEPES pH=7.4, 0.1% Triton-X-100, 5 mmol/L EGTA, 5 mmol/L EDTA, 50 mmol/L NaCl, 50 mmol/L NaF, 50 mmol/L sodium pyrophosphate, 1 mmol/L sodium orthovanadate, 10 mg/ml leupeptin and 1 mmol/L PMSF. Lysates were precleared by centrifugation, and protein concentration was measured by DC protein assay (Bio-Rad). PAK antibody was added to equal amounts of protein per sample and incubated for 12 hr at 4 °C. Antibody complexes were collected by addition of protein A-agarose for 3 hr. Precipitates were washed 5 times in cell lysis buffer, resuspended in SDS-sample buffer and boiled for 10 min. After centrifugation for 10 min at 10,000xg the supernatants were size fractionated by SDS-PAGE and PAK activity was assayed by [$^{32}$P] incorporation into MBP using an in-gel kinase assay as previously described (4).

**PAK-immunocomplex in vitro kinase assay** - CHO-AT1 cells were co-transfected by Myc-tagged wild type PAK1 (pCMV6M-PAK1) or PAK-A13 (pCMV6M-PAK-A13) and the indicated pEBB-Nck plasmids for 24 hr. Cells were then growth arrested for 24 hr prior to stimulation by 1 µM AngII for 30 min. Cells were lysed with lysis buffer (see above). Myc-tag antibody 9E10 (Upstate Biotechnology) was added to equal amounts of protein per sample and incubated for 4 hr at 4 °C. Antibody complexes were collected by addition of protein A-agarose for 2 hr. Precipitates were washed 3 times in cell lysis buffer and 2 times in kinase reaction buffer. Subsequently, samples were incubated in 30 µl kinase reaction buffer containing 50 mM HEPES pH=7.4, 10 mM MgCl$_2$, 2 mM MnCl$_2$, 50 µM ATP and 5 µCi of [$\gamma$-$^{32}$P]ATP for 30 min at 30 °C in the presence 1 µg MBP as substrate. The *in vitro* kinase reaction was stopped by addition of SDS-sample buffer and heating of samples to 95 °C for 10 min. After centrifugation for 10 min at 10,000 x g the supernatants were size fractionated by SDS-PAGE and MBP phosphorylation was assessed by autoradiography.
PAK and JNK activation by AngII is dependent on Rac1 and Nck

**JNK activity assay** - JNK1 was immunoprecipitated from control and AngII-stimulated CHO-AT1 cell lysate 48 hr after transfection of the indicated plasmids. Agarose beads were collected by centrifugation and washed three times with lysis buffer \((50 \text{ mM HEPES, } pH = 7.5, 150 \text{ mM NaCl, } 1.5 \text{ mM MgCl}_2, 1 \text{ mM EGTA, } 10 \% \text{ glycerol and } 1 \% \text{ Triton X-100})\) and two times with kinase reaction buffer. Beads were then incubated for 30 min at 30 °C in 30 µl kinase reaction buffer containing 20 mM HEPES, 10 mM MgCl₂, 50 µM ATP in the presence of 2 µg c-Jun (1-79) as substrate. The reaction was terminated by the addition of SDS sample buffer, proteins were separated by SDS-PAGE, and JNK activity was assessed by Western blotting with phospho-c-Jun specific antibodies (Cell Signaling Technology).

**GST-PAK-PBD binding assay** - PAK-PBD binding assay was performed essentially as previously described (34). In brief, the p21-binding domain of human PAK1, comprising amino acids 68-166, was subcloned into the bacterial expression vector pGEX-2TK (Amersham Pharmacia Biotech, Inc.) and was expressed in E.coli as GST-PAK-PBD fusion protein according to the manufacture’s protocol. 15 µl of a 50 % slurry of GST-PAK-PBD glutathione-Sepharose 4B was added to cell lysates of VSMC constantly rotating at 4 °C for 60 min. Bound proteins were collected by centrifugation and pellets were washed three times in cell lysis buffer \((25 \text{ mM HEPES } pH= 7.5, 150 \text{ mM NaCl, } 25 \text{ mM NaF, } 10 \% \text{ glycerol, } 0.25 \% \text{ sodium deoxycholate, } 10 \text{ mM MgCl}_2, 1 \text{ mM EDTA, } 1 \% \text{ Triton-X100})\) and finally suspended in SDS sample buffer. Proteins were size fractionated by SDS-PAGE and binding of Rho-family GTPases was determined by Western blotting with Rac1 and Cdc42 antibodies.

**Generation of Nck-SH domain GST fusion proteins** - GST fusion proteins containing Nck-SH2 and the three Nck-SH3 domains were generated by subcloning of the corresponding PCR fragments from a template Nck cDNA (32) into pGEX-2TK (Amersham Pharmacia Biotech,
PAK and JNK activation by AngII is dependent on Rac1 and Nck Inc.). Nck-SH2 encoded amino acids 275-377, Nck-SH3-1 amino acids 2-68, Nck-SH3-2 amino acids 98-168 and Nck-SH3-3 amino acids 190-256. Nck-SH fragments subcloned into pGEX-2TK were sequenced to exclude mutations due to PCR amplification. GST fusion proteins were isolated using glutathione-Sepharose 4B according to the manufacture`s instructions (Amersham Pharmacia Biotech, Inc.). Fusion proteins were checked by Coomassie Blue staining and yielded single bands of expected size for all GST-Nck-SH domains.

[^3H]-Thymidine incorporation into DNA - The effect of AngII on[^3H] thymidine incorporation into cell DNA was assessed as previously described (35). In brief, CHO-AT1 cells were grown to confluence in 24-well plates, growth arrested in serum-free medium for 24 hr prior to stimulation with 1 µM AngII for 24 hr. 20 hr after addition of AngII 3 µCi/ml[^3H] thymidine was added. 4 hr later the experiment was stopped by aspirating the medium and subjecting the cultures to sequential washes with Dulbecco’s phosphate-buffered saline (PBS) containing 1 mM CaCl₂, 1 mM MgCl₂, 10% trichloroacetic acid (w/v) and ethanol/ether (2:1, v/v). Acid-insoluble[^3H] thymidine was extracted with 0.5 M NaOH (250 µl per well) and 100 µl of this solution was mixed with 5 ml scintillator liquid (Packard, Ultimagold, Groningen, The Netherlands) and quantified using a liquid scintillation counter (LS 3801; Beckman). 50 µl of the residual solution was used for determination of protein using the Bio-Rad protein assay according to the method of Bradford (36). Three independent experiments were performed where triplicate values for each condition were obtained. Data were calculated as cpm/µg protein.

Densitometry and Statistics - For quantification of Western blots or[^32P]-incorporation into MBP films were scanned and analyzed by densitometry using arbitrary units. Activation is presented as the fold increase over the respective control (mean ± S.E.). Statistical analysis was performed by
PAK and JNK activation by AngII is dependent on Rac1 and Nck

Student`s t-Test (unpaired and two-tailed) using StatView 5.0 software. A p-value of < 0.05 was considered significant.

RESULTS

AngII stimulates Rac1 in VSMC

To determine whether AngII stimulated Cdc42 or Rac in VSMC we used the GST-PAK-PBD binding assay recently developed by Bagrodia et al. (37). Cdc42 and Rac1 were expressed in VSMC as determined by Western-blotting (data not shown). Addition of GTP-γS to total cell lysates of VSMC increased binding of endogenous Rac1 and Cdc42 to GST-PAK-PBD, demonstrating functional integrity of the fusion protein used (data not shown). AngII stimulated binding of Rac1 to GST-PAK-PBD in a time-dependent manner, peaking at 1 min (3.1 ± 0.13 fold increase, n=3) and showing sustained activation up to 10 min. (Fig. 1a, lower panel). The dependence of Rac1 binding to GST-PAK-PBD on AngII concentration was determined at 1 min. Maximal stimulation was observed at a concentration of 1 µM AngII (2.6 ± 0.62 fold increase, n=3) (Fig. 1b, lower panel). However, AngII-induced stimulation of Cdc42 was not observed (data not shown).

AngII-induced activation of Rac1 is dependent on tyrosine kinases and PKC

Previous results obtained in VSMC (5) demonstrated that a tyrosine kinase other than src is involved in AngII-mediated activation of PAK and JNK. To test for involvement of tyrosine kinases in Rac1 activation by AngII, VSMC were pretreated by 100 µM Genistein for 16 hr or 10 µM PP1 for 30 min prior to stimulation by 1 µM AngII and Rac1 binding to GST-PAK-PBD was determined. We have previously shown that 10 µM PP1 effectively inhibited src activation by AngII in VSMC (33). Genistein inhibited AngII-mediated binding of Rac1 to GST-PAK-PBD (fold increase of DMSO = 2.47 ± 0.31 vs. genistein = 1.21 ± 0.12, p = 0.02, Fig. 2a, lower panel), whereas PP1 had no significant effect (fold increase of DMSO
PAK and JNK activation by AngII is dependent on Rac1 and Nck

= 2.41 ± 0.31 vs. PP1 = 2.63 ± 0.37, p = 0.74, Fig. 2b, lower panel), indicating that a tyrosine kinase other than src is involved in AngII-induced Rac1 activation.

We next examined the effect of PKC inhibition on Rac1 activation by AngII. Down-regulation of phorbol-ester sensitive PKC isoforms (PKC−α,−β,−γ,−δ,−ε,−θ and −η) by pretreatment of VSMC with 1 µM PDBU for 24 hr inhibited AngII-mediated activation of Rac1 (fold increase of DMSO = 2.8 ± 0.63 vs. PDBU 0.97 ± 0.21, p = 0.05, Fig. 3a, lower panel). However, the PKC inhibitor GF109203X (GFx), which is thought to preferentially inhibit PKC−α,−β,−γ (38), did not have a major effect on Rac1 activation by AngII (fold increase of DMSO = 2.8 ± 0.63 vs. GFx = 2.1 ± 0.25, p = 0.48, Fig. 3b, lower panel).

Nck-PAK interaction

PAK has been shown to bind Nck by interaction of its first proline rich region with the second Nck-SH3 domain (19, 20). Whereas Galisteo et al. (19) reported constitutive Nck-PAK interaction in L6 cells treated by PDGF, Bokoch et al. (20) observed an increase in the amount of PAK bound to Nck after stimulation of Swiss 3T3 cells with PDGF. In contrast, Zhao et al. (39) reported that PAK autophosphorylation negatively regulated Nck-PAK interaction in COS-7 cells transfected by recombinant PAK. To determine whether PAK interacted with Nck in VSMC, we immunoprecipitated Nck from control and AngII-stimulated VSMC and evaluated binding of PAK by immunoblotting. At baseline PAK was bound to Nck, showing no increase after AngII treatment of VSMC (Fig. 4a). To further characterize Nck-PAK interaction in VSMC we performed in vitro binding studies using GST fusion proteins comprising the various Nck-SH domains and immunoblotted for PAK. As previously reported (19, 20), Nck-PAK interaction in VSMC was mediated by binding of PAK to Nck-SH3-2 domain (Fig. 4b). Essentially the same results as shown in figure 4 were obtained for CHO-AT1 cells (data not shown).
AngII induces translocation of the Nck-PAK complex to the membrane fraction

Recruitment of PAK to the plasma membrane has been previously shown to facilitate its activation (40, 41). Lu et al. (40) have demonstrated in 293T cells that translocation of PAK can be mediated by the adaptor molecule Nck. In order to evaluate translocation of Nck and PAK by AngII treatment of VSMC, we prepared cytosolic and particulate cell fractions by differential centrifugation of unstimulated and AngII- or PDGF- stimulated VSMC and performed Western blotting using Nck and PAK antibodies. AngII induced an approximately 2-fold increase of immunoreactive Nck and PAK in the particulate fraction after 5 min (Fig. 5a and 5b). PDGF treatment of VSMC for 5 min showed a slightly lower increase of Nck in the particulate fraction compared to AngII (Fig. 5a and 5b), however, PAK immunoreactivity increased by about 3.5 fold over control (Fig. 5a and 5b).

To further evaluate the relevance of membrane localisation for AngII-induced PAK activation, we determined PAK activity by an immunocomplex MBP-in-gel kinase assay from cytosolic and membrane fractions of VSMC that had been stimulated by 100 nM AngII for 15 min. In the cytosolic fraction AngII stimulated PAK activity by 1.44 ± 0.03 fold (n=3), whereas there was a 3.6 ± 0.3 (n=3) fold increase in the membrane fraction (p = 0.02, fold increase cytosolic vs. membrane fraction) (Fig. 5c).

AngII stimulates tyrosine phosphorylation of Nck-associated proteins

Tyrosine kinases are important mediators of AngII-induced Rac1 (see above), PAK and JNK activation (5). Therefore, we were interested to determine whether Nck bound tyrosine-phosphorylated proteins upon AngII stimulation of VSMC. Nck immunoprecipitates of AngII-stimulated VSMC showed slightly increased association of a 100 kD tyrosine phosphorylated protein (Fig. 6a, lower arrow). Tyrosine phosphorylation of a 150 kD protein was only inconsistently seen (Fig. 6a, upper arrow). To further define interaction of the 100 kD protein with Nck, we generated a GST-Nck-SH2 fusion protein. In vitro binding studies...
PAK and JNK activation by AngII is dependent on Rac1 and Nck using GST-Nck-SH2 did not detect association of the 100 kD tyrosine phosphorylated protein with Nck-SH2 (Fig. 6b) (nor with the various Nck-SH3 domains, data not shown). Thus binding of the 100 kD protein to Nck is either indirectly or is mediated by interaction with an non-SH domain region of Nck. However, we detected increased association of 25 kD and 30 kD tyrosine phosphorylated proteins with Nck-SH2 upon AngII stimulation of VSMC, that were not detected by immunoprecipitation (Fig. 6a and 6b).

Binding of Shc (30) and Grb2 (29) to transactivated EGF receptor has been shown to be a critical step in ERK1/2 activation by AngII. Furthermore, Heenemann et al. (42) recently demonstrated in VSMC AngII-induced binding of Shc to transactivated PDGF receptor. By analogy, we were interested to examine whether AngII stimulated binding of Nck to transactivated EGF- or PDGF receptor. Western blotting of Nck immunoprecipitates from unstimulated and AngII-stimulated VSMC with EGF receptor- or PDGF receptor antibodies yielded negative results (data not shown). Corresponding in vitro binding studies using GST-Nck-SH2 fusion proteins also showed no binding of EGF receptor or PDGF receptor to Nck-SH2 upon AngII stimulation (data not shown), whereas PDGF treatment of VSMC stimulated binding of GST-Nck-SH2 to PDGF receptor (Fig. 6b). To further substantiate that EGF- or PDGF receptor transactivation plays no role in AngII-induced PAK stimulation, we pretreated VSMC for 30 min with 250 nM AG1478 (to inhibit EGF receptor) or 10 µM AG1296 (to inhibit PDGF receptor) and determined AngII-induced PAK activity by an immunocomplex MBP-in-gel kinase assay. The concentrations used for AG1478 and AG1296 have been previously shown to effectively inhibit EGF- and PDGF receptor tyrosine kinase activity in VSMC (30). Neither inhibition of EGF- nor PDGF receptor by the indicated tyrphostins did influence AngII-induced PAK activation (Fig. 7).

Overexpression of dominant-negative Nck inhibits AngII-induced activation of PAK and JNK in CHO-AT1 cells
PAK and JNK activation by AngII is dependent on Rac1 and Nck

We have previously shown (5), that overexpression of kinase dead PAK (PAK-K299R) did inhibit AngII-mediated stimulation of JNK in CHO cells stably expressing the angiotensin II type I receptor (CHO-AT1 cells). To further elucidate the role of Nck in AngII signal transduction, we expressed dominant-negative Nck mutants, exhibiting inactivating mutations in either the Nck-SH2 domain (Nck-K308) or all Nck-SH3 domains (Nck-KSH3all). The plasmids containing the various dominant-negative Nck mutants were kindly provided by Bruce Mayer and have been extensively characterized by his group (32). CHO-AT1 cells were co-transfected with Myc-tagged human wild type PAK1 (pCMV-PAK1) and either control vector (pEBB-βGal), pEBB-Nck-K308 or pEBB-Nck-KSH3all. Expression of transfected plasmids was checked by immunoblotting using Myc-tag antibodies (for PAK expression) or monoclonal Nck-antibody (data not shown). 48 hr after transfection cells were stimulated by 1 µM AngII for 30 min and PAK was immunoprecipitated by Myc-tag antibody (9E10, UBI). Subsequently, an in vitro kinase assay was performed using MBP as substrate. AngII-stimulated PAK activity in CHO-AT1 cells could be significantly inhibited by co-expression of Nck-KSH3all (fold increase of β-Gal = 2.47 ± 0.4 vs. Nck-KSH3all = 1.01 ± 0.05, p = 0.03, Fig. 8) and by Nck-K308 (fold increase of β-Gal = 2.47 ± 0.4 vs. Nck-K308 = 1.04 ± 0.10, p = 0.03, Fig. 8). To determine the effect of dominant-negative Nck on AngII-mediated JNK activation, we performed a JNK-immunocomplex in vitro kinase assay using c-Jun as substrate. Activation of JNK was determined by immunoblotting with phospho-c-Jun specific antibodies. Parallel to inhibition of PAK activation, we found inhibition of AngII-induced JNK by co-expression of Nck-KSH3all (fold increase of β-Gal = 2.87 ± 0.49 vs. Nck-KSH3all = 1.57 ± 0.24, p = 0.05, Fig. 8) and Nck-K308 (fold increase of β-Gal = 2.87 ± 0.49 vs. Nck-K308 = 1.03 ± 0.05, p = 0.06, Fig. 8).

Since Nck-SH3 domains are known to interact with various other molecules (39) (e.g. dynamin, NIK, SAM68, WASP) that might indirectly influence AngII-induced PAK activation, we examined the ability of the PAK-A13 mutant (which does not bind Nck) to be
PAK and JNK activation by AngII is dependent on Rac1 and Nck stimulated by AngII. CHO-AT1 cells were transfected with pCMV6M-PAK-wt or pCMV6M-PAK-A13 and AngII-induced PAK activity was determined by PAK autophosphorylation and by Myc-tag immunocomplex in vitro kinase assay. AngII-induced activation of PAK was completely blocked by expression of the PAK-A13 mutant (Fig. 9).

Recently, Wen et al. (43) demonstrated decreased ERK1/2 activity in CHO-AT1 cells transfected by kinase-dead PAK1, which had been stimulated by 12-HETE. To test for specificity of JNK inhibition, we determined ERK1/2 activity in AngII-stimulated CHO-AT1 cells expressing kinase-dead PAK1, Nck-K308 or Nck-KSH3all by Western-blotting using phospho-ERK1/2 specific antibodies. Stimulation of ERK1/2 by AngII and PMA was neither inhibited by expression of kinase-dead PAK1 nor by expression of Nck-K308 or Nck-KSH3all (data not shown). Activation of p38 by AngII could not be detected in CHO-AT1 cells by an immunocomplex in vitro kinase assay using ATF2 as substrate, where activity was assessed by Western blotting with phospho-ATF2 specific antibodies (data not shown).

Inhibition of JNK by overexpression of dominant-negative Nck diminishes $[^3]$H-thymidine incorporation in CHO-AT1 cells

The role of JNK in AngII-induced growth responses has not been defined so far. By overexpression of Nck-K308 and Nck-KSH3all we specifically inhibited AngII-stimulated JNK with no effect on ERK1/2. To define the role of JNK in AngII-induced DNA synthesis, we determined $[^3]$H thymidine incorporation in AngII-stimulated CHO-AT1 cells that had been co-transfected with wild type PAK1 and the various dominant-negative Nck plasmids. AngII led to a 2.4 ± 0.4 fold increase in $[^3]$H thymidine incorporation under control conditions (transfection of pEBB-β-Gal). Overexpression of Nck-KSH3all and Nck-K308 both inhibited AngII-stimulated DNA synthesis (fold increase over resp. control for Nck-KSH3all = 1.41 ± 0.02, p = 0.02 vs. β-Gal, and for Nck-K308 = 1.04 ± 0.08, p = 0.08 vs. β-Gal, Fig. 10).
DISCUSSION

In the present study, we report for the first time that AngII stimulates the GTPase Rac1 in a tyrosine kinase- and PKC-dependent manner. Furthermore, we demonstrate that AngII-induced activation of PAK and JNK is mediated by the adaptor molecule Nck. In addition, we show that specific inhibition of JNK by overexpression of dominant-negative Nck diminishes AngII-stimulated DNA synthesis in CHO-AT1 cells.

Small G-proteins of the Ras superfamily are known to be critical regulators of MAP kinase pathways. Ras has been shown to be an important regulator of ERK1/2, whereas Rac and Cdc42 are thought to mainly stimulate JNK and p38. In VSMC, AngII has been previously shown to regulate ERK1/2 via activation of Ras (12). Recently, Haendeler et al. (44) also implicated Ras and to a lesser extent Rap1 in AngII induced ERK1/2 activation. However, small G-proteins of the Rho family have not been demonstrated so far to regulate AngII-induced JNK activation in VSMC. In the present report, we demonstrated binding of (GTP-bound) Rac1 to PAK-PBD in VSMC stimulated by AngII. Furthermore, our data indicate that a tyrosine kinase other than src is involved in PAK and JNK activation by AngII. In addition, downregulation of phorbol-ester sensitive PKC-isoforms inhibited Rac1 activation by AngII. These results are in agreement with formerly published data obtained in VSMC (5) considering PAK and JNK activation by AngII. This indicates that AngII-induced PAK and JNK activation in VSMC is mediated by Rac1. A critical role for Rac1 in AngII signaling is further supported by recent findings from Murasawa et. al (45), who showed in cardiac fibroblasts that overexpression of dominant-negative Rac1, but not Cdc42, inhibited AngII-mediated activation of PAK and JNK.

Targeting of proteins to specific cellular sites is increasingly recognized as a mechanism to regulate signal transduction pathways. A critical role for recruitment of PAK to the plasma membrane has been established by Lu et al. (40, 46) and Bokoch et al. (41), who demonstrated that targeting PAK directly to the plasma membrane facilitated its activation.
PAK and JNK activation by AngII is dependent on Rac1 and Nck

Bokoch et al. (41) reported increased activity of membrane targeted PAK independently of its ability to interact with Rac or Cdc42 and suggested that binding to membrane lipids facilitates stimulation of PAK. In contrast, data from Lu et al. (46) showed that activation of membrane localized PAK could be inhibited by expression of proteins that inhibit Rho-family GTPases (e.g. dominant-negative Cdc42D57Y), indicating that translocation of PAK to the plasma membrane serves its interaction with activated Cdc42 or Rac. The adaptor molecule Nck has been identified as a putative signaling molecule to mediate translocation of PAK to the plasma membrane. First of all, Nck has been previously implicated in tyrosine kinase receptor signaling leading to activation of PAK (19) and JNK (23, 28). Upon stimulation of tyrosine kinase receptors a preformed Nck-PAK complex translocates to the plasma membrane by binding of Nck-SH2 domain to phosphotyrosine residues of the activated receptor (19, 25, 26, 28). Second, Lu et al. (40) demonstrated that recruitment of PAK to the plasma membrane by a myristolated Nck-SH3-2 domain facilitated activation of PAK. Importantly, overexpression of a non-myristolated Nck-SH3-2 domain did not mediate PAK activation, indicating, that merely facilitating Nck-PAK interaction is not sufficient for PAK activation (40).

In the present report, we demonstrated in VSMC AngII-induced translocation of Nck and PAK to the particulate fraction. Furthermore, compared to the cytosolic fraction, we detected significantly enhanced PAK activity in the membrane fraction of AngII-treated VSMC. Thus, our data support a critical role for recruitment of PAK to the plasma membrane, where stimulation by activated Rac1 presumably takes place. We hypothesized, that in analogy to ERK1/2 activation by AngII (29, 30, 47), AngII-mediated transactivation of a tyrosine kinase receptor might promote binding of Nck, thus facilitating membrane localisation of PAK. However, we could not detect AngII-mediated binding of Nck to either EGF- or PDGF receptor. Furthermore, specific inhibition of EGF- and PDGF receptor kinase by tyrphostins did not affect AngII-mediated PAK activation in VSMC. These results are in agreement with recent data from Eguchi et al. (48), who showed that AngII-induced JNK
PAK and JNK activation by AngII is dependent on Rac1 and Nck activation in VSMC was not dependent on transactivation of the EGF receptor. Surprisingly, the tyrosine phosphorylated 100 kD protein that we detected in Nck-immunoprecipitates of AngII-stimulated VSMC did not bind to a GST-Nck-SH2 fusion protein. However, AngII stimulated binding of 30 kD and 25 kD tyrosine phosphorylated proteins to GST-Nck-SH2 that were not detected in Nck-immunoprecipitates. These data indicate that the polyclonal Nck-antibody used for immunoprecipitation blocked the Nck-SH2 domain. To date, the role of Nck-SH2 associated 25 kD and 30 kD phosphotyrosine proteins in AngII-mediated PAK activation remains unclear. Identification of these molecules and characterization of their function will be the purpose of future studies.

To further define the role of Nck in AngII signaling, we employed expression of dominant-negative Nck mutants in CHO-AT1 cells. Our data indicate, that blocking Nck-SH2 domain interacting proteins in CHO-AT1 cells by overexpression of Nck-KSH3all, or sequestration of PAK by an inactivated Nck-SH2 domain (Nck-K308) significantly inhibited activation of PAK and JNK by AngII. Conversely, a PAK mutant that lacks Nck binding (PAK-A13), could not be stimulated by AngII. Although we cannot completely rule out that other proteins interacting with either Nck-SH3 domains or the PAK amino terminus play a role in AngII-mediated PAK and JNK activation, these results strongly support a role for Nck in AngII-mediated activation of PAK and JNK. Furthermore, they are the first to demonstrate that a G-protein coupled receptor requires Nck for PAK activation.

Identification of Nck as an upstream mediator of JNK enabled us to investigate the role of JNK in AngII-mediated growth responses in more detail. Several in vivo studies have previously demonstrated activation of JNK by AngII under conditions that led to media hypertrophy (49, 50) or cardiac hypertrophy (51). However, JNK has also been implicated in apoptosis (52) and it becomes increasingly clear that regulation of apoptosis by AngII is critical to hypertrophic growth in cardiovascular tissue (53). In the present study, we used CHO-AT1 cells as a model system to investigate the effect of JNK inhibition on DNA
PAK and JNK activation by AngII is dependent on Rac1 and Nck synthesis. Overexpression of dominant-negative Nck molecules inhibited JNK activation by AngII, but did not affect ERK1/2 activation. Inhibition of JNK significantly decreased AngII-induced DNA synthesis, indicating a growth promoting rather than proapoptotic role of JNK in AngII signaling, at least in CHO-AT1 cells. In CHO-AT1 cells and VSMC AngII stimulated ERK1/2 and JNK, whereas it did not activate p38. Ushio-Fukai et al. (7) recently demonstrated a stimulatory role for p38 in AngII-induced protein and DNA synthesis in VSMC. However, in contrast to our results (5) they could not find JNK activation by AngII in VSMC (7). Viedt et al. (54) on the other hand, reported AngII-induced stimulation of ERK1/2, JNK and p38 in VSMC. To date, it remains unclear why these differences exist in the same cell system. However, since JNK and p38 activate common transcription factors, such as ATF2 (55), it can be speculated that they might substitute for each other under different conditions. Since we could not detect AngII-mediated activation of p38 in CHO-AT1 cells and VSMC, our results do not exclude a role for p38 in AngII-induced growth responses in general. The development of specific pharmacological JNK inhibitors will be of great help to more specifically address the roles of ERK1/2, JNK and p38 on AngII promoted growth in cardiovascular target tissues such as VSMC.

The present report identifies Rac1 and Nck as upstream mediators of PAK (and hence JNK) in AngII signaling. These findings, together with previous results (5, 45), suggest the following model for AngII-mediated JNK activation in VSMC: AngII stimulation of VSMC leads to rapid activation of Rac1. Translocation of PAK to the "activation compartment" is mediated by Nck, thus facilitating stimulation of PAK by activated Rac1. Future studies are now aimed at identifying Nck-interacting proteins that will hopefully further increase our understanding of AngII-induced growth processes in cardiovascular tissues.
PAK and JNK activation by AngII is dependent on Rac1 and Nck

ACKNOWLEDGEMENT

This work was supported by grants from the German Research Foundation to U.S. (SCHM 1174/3-1) and the BONFOR program to K.T. (BONFOR 110/24). We thank Dirk Bokemeyer for critical reading of the manuscript.

REFERENCES


PAK and JNK activation by AngII is dependent on Rac1 and Nck


PAK and JNK activation by AngII is dependent on Rac1 and Nck


PAK and JNK activation by AngII is dependent on Rac1 and Nck


PAK and JNK activation by AngII is dependent on Rac1 and Nck


PAK and JNK activation by AngII is dependent on Rac1 and Nck


**FOOTNOTES**

1 The abbreviations used are: AngII, angiotensin II; AT1R, angiotensin type 1 receptor; EGF, epidermal growth factor; ERK1/2, extracellular regulated kinase; JNK, c-Jun NH2-terminal
PAK and JNK activation by AngII is dependent on Rac1 and Nck kinase; PKC, protein kinase C, MAP kinase, mitogen activated protein kinase; PAK, p21-activated kinase; PBD, p21-binding domain; PDGF, platelet derived growth factor; SH domain, src homolgy domain; VSMC, vascular smooth muscle cells;

FIGURE LEGENDS

Fig. 1. AngII stimulates Rac1 in VSMC: time course and concentration dependence.

A) VSMC were stimulated by 1 µM AngII for the indicated times and binding of activated Rac1 to GST-PAK-PBD (PAK-PBD) was determined by Western blotting (lower panel). B) VSMC were stimulated by 0.1 - 1000 nM AngII for 1 min and binding of activated Rac1 to GST-PAK-PBD (PAK-PBD) was determined by Western blotting (lower panel). Equal loading of proteins for the precipitation assays was assessed by Rac1-Western blotting of 10 µl total cell lysate (TCL) (upper panels). Arrows indicate Rac1.

Fig. 2. AngII-induced Rac1 activation is dependent on tyrosine kinases.

VSMC were pretreated by 100 µM genistein for 16 hr (A) or 10 µM PP1 for 15 min (B) and then stimulated by 1 µM AngII for 1 min. Binding of activated Rac1 to GST-PAK-PBD (PAK-PBD) was assessed by Western blotting (lower panels). Equal loading of proteins for the precipitation assays was assessed by Rac1-Western blotting of 10 µl total cell lysate (TCL) (upper panels). Arrows indicate Rac1. C) Quantitative densitometry of Rac1 immunoblots was performed. Values shown are mean ± S.E. of 3 independent experiments. * p < 0.05 vs. DMSO.

Fig. 3. Rac1 stimulation by AngII is dependent on PKC activation.

VSMC were pretreated by 1 µM PDBU for 24 hr (A) or 1 µM GF109203X (GFx) for 10 min (B) and then stimulated by 1 µM AngII for 1 min. Binding of activated Rac1 to GST-PAK-PBD (PAK-PBD) was assessed by Western-blotting. Equal loading of proteins for the
PAK and JNK activation by AngII is dependent on Rac1 and Nck precipitation assays was assessed by Rac1-Western blotting of 10 µl total cell lysate (TCL) (upper panels). Arrows indicate Rac1. C) Quantitative densitometry of Rac1 immunoblots was performed. Values shown are mean + S.E. of 3 independent experiments. * p < 0.05 vs. DMSO.

**Fig. 4: Nck-PAK interaction.**

A) Growth arrested VSMC were treated by 100 nM AngII for the indicated times. Nck was immunoprecipitated by polyclonal Nck-antibody and PAK binding was detected by Western blotting with polyclonal PAK1 antibody (sc-881). N indicates immunoprecipitation by rabbit pre-immune serum and TCL, total cell lysate. PAK binds constitutively to Nck in VSMC. B) Total cell lysates of VSMC were prepared and *in vitro* binding assays using GST or the indicated GST-Nck-SH domain fusion proteins were performed. Bound proteins were visualized by polyclonal PAK1 antibody. Arrows to the right indicate PAK in VSMC that migrates as a doublet. Endogenous PAK binds to the second Nck-SH3 domain.

**Fig. 5: Translocation of Nck and PAK to the plasma membrane.**

A) Membrane fractions of VSMC that were either left untreated (Contr.), or stimulated by 100 nM AngII for 10 min (AngII), or 50 ng/ml PDGF-AB for 10 min (PDGF) were prepared as described under EXPERIMENTAL PROCEDURES. 20 µg of total protein were size fractionated by 7.5% SDS-PAGE, transferred to nitrocellulose membrane and blotted by Nck (upper panel) or PAK (lower panel). B) The relative increase of immunoreactive Nck (open bars) or PAK (closed bars) over control in the membrane fraction of AngII and PDGF stimulated VSMC was determined by densitometry. Values shown are mean + S.E. of 3 independent experiments. C) PAK activity was determined by an immunocomplex MBP-in-gel kinase assay form cytosolic and membrane fractions of VSMC that were either left untreated (Contr.), or stimulated by 100 nM AngII for 15 min (AngII). Phosphorylation was
PAK and JNK activation by AngII is dependent on Rac1 and Nck assessed by autoradiography. The result shown is representative of 3 independent experiments.

**Fig. 6. AngII induces tyrosine phosphorylation of Nck-associated proteins.**

A) Growth arrested VSMC were either left untreated (Contr.) or stimulated by 100 nM AngII for the indicated times. Nck was immunoprecipitated by polyclonal anti-Nck, bound proteins were size fractionated by 7.5% SDS-PAGE, transferred to nitrocellulose membrane and blotted by anti-phosphotyrosine antibodies (PY99). N indicates immunoprecipitation by rabbit pre-immune serum. AngII induced tyrosine phosphorylation of a 100 kD protein (lower arrow). Increased tyrosine phosphorylation of an approximately 150 kD protein was only inconsistently seen (upper arrow). Molecular weight markers are indicated to the right (kD).

B) Growth arrested VSMC were either left untreated (Contr.), stimulated by 100 nM AngII for the indicated times or treated by 50 ng/ml PDGF-AB for 5 min (PDGF). Cell lysates were prepared and an in vitro binding assay using GST-Nck-SH2 or GST alone was performed. Proteins were size-fractionated by SDS-PAGE transferred to nitrocellulose membrane and an anti-phosphotyrosine immunoblot was performed. AngII stimulated binding of a 30 kD protein to Nck-SH2 (B, middle arrow). A longer exposure of the blot revealed additional binding of a 25 kD protein (B, lower arrow). PDGF-AB treatment stimulated binding of a 180 kD phosphotyrosine protein to Nck-SH2 that corresponded to PDGF receptor (B, upper arrow). Molecular weight markers are indicated to the right (kD).

**Fig. 7: Inhibition of EGF- and PDGF receptor kinase does not affect AngII-induced activation of PAK.** Growth arrested VSMC were pretreated for 30 min by 250 nM AG 1478 to inhibit EGF receptor kinase, or by 10 µM AG 1296 to inhibit PDGF receptor kinase. DMSO was used as a control. AngII-induced PAK activity was determined by an immunocomplex MBP-in-gel kinase assay as described under EXPERIMENTAL
PAK and JNK activation by AngII is dependent on Rac1 and Nck

PROCEDURES. Phosphorylation was assessed by autoradiography. The result shown is representative of 3 independent experiments.

Fig. 8. Overexpression of dominant-negative Nck inhibits AngII-induced stimulation of PAK and JNK. A) CHO-AT1 cells were transfected by Myc-tagged wild type PAK1 together with either control vector (pEBB-β-Gal), pEBB-Nck-KSH3all or pEBB-Nck-K308. PAK activity was examined by an immunocomplex MBP in vitro kinase assay using anti-Myc-tag antibodies as described under EXPERIMENTAL PROCEDURES. Phosphorylation of MBP was determined by autoradiography (A, upper panel). JNK activity was assessed by an in vitro kinase assay using GST-c-Jun as substrate and performing Western blotting with phospho-c-Jun (p-c-Jun) specific antibodies as described under EXPERIMENTAL PROCEDURES (A, lower panel). B) Quantitative densitometry was performed as described under EXPERIMENTAL PROCEDURES. Results shown are mean ± S.E. * p < 0.05 vs. β-Gal.

Fig. 9: A PAK mutant that lacks Nck binding cannot be stimulated by AngII. CHO-AT1 cells were transfected by Myc-tagged wild type PAK1 (PAK-wt) or by Myc-tagged PAK1-A13 (PAK-A13). PAK activity was examined by PAK autophosphorylation and by an immunocomplex MBP in vitro kinase assay using anti-Myc-tag antibodies as described under EXPERIMENTAL PROCEDURES. Phosphorylation of PAK (upper panel) and MBP (lower panel) was assessed by autoradiography. The result shown is representative of 3 independent experiments.

Fig. 10. Overexpression of dominant-negative Nck inhibits AngII-induced DNA synthesis in CHO-AT1 cells. CHO-AT1 cells were co-transfected by wild type PAK1
PAK and JNK activation by AngII is dependent on Rac1 and Nck together with pEBB-β-Gal, pEBB-Nck-KSH3all or pEBB-Nck-K308. [3H] thymidine incorporation of control and AngII-stimulated CHO-AT1 cells was determined and the fold increase over respective control was calculated as described under EXPERIMENTAL PROCEDURES. * p < 0.05 vs. β-Gal.
Fig. 1
Fig. 2

**A**

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>Genistein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contr.</td>
<td>AngII</td>
<td>Contr.</td>
</tr>
<tr>
<td>TCL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK-PBD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>PP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contr.</td>
<td>AngII</td>
<td>Contr.</td>
</tr>
<tr>
<td>TCL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK-PBD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C**

![Graph showing fold increase over control for DMSO, Genistein, and PP1](image)

*Fig. 2*
Blot: Rac1

A

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>PDBU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contr. AngII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contr. AngII</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TCL

PAK-PBD

B

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>GFx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contr. AngII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contr. AngII</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TCL

PAK-PBD

C

![Bar graph showing fold increase over response control for DMSO, PDBU, and GFx](http://www.jbc.org/)

Fig. 3
### Fig. 4

#### A

<table>
<thead>
<tr>
<th>AngII [min]</th>
<th>Contr.</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
</table>

**Blot: PAK**

[Image of blot showing PAK bands at different AngII time points]

**IP:**
- Nck
- N
- TCL

#### B

- GST
- SH2
- SH3-1
- SH3-2
- SH3-3

[Image of blot showing PAK bands with different SH domains]

[Fig. 4]
**A**

Contr. AngII PDGF

Blot:

Nck

PAK

Membrane

**B**

![Bar graph showing relative increase over resp. control](image)

**C**

Contr. AngII Contr. AngII

MEMBRANE CYTOSOL

PAK

Fig. 5
Fig. 6
Fig. 7
**Fig. 8**

**A**

![Western blot images](image)

- **PAK** and **JNK** are shown in the presence of AngII and contr. AngII with or without Nck-KSH3all and Nck-K308.

- MBP and p-c-Jun are indicated by arrows.

**B**

**Bar chart**

- Fold increase over resp. control.

- **PAK** and **JNK** are compared.

- AngII, contr. AngII, Nck-KSH3all, and Nck-K308 conditions are shown.

- Asterisks indicate statistical significance.
Fig. 9

Autoradiogram

Contr. AngII Contr. AngII

PAK-wt PAK-A13

PAK MBP
Fig. 1 CHO-AT1 cells were treated by Lipofectamine only (contr.) or were transfected by Lipofectamine with pCMV6M-PAK-wt (PAK-wt). After stimulation by 1 µM AngII for 30 min, PAK activity was assessed by Myc-tag-immunocomplex MBP in vitro kinase assay, as described in the method section of the manuscript. AngII stimulated MBP phosphorylation only in transfected cells, whereas there was only very little background activity immunoprecipitated by the Myc-tag antibody in non-transfected cells.

Fig. 2 CHO-AT1 cells were transfected by Lipofectamine with pCMV6M-PAK-wt (PAK-wt). After stimulation by 1 µM AngII for 30 min, MBP phosphorylation was assessed by Myc-tag-immunocomplex (Myc-tag) or Dynamin-immunocomplex (using monoclonal Dynamin-Ab from Transduction Lab) MBP in vitro kinase assay, as described in the method section of the manuscript. AngII stimulated MBP phosphorylation was only detected in Myc-tag immunoprecipitates, whereas there was no AngII-stimulated activity towards MBP in the Dynamin-immunoprecipitates.
Angiotensin II-induced stimulation of p21-activated kinase and c-Jun NH2-terminal kinase is mediated by Rac1 and Nck

Udo Schmitz, Kerstin Thömmes, Imke Beier, Wolfgang Wagner, Agapios Sachinidis, Rainer Düsing and Hans Vetter

J. Biol. Chem. published online March 28, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102450200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts