Basic Res Cardiol 97: 334 – 342 (2002)
DOI 10.1007/s00395-002-0357-8

ORIGINAL CONTRIBUTION

Angiotensin II-augmented migration of VSMCs towards PDGF-BB involves Pyk2 and ERK 1/2 activation

Florian Blaschke*
Philipp Stawowy*
Kai Kappert
Stephan Goetze
Ulrich Kintscher
Brigitte Wollert-Wulf
Eckart Fleck
Kristof Graf

Received: 16 July 2001
Returned for 1. revision: 2 August 2001
1. Revision received: 12 December 2001
Returned for 2. revision: 21 January 2002
2. Revision received: 14 February 2002
Accepted: 13 March 2002

* Both authors contributed equally to this work

F. Blaschke · P. Stawowy · K. Kappert
S. Goetze · U. Kintscher · B. Wollert-Wulf
E. Fleck · PD Dr. K. Graf (✉)
Deutsches Herzzentrum Berlin
Department of Medicine/Cardiology
Augustenburger Platz 1
13353 Berlin, Germany
Tel.: +49 30-4593 2413
Fax: +49 30-4593 2415
E-Mail: graf@dhzb.de

Abstract Activation of the local and systemic renin-angiotensin system is directly and indirectly involved in mechanisms of vascular remodeling during chronic hypertension. This study investigated the effect of angiotensin II (AII) on rat vascular smooth muscle cell (VSMC) migration towards platelet-derived growth factor-BB (PDGF-BB) *in vitro*. Pre-treatment with AII (1 μM) for 48 or 72 h induced a significant increase in PDGF-BB-directed migration by 77 ± 21 % and 58 ± 24 %, respectively (both $p < 0.01$). This effect was concentration dependent and inhibited by the selective angiotensin receptor type I (AT_1) blocker DUP 753. PDGF-directed migration of VSMCs was significantly inhibited by antibodies against β_3-and β_5-integrins, indicating an important role of these integrins in VSMC migration. However, AII augmented migration was not accompanied by an increased expression of β_3- and β_5-integrin mRNA and protein levels in VSMCs. Inhibition of the mitogen-activated protein kinase ERK 1/2 with PD 98059 (30 μM) completely abolished the effect of AII on PDGF-BB-directed VSMC migration ($p < 0.01$). The proline-rich tyrosine kinase 2 (Pyk2) and focal adhesion kinase (FAK) are cytoskeleton-associated protein kinases participating in integrin-dependent signaling. Therefore, expression and phosphorylation of these kinases was determined 48 h after AII treatment, revealing a significant increase in Pyk2 and FAK protein levels (up to 2-fold, both $p < 0.05$) and increased phosphorylation of Pyk2 (2-fold, $p < 0.05$) and ERK 1/2 (4-fold, $p < 0.05$) as compared to controls. Furthermore, immunofluorescence and Western blot analysis demonstrated a translocation of Pyk2 from the plasma membrane to the cytosol, as well as a perinuclear enrichment of ERK 1/2 protein 48 h after AII treatment. In conclusion, our data suggest that changes in the levels of Pyk2 and ERK 1/2 phosphorylation, responsible for integrin-dependent signaling, as well as their subcellular translocation are important for the enhanced chemotactic response of VSMCs after AII pre-treatment.

Key words Vascular smooth muscle cell – mitogen-activated protein kinase – Pyk2 – migration – integrins

Introduction

Angiotensin II (AII) has various biological functions besides its role as a potent vasoconstrictor. AII has been shown to stimulate the growth of vascular smooth muscle cells (VSMCs) *in vitro* and to enhance neointimal hyperplasia *in vivo*, when elevated in circulation (1, 2). The receptors for AII have been classified into two subtypes: the angiotensin type I (AT_1) receptor and the type II (AT_2) receptor. Both are members of the G-protein coupled receptor superfamily that have seven transmembrane helices (3, 4). The AT_1 receptor has been identified to mediate effects such as vasoconstriction, cardiovascular hypertrophy and aldosterone secretion (5). Signal transduction following activation of AT_1 receptor is complex and includes stimulation of phospholipase C (PLC), which generates diacylglycerol and inositol 1,4,5-triphosphate (IP_3). This leads to increased intracellular calcium, activation of protein kinase C (PKC) and activation of the RAS-RAF-MEK-ERK kinase pathway (6, 7). Furthermore, AII causes a rapid phosphorylation of multiple tyrosine kinases such as focal adhesion kinase (FAK), paxillin, $STAT_1$ and Pyk2 (8–12). AII induces migration and DNA synthesis of VSMCs via the AT_1 receptor, involving the activation of the mitogen-activated protein kinase ERK 1/2 (13, 14). Cell adhesion and migration, which play an important role in vascular remodeling during hypertension and atherosclerosis, are controlled by several growth factors. Platelet-derived growth factor-BB (PDGF-BB), fibroblast growth factor-2 (FGF-2) and AII are factors known to contribute to these processes. VSMC migration also depends on the composition of the extracellular matrix and the expression of specific cell surface receptors, which include the family of integrin receptors. It has been demonstrated that integrin receptors are essential for migration of VSMCs (15, 16). Recent studies demonstrate that growth factors can enhance integrin-dependent motility and chemotaxis of vascular cells: FGF-2 potentiates cell motility and migration of VSMCs towards PDGF-BB (17) and AII enhances adhesion and spreading of human VSMCs on collagen I and fibronectin via activation of β_1-integrins (18).

In the present study we demonstrate that pre-treatment of VSMCs with AII, but not PDGF-BB, increases VSMC migration towards PDGF-BB, which is potentially mediated by chemotactic signaling involving Pyk2 and ERK 1/2 phosphorylation and translocation.

Methods

Materials

Angiotensin II was obtained from Bachem, phorbol 12-myristate 13-acetate (PMA) and PD 123319 from Sigma, PDGF-BB from Biosource, DUP 753 from Merck Laboratories and PD 98059 from Calbiochem. Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane, horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies and ECL Western blotting detection reagents were from Amersham Life Science. Antibodies were purchased from the following vendors: anti-β_1-integrin (AB1937) and anti-α_5-integrin (AB1926) from Chemicon; anti-β_3-integrin (F11), anti-α_1-integrin (F19), anti-α_2-integrin (N19), anti-β_5-integrin (P1F6), anti-Pyk2, anti-FAK and anti-phosphotyrosine (pTyr) from Santa Cruz; secondary FITC-conjugated anti-goat, anti-mouse and anti-rabbit from Sigma; antibodies against phosphorylated and total ERK 1/2 from Promega; anti-phosphorylated Pyk2 (pY402) from Biosource International, and anti-α-smooth-muscle actin from Dako. Reagents for immunofluorescence were purchased from Vector Laboratories.

Cell culture

Rat vascular smooth muscle cells were prepared from the thoracic aorta of 2 – 3 month old Sprague-Dawley rats by the explant technique as described previously (19, 20). The cells were cultured in DMEM containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 200 mM glutamine. For all experiments, early passaged (3 to 6) VSMCs were grown to 60 – 70% confluence and made quiescent by serum starvation in serum-free medium for 24 h. When used, inhibitors or blocking antibodies were added 60 min before stimulation. PMA (0.1 μM) was added 24 h before the start of the experiment to completely downregulate PKC activity. Each individual experiment represented in the *n* value was performed with an independent preparation of VSMCs. Cell purity (> 95%) and identity was assessed by immunostaining with an antibody against α-smooth muscle actin.

Flow cytometry

Integrin cell surface expression was evaluated by indirect immunofluorescence using flow cytometry (18). Briefly, VSMCs were incubated in PBS containing 5% bovine serum albumin (BSA) to suppress nonspecific binding,

followed by incubation on ice for 60 min with control or integrin-specific antibodies at predetermined saturating concentrations. Cells were incubated with appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 20 min on ice (dilutions: anti-goat 1:100, anti-mouse 1:30, anti-rabbit 1:40). Cells were then fixed with 4% paraformaldehyde, washed and resuspended in PBS and then analyzed using a Becton-Dickinson FACScalibur flow cytometer.

Migration

Migration experiments were performed in transwell chambers with gelatin-coated polycarbonate membranes as described previously (13). For inhibition studies, cells were pre-treated for 60 min with PD 98059 (30 μM), DUP 753 (100 μM), PD 123319 (100 μM) or vehicle (0.2% FCS/DMEM), followed by the addition of AII (1 μM). 100 μl cell suspension (final concentration 500,000 cells/well) were added to the upper compartment at 37 °C. Migration was induced by addition of PDGF-BB at a final concentration of 20 ng/ml to the lower compartment. After a 4 h migration period, non-migrating cells were removed with a cotton swab from the upper surface. The number of cells that had migrated to the lower surface of the membrane was determined per x200 high power field (HPF). Four randomly chosen HPFs were counted per membrane. Experiments were performed in quadruplicate and repeated at least three times.

Western blot analysis

Cells were made quiescent by serum starvation for 24 h and then stimulated with AII (1 μM) or PDGF-BB (20 ng/ml) for the indicated time intervals. Proteins were extracted in RIPA-buffer supplemented with proteinase inhibitors. For subcellular fractionation analysis, nuclear and cytosolic fractions were prepared by the method of Dignam et al. (21). To assure that the seperation was accurate, cytosolic and nuclear fractions were controlled by detection of tubulin. Proteins (30 – 50 μg) were separated by 10% SDS-PAGE gel electrophoresis as described previously (5). Proteins were blotted onto nitrocellulose membranes followed by incubation with antibodies that recognize either a) ERK 1/2 when phosphorylated on threonine 202 and tyrosine 204 (pERK), or b) Pyk2, when phosphorylated at tyrosine 402 (pPyk2). Antibodies were used at a concentration of 1:500 (Pyk2), 1:1000 (pPyk2), 1:2000 (ERK 1/2) and 1:5000 (pERK) at 4 °C overnight. The ECL system was used for visualization. Band intensity was analyzed by densitometry using the NIH Image Program 1.62 on a Macintosh PC and expressed in arbitrary units (A.U.). Experiments were performed at least three times with separate preparations of VSMCs.

Immunoprecipitation

Equal amounts of protein (500 μg) were incubated with an antibody against phosphotyrosine residues (pTyr). Immune complexes were then precipitated using protein G agarose (4 °C, overnight). Beads were centrifuged, washed, resuspended in sample buffer and analyzed by 10% SDS-PAGE and Western blotting as described above.

Immunofluorescence

Immunofluorescence was used to investigate the subcellular distribution of ERK 1/2 or Pyk2 in response to AII. Cells were grown on gelatin-coated chamber slides and made quiescent by serum starvation for 24 h. AII (1 μM) or PDGF (20 ng/ml) stimulated cells (48 h) or untreated cells were fixed in 4% paraformaldehyde/PBS for 15 min at room temperature, and then permeabilized with 0.5% Triton X-100/PBS for 5 min at 4 °C. After blocking (2% BSA/10% horse serum) cells were incubated with anti-ERK 1/2 or anti-Pyk2 antibody (4 °C, overnight) followed by a secondary fluorescein-conjugated antibody for 60 min at 37 °C. Slides were mounted in Vectashield mounting medium and specimens were examined and photographed using a fluorescence microscope.

Statistical analysis

Analysis of variance (ANOVA) and paired or unpaired t-test were performed for statistical analysis as appropriate. P-values less than 0.05 were considered to be statistically significant. Results are expressed as mean ± S.E.M.

Results

AII pre-treatment increases PDGF-BB-directed migration

Rat aortic VSMCs were made quiescent by 24 h treatment in serum-free culture medium. Treatment of VSMCs for 48 h with AII (1 μM) increased the number of migrated cells towards PDGF-BB (20 ng/ml) in a transwell chamber migration system by 77 ± 21% over control ($n = 6$, $p < 0.01$, Fig. 1A). The AT_1 receptor blocker DUP 753 completely inhibited AII-augmented migration ($p < 0.01$), whereas blockade of the AT_2 receptor with PD 123319 had no effect (Fig. 1A). In contrast to AII, pretreatment with PDGF-BB (20 ng/ml) for 24 to 72 h did not increase the chemotactic response of VSMCs. The migration assays demonstrated that the increase of migrated

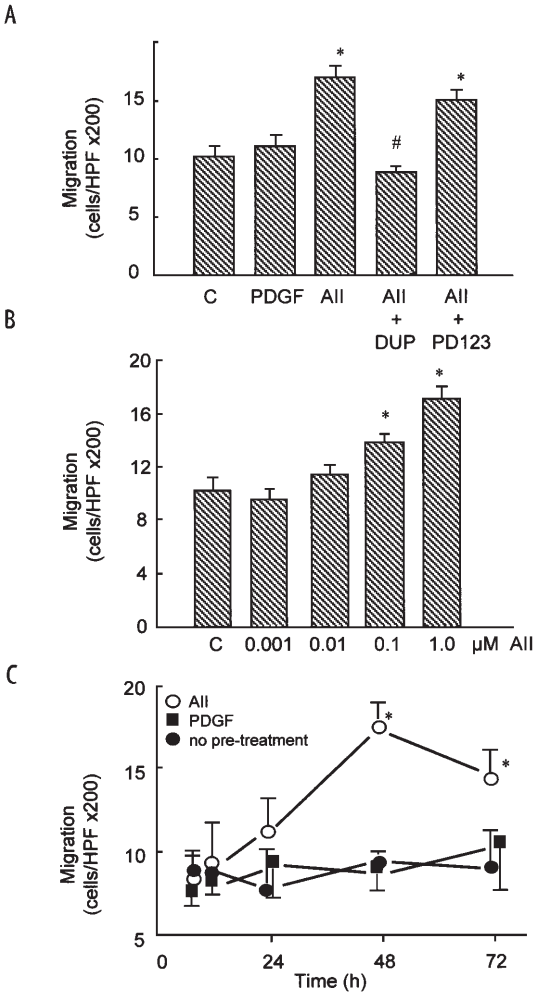

Fig. 1 **A** Pre-treatment with AII (1 μM) for 48 h induced a significant increase (*p < 0.01 vs. control, C) in PDGF-BB-directed migration in a transwell chamber system. Treatment with PDGF-BB for 48 h did not increase the rate of migration. The AT_1 receptor blocker DUP 753 (DUP, 100 μM) completely inhibited the AII-mediated effect (# p < 0.01 vs. AII alone), whereas the AT_2 receptor blocker PD 123319 (PD 123, 100 μM) had no effect. **B** The increase in PDGF-BB-directed migration after pre-treatment with AII is concentration dependent. **C** The effect of treatment with AII was investigated in a time course experiment. VSMCs were treated for various time intervals between 4 and 72 h with AII (1 μM, hollow circles), PDGF-BB (20 ng/ml, filled squares) or control diluent (black circles). A significant increase in PDGF-BB-directed migration was observed after pre-treatment with AII between 48 and 72 h, whereas treatment with PDGF-BB or diluent did not further augment the chemotactic response of VSMCs. All experiments (**A-C**) were performed in quadruplicate with three different sets of cells (*p < 0.01 vs. untreated controls, # p < 0.01 vs. AII alone, mean ± S.E.M.).

cells towards PDGF-BB after pre-treatment with AII for 48 h is dependent on the AII-concentration used (Fig. 1B). AII induced a significant increase in migration by 43 ± 10% at 100 nM (n = 8, p < 0.05) and 77 ± 12% at 1 μM (n = 9, p < 0.01). The effect of AII was also time-dependent: while no significant increase was observed after treatment with AII for 24 h, treatment for 48 or 72 h with AII (1 μM) resulted in significantly augmented migration (Fig. 1C). The AII-mediated increase in migration was inhibited by co-incubation with the ERK 1/2 MAPK pathway inhibitor PD 98059 (30 μM). The effect of AII on PDGF-BB-directed migration was also inhibited after downregulation of protein kinase C with PMA (0.1 μM) for 24 h (Fig. 2A). To confirm that the effect of PD 98059 on VSMC migration was not caused by an induction of apoptosis, migration of untreated VSMCs and cells that had been pre-treated with PD 98059 for 48 h were compared. PD 98059 pre-treatment had no effect on migration of VSMCs towards PDGF-BB (Fig. 2A). Furthermore, PD 98059 did not change intracellular α-smooth muscle actin organization, as investigated by immunofluorescence (data not shown). No significant amount of apoptosis and cell necrosis was observed by

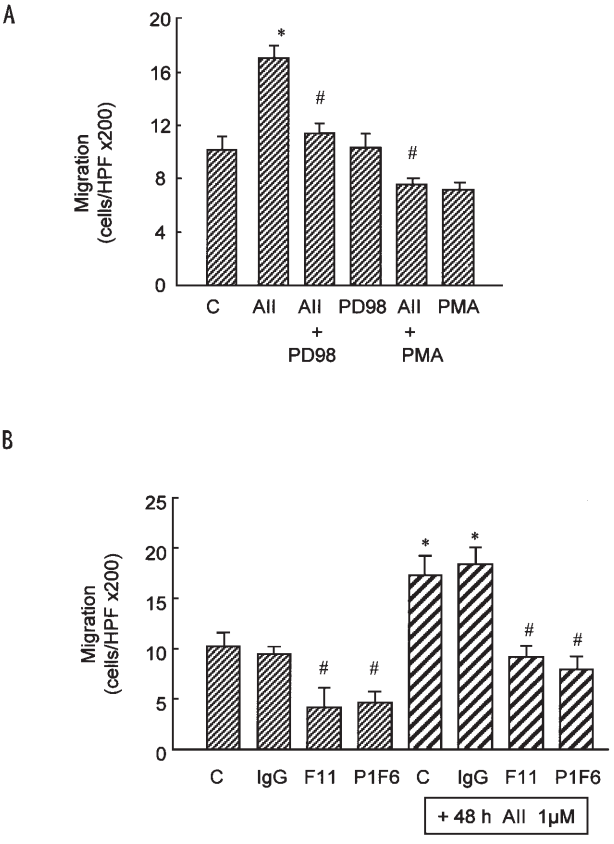

Fig. 2 **A** To investigate the role of ERK 1/2 and PKC, experiments with AII were performed after downregulation of PKC activity by PMA (0.1 μM) for 24 h or in the presence of the MEK inhibitor PD 98059 (PD98, 30 μM). Downregulation of PKC by PMA or co-incubation with PD 98059 inhibited the augmented migration-response towards PDGF-BB in cells that were pre-treated with AII (1 μM). Experiments were performed in quadruplicate with different sets of cells (*p < 0.01 vs. untreated controls, # p < 0,01 vs. AII alone, mean ± S.E.M.). **B** Migration towards PDGF-BB was significantly inhibited in the presence of blocking antibodies against anti-β_3-integrin (clone F11, 25 μg/ml) or anti-β_5-integrin (clone P1F6, 25 μg/ml). Non-immune IgGs (25 μg/ml) or diluent served as controls. The same experiment was conducted after 48 h AII (1 μM) treatment of VSMCs, revealing comparable results. Experiments were performed in quadruplicate with three different sets of cells (*p < 0.01 vs. untreated control cells, # p < 0.01 vs. AII alone, mean ± S.E.M.).

blocking the ERK 1/2 MAPK pathway. These results are in accordance with a recent publication of Das et al., demonstrating that long-term treatment with PD 98059 did not induce apoptosis (22).

AII does not change the expression of integrins involved in cell migration

The chemotactic response of VSMCs is known to be partially mediated via β_3- and β_5-integrins. Preincubation with the blocking antibody F11, which inhibits β_3-integrin, and P1F6, which blocks β_5-integrin, demonstrated similar inhibitory effects on VSMCs migration. Both antibodies demonstrated about 50 % inhibition (both $p < 0.01$) of migration in response to PDGF-BB in untreated controls and in cells pre-treated with AII for 48 h (Fig. 2B). This confirms the importance of these integrins in VSMC migration, but does not explain the mechanism how AII augmented the migration towards PDGF-BB. Therefore, the surface expression of integrin receptors was investigated by flow cytometry. No significant changes of α_1-, α_2-, β_1-, β_3- and β_5-integrin expression in VSMCs after 48 h AII treatment were detected (Fig. 3), which is in line with previous results from our laboratory (13). We also tested whether the inhibitory effect of the blocking antibodies on PDGF-BB-directed migration was changed after AII treatment. However, quantity of inhibition (in percentage) was not significantly changed in AII pre-treated cells, as compared to controls: β_3-inte-

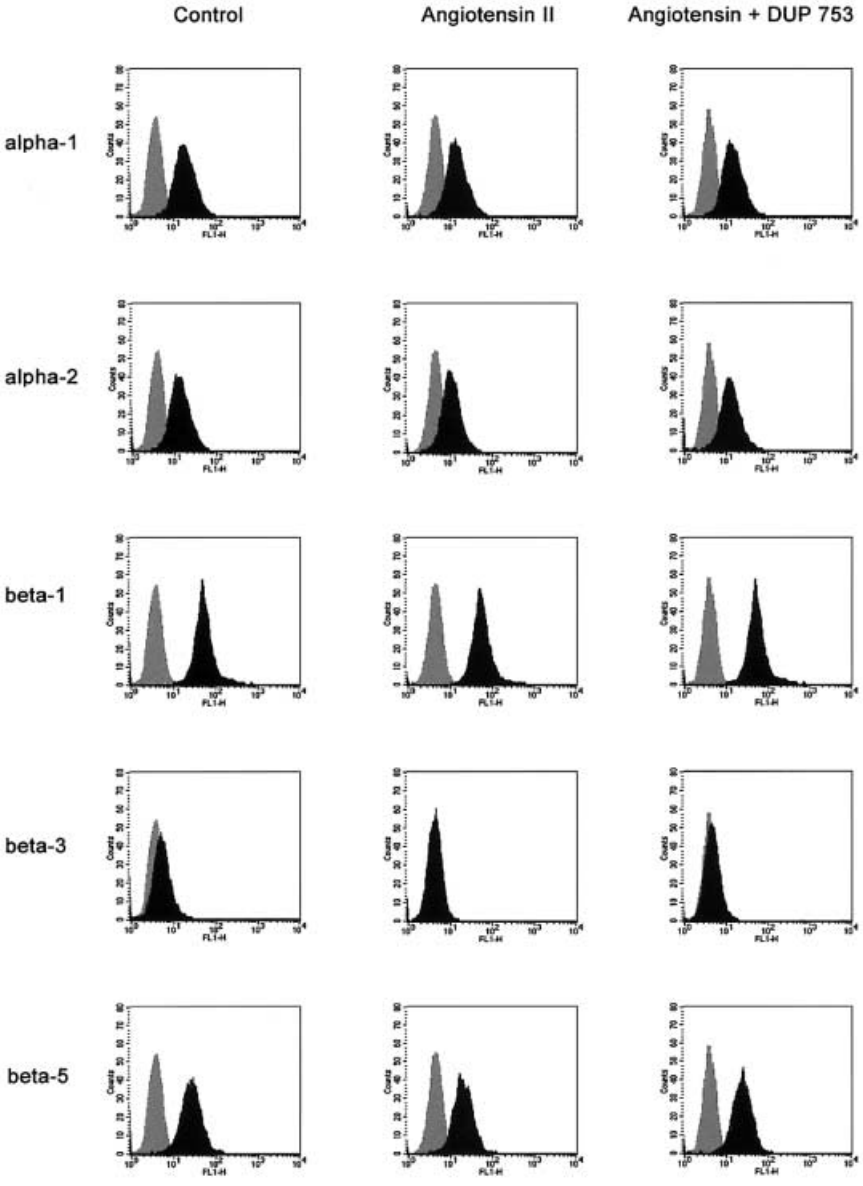

Fig. 3 Flow cytometry analysis demonstrated no effect of AII treatment (48 h, 1 μM) on the expression of integrins by VSMCs. Cells were incubated with a non-specific mouse IgG (gray area) or with specific anti-α_1-integrin (clone F19), anti-α_2-integrin (clone N19), anti-β_1-integrin (clone AB1937), anti-β_3-integrin (clone F11) or anti-β_5-integrin (clone AM1926) (at predetermined saturating concentrations). The x-axis corresponds to fluorescence intensity on a logarithmic scale and the y-axis to the cell number.

grin inhibition by F11 in controls 61.5 ± 15.2% (n = 4), in AII treated cells 53.7 ± 6.8% (n = 3); β_5-integrin inhibition by P1F6 in controls 50.8 ± 8.7% (n = 4), in AII treated cells 56.0 ± 9.1% (n = 3).

AII modulates intracellular protein kinase expression and phosphorylation

AII is known to rapidly phosphorylate FAK, Pyk2 and ERK 1/2 (20, 23, 27, 28). The proline-rich tyrosine kinase Pyk2 and FAK are cytoskeleton-associated protein kinases which are involved in integrin signaling. ERK 1/2 and FAK are important for VSMC migration (6, 8). As shown in Fig. 4, the cellular levels of ERK 1/2 were not changed after 48 h treatment with either AII (1 μM), PDGF-BB (20 ng/ml), or AII (1 μM) plus DUP 753 (100 μM) as compared to controls. AII alone induced an 82% increase of Pyk2 protein expression (control: 51.1 ± 7.3 A.U., AII: 93.4 ± 9.8 A.U. $p < 0.05$, n = 4 from different VSMC preparations) and 55% increase in FAK protein content as determined by Western blotting and image analysis (control: 40.7 ± 4.3 A.U., AII: 63.0 ± 6.4, A.U., $p < 0.05$, n = 3 from different VSMC preparations). Treatment with PDGF-BB or AII alone after pre-incubation with the AT_1 receptor blocker DUP 753 had no effect on protein levels of FAK, Pyk2 or ERK 1/2. A modest but significant increase in the phosphorylation state of ERK 1/2 by 4-fold and Pyk2 by 2-fold (n = 4, both $p < 0.05$) was detected with phospho-specific antibodies 48 h after AII treatment (Fig. 4). In contrast, phosphorylation of FAK was not significantly changed by AII pre-treatment. Also, 48 h pre-treatment with PDGF-BB or AII plus DUP 753 did not cause significant changes in FAK, Pyk2 or ERK 1/2 phosphorylation.

AII induces translocation of ERK 1/2 and Pyk2

VSMCs were stimulated with AII (1 μM), PDGF-BB (20 ng/ml) or diluent for 8 and 48 h to examine the subcellular distribution of FAK, Pyk2 and ERK 1/2 protein by immunofluorescence. Pyk2 immunoreactivity was translocated from the plasma membrane to the cytosol after 8 and 48 h AII treatment (Fig. 5), while AII led to a translocation of ERK 1/2 from the cytoplasm to perinuclear/nuclear regions. These changes in immunoreactivity of Pyk2 and ERK 1/2 were not observed in PDGF-BB-treated VSMCs (data not shown). On the other hand FAK showed a typical perimembranous localization in VSMCs, which was not significantly changed after treatment with AII or PDGF-BB (data not shown). Treatment of VSMCs with either AII or PDGF-BB did not change α-smooth muscle actin staining. Translocation of ERK 1/2 protein after long-term AII treatment was confirmed by subcellular fractionation studies and immunoblotting: nuclear translocation of ERK 1/2 was observed after 30 min AII stimulation with a further increase in nuclear

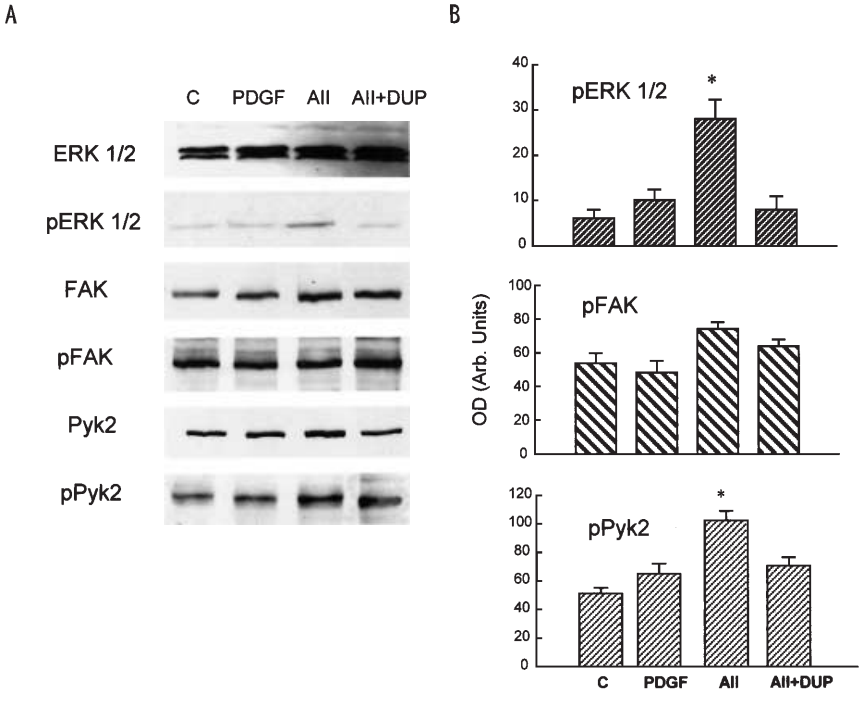

Fig. 4 **A** AII treatment for 48 h causes enhanced ERK 1/2 and Pyk2 phosphorylation. VSMCs were treated with AII (1 μM), PDGF-BB (20 ng/ml) or a combination of AII and the AT_1 receptor blocker DUP 753 (DUP, 100 μM) for 48 h. Proteins were analyzed for expression of ERK 1/2, FAK and Pyk2 and their phosphorylated forms pERK 1/2, pFAK and pPyk2 using specific antibodies. The Western blots shown are representatives of experiments that were performed with cells from four different preparations. **B** Densitometric analysis of four experiments obtained with phospho-specific antibodies against pERK 1/2, pFAK and pPyk2. Only treatment with AII (1 μM) resulted in a significant phosphorylation of ERK 1/2 and Pyk2 after 48 h (*$p < 0.05$ vs. untreated controls, mean ± S.E.M.).

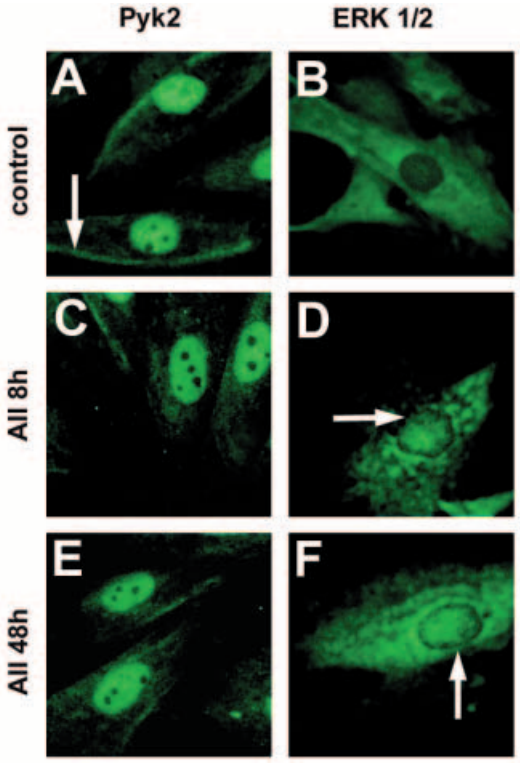

Fig. 5 Immunofluorescence staining with ERK 1/2 antibody revealed an AII-induced translocation of ERK 1/2 from the cytoplasm to perinuclear/nuclear regions. Pyk2 translocated from the plasma membrane to the cytosol. Cells were stimulated with AII (1 μM) for 8 and 48 h and localization of total Pyk2 protein (**C, E**) (anti-Pyk2 at 1:200) and total ERK 1/2 (**D, F**) (anti-ERK 1/2 at 1:200) and was compared to unstimulated controls (**A, B**).

ERK 1/2 protein after 8 h (8-fold, $p < 0.05$). Total ERK 1/2 protein levels in the whole cell extract were not changed after AII stimulation (Fig. 6).

Discussion

The present study demonstrates that pre-treatment with AII enhances the migration of VSMCs towards PDGF-BB. This effect was concentration-dependent and occurred at physiologically relevant concentrations. The AT_1 receptor blocker DUP 753 completely abolished these AII-induced effects, whereas the AT_2 receptor antagonist PD 123319 had no effect.

Incubation of VSMCs with the pharmacological MEK inhibitor PD 98059, which blocks the activation of ERK 1/2, abrogated these AII-induced effects, indicating an involvement of this pathway in these mechanisms. Interestingly, downregulation of PKC with PMA also abolished the AII-dependent increase in PDGF-BB-directed migration, confirming that the increase in migration involves PKC dependent signal transduction. AII is known to induce the synthesis and release of different cytokines and growth factors in VSMCs, including interleukin 6 (IL-6) and transforming growth factor-β (TGF-β), which could constitute a possible mechanism for AII promigratory actions. Increases of IL-6, observed after 30 min of AII stimulation with a second peak at 12 to 24 h, have been reported to be completely abolished by the MEK inhibitor PD 98059, whereas downregulation of PKC with PMA had no effect on the expression (23). Since we found that the AII-mediated increase in migration towards PDGF-BB was inhibited by PD 98059 and PMA, different or additional pathways appear to be involved in AII-induced IL-6 expression and the promigratory effect of AII. This is underscored by our observation that PDGF-BB, which is also known to induce IL-6 synthesis in different cell types (24), did not increase PDGF-BB-directed migration. We have recently shown that PDGF-BB-directed migration of human and rat VSMCs is ERK 1/2 dependent (13). Treatment with growth factors can

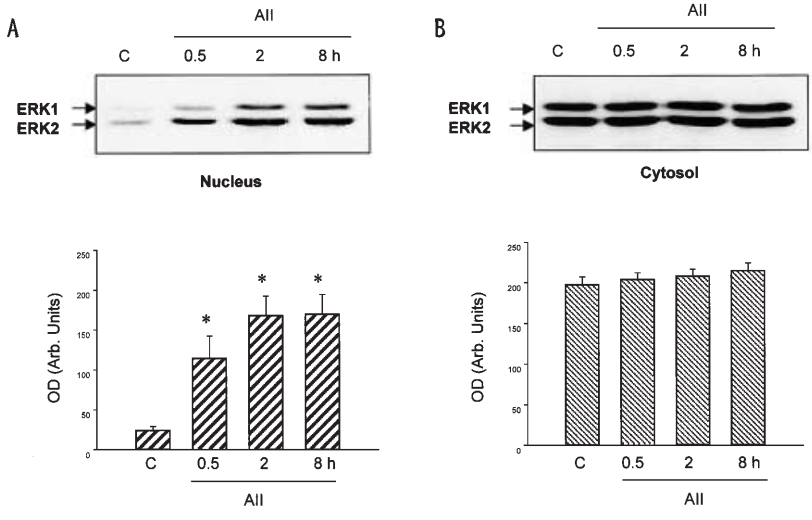

Fig. 6 AII induces nuclear translocation of ERK 1/2 without affecting total ERK 1/2 protein levels. Serum-starved VSMCs were treated with AII (1 μM) for 0.5, 2 and 8 h and nuclear (**A**) and whole cell extracts (**B**) were immunoblotted with an anti-ERK 1/2 antibody. The Western blot shows representative immunoblots from three separate experiments. Data from densitometric analysis are expressed as mean ± S.E.M., *$p < 0.05$ vs. unstimulated controls.

enhance cell motility and chemotaxis of vascular cells. Pickering and coworkers (17) demonstrated that FGF-2 potentiates migration of VSMCs via an activation and increase of β_1-integrins, as well as intracellular reorganization of stress fibers. We and others have reported that the chemotactic response of VSMCs is mediated via β_3- and β_5-integrins (25, 26). The specific blocking antibody F11, which inhibits β_3-integrins and P1F6, which blocks β_5-integrins, significantly inhibited PDGF-BB-directed migration. This effect was also observed in AII-treated cells. In the present and in previous studies (18), we did not detect changes in the expression of integrin receptors in AII-treated cells, which is in contrast to the work of Pickering et al. (17), who observed an upregulation of β_1-integrin receptors after FGF-2 pre-treatment. These different observations may be due to a FGF-2-specific effect. FGF-2 pre-treatment also caused reorganization of stress fiber composition in VSMCs which potentially contributed to increased cell migration. Similar changes have also been reported in VSMCs after AII treatment (27). AII stimulated bundling of actin filaments to form stress fibers, the assembly of focal adhesions and protein tyrosine phosphorylation at focal adhesions such as p130Cas, an adapter protein to Pyk2, and paxillin, which binds to the cytoplasmatic tail of integrins (27). Using flow cytometry we did not detect a relevant upregulation of integrin expression on the cell surface of VSMCs. Unfortunately, a potential modulation of the affinity/avidity of integrin receptors can not be assessed by this method. However, changes in the affinity/avidity of integrin receptors in AII-treated cells or alterations in post-receptor signaling pathways might still play an important role in the mechanisms of AII-augmented VSMC migration.

Therefore, we investigated the involvement of protein kinases which participate in the signaling of integrins and AII. Treatment with AII for 48 h resulted in a significant increase in Pyk2 and FAK protein concentration. We also observed a significant increase in Pyk2 phosphorylation after 48 h of AII treatment, whereas FAK phosphorylation was not affected. In previous studies ERK 1/2 has been demonstrated to be a downstream target of FAK and Pyk2 (28, 29). While ERK 1/2 protein concentrations were not affected, its phosphorylation was significantly increased after 48 h of AII treatment. Co-treatment with the AT_1 receptor blocker DUP 753 completely abolished the AII-induced effects. Treatment with PDGF-BB for 48 h did not significantly increase Pyk2 and FAK concentrations, nor did it result in increased ERK 1/2 or Pyk2 phosphorylation. Our data demonstrate that the AII-induced migration is associated with an increase in FAK and Pyk2 protein concentration and a prolonged activation of ERK 1/2 and Pyk2.

AII can rapidly stimulate tyrosine phosphorylation of FAK, Pyk2 and the activation of ERK 1/2 in cultured VSMCs (11, 12, 30). The non-receptor tyrosine kinase Pyk2 appears to function at a point of convergence of integrins and certain G-protein coupled receptor signaling cascades. It associates with focal adhesion proteins such as paxillin and p130 cas, which are a linkage to the PI3-kinase pathway and Akt (29). Recent studies demonstrated that integrin activation results in focal adhesion targeting of Pyk2 in an integrin-clustering-dependent manner (31). Activation of FAK and ERK 1/2 are required for migration of VSMCs (13, 32). It has been suggested that Pyk2 is involved in VSMC migration (33). Furthermore, Pyk2 is essential for AII signaling and AII-mediated protein synthesis. Pyk2 antisense oligodeoxynucleotides (ODNs) did significantly reduce ERK 1/2 activation and protein synthesis induced by AII (28), indicating an important role of Pyk2 in AII-mediated ERK 1/2-dependent signal transduction. Our data indicate that the increase in total Pyk2 after 48 h AII stimulation is accompanied by an increased phosphorylation state of Pyk2. The phosphorylation of Pyk2 is PKC dependent (28) which might explain the observed inhibition of AII-augmented migration after PKC downregulation by PMA in the present study. Subcellular analysis of ERK 1/2 and Pyk2 by immunofluorescence after long-term AII stimulation demonstrated a translocation of ERK 1/2 to the perinuclear/nuclear region and a change in Pyk2 distribution from the plasmamembrane to cytosolic compartments. Due to the semiquantitative aspect of immunofluorescence, Western blot experiments with nuclear fractions were performed to strengthen the translocation data. This translocation might contribute to the described increase in migration. The data suggest that AII facilitates and increases cellular responsiveness via translocation of intracellular ERK 1/2 and Pyk2.

In conclusion, the present data underscore the importance of ERK 1/2 and Pyk2 in regulating AT_1 receptor-dependent intracellular mechanisms in response to AII treatment. Our results suggest that AII modulates integrin-dependent functions in VSMCs by altering expression, phosphorylation and the subcellular localization of the protein kinases Pyk2 and ERK 1/2.

Acknowledgment This study was supported by a grant from the Deutsche Forschungsgemeinschaft to K.G. (GR 1368/2-2). The authors gratefully acknowledge the technical assistance of Chantel Spencer.

▶

References

1. Daemen MJ, Lombardi DM, Bosman FT, Schwartz SM (1991) Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. Circ Res 68: 450–6
2. Dubey RK, Jackson EK, Luscher TF (1995) Nitric oxide inhibits angiotensin II-induced migration of rat aortic smooth muscle cell. Role of cyclic-nucleotides and angiotensin1 receptors. J Clin Invest 96: 141–9
3. Mukoyama M, Nakajima M, Horiuchi M, Sasamura H, Pratt RE, Dzau VJ (1993) Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. J Biol Chem 268: 24539–42
4. Sasaki K, Yamano Y, Bardhan S, Iwai N, Murray JJ, Hasegawa M, Matsuda Y, Inagami T (1991) Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. Nature 351: 230–3
5. Dietz R, von Harsdorf R, Gross M, Kramer J, Gulba D, Willenbrock R, Osterziel KJ (1998) Angiotensin II and coronary artery disease, congestive heart failure, and sudden cardiac death. Basic Res Cardiol 93 (Suppl 2): 101–8
6. Haendeler J, Berk BC (2000) Angiotensin II mediated signal transduction. Important role of tyrosine kinases. Regul Pept 95: 1–7
7. Marrero MB, Schieffer B, Paxton WG, Duff JL, Berk BC, Bernstein KE (1995) The role of tyrosine phosphorylation in angiotensin II-mediated intracellular signalling. Cardiovasc Res 30: 530–6
8. Eguchi S et al. (1999) Involvement of PYK2 in angiotensin II signaling of vascular smooth muscle cells. Hypertension 33: 201–6
9. Leduc I, Meloche S (1995) Angiotensin II stimulates tyrosine phosphorylation of the focal adhesion-associated protein paxillin in aortic smooth muscle cells. J Biol Chem 270: 4401–4
10. Marrero MB, Schieffer B, Paxton WG, Heerdt L, Berk BC, Delafontaine P, Bernstein KE (1995) Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. Nature 375: 247–50
11. Polte TR, Naftilan AJ, Hanks SK (1994) Focal adhesion kinase is abundant in developing blood vessels and elevation of its phosphotyrosine content in vascular smooth muscle cells is a rapid response to angiotensin II. J Cell Biochem 55: 106–19
12. Sabri A, Govindarajan G, Griffin TM, Byron KL, Samarel AM, Lucchesi PA (1998) Calcium- and protein kinase C-dependent activation of the tyrosine kinase PYK2 by angiotensin II in vascular smooth muscle. Circ Res 83: 841–51
13. Graf K, Xi XP, Yang D, Fleck E, Hsueh WA, Law RE (1997) Mitogen-activated protein kinase activation is involved in platelet-derived growth factor-directed migration by vascular smooth muscle cells. Hypertension 29: 334–9
14. Xi XP, Graf K, Goetze S, Hsueh WA, Law RE (1997) Inhibition of MAP kinase blocks insulin-mediated DNA synthesis and transcriptional activation of c-fos by Elk-1 in vascular smooth muscle cells. FEBS Lett 417: 283–6
15. Clark EA, Brugge JS (1995) Integrins and signal transduction pathways: the road taken. Science 268: 233–9
16. Hynes RO (1999) Cell adhesion: old and new questions. Trends Cell Biol 9: M33–7
17. Pickering JG et al. (1997) Fibroblast growth factor-2 potentiates vascular smooth muscle cell migration to platelet-derived growth factor: upregulation of alpha2beta1 integrin and disassembly of actin filaments. Circ Res 80: 627–37
18. Kappert K, Schmidt G, Doerr G, Wollert-Wulf B, Fleck E, Graf K (2000) Angiotensin II and PDGF-BB stimulate beta(1)-integrin-mediated adhesion and spreading in human VSMCs. Hypertension 35: 255–61
19. Chamley-Campbell J, Campbell GR, Ross R (1979) The smooth muscle cell in culture. Physiol Rev 59: 1–61
20. Graf K, Schaper C, Grafe M, Fleck E, Kunkel G (1998) Glucocorticoids and protein kinase C regulate neutral endopeptidase 24.11 in human vascular smooth muscle cells. Basic Res Cardiol 93: 11–7
21. Dignam JD, Lebovitz RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11: 1475–89
22. Das M, Bouchey DM, Moore MJ, Hopkins DC, Nemenoff RA, Stenmark KR (2001) Hypoxia-induced proliferative response of vascular adventitial fibroblasts is dependent on g protein-mediated activation of mitogen-activated protein kinases. J Biol Chem 276: 15631–40
23. Funakoshi Y, Ichiki T, Ito K, Takeshita A (1999) Induction of interleukin-6 expression by angiotensin II in rat vascular smooth muscle cells. Hypertension 34: 118–25
24. Franchimont N, Durant D, Rydziel S, Canalis E (1999) Platelet-derived growth factor induces interleukin-6 transcription in osteoblasts through the activator protein-1 complex and activating transcription factor-2. J Biol Chem 274: 6783–9
25. Kappert K et al. (2001) Integrins alphavbeta3 and alphavbeta5 mediate VSMC migration and are elevated during neointima formation in the rat aorta. Basic Res Cardiol 96: 42–9
26. Slepian MJ, Massia SP, Dehdashti B, Fritz A, Whitesell L (1998) Beta3-integrins rather than beta1-integrins dominate integrin-matrix interactions involved in postinjury smooth muscle cell migration. Circulation 97: 1818–27
27. Ishida T, Ishida M, Suero J, Takahashi M, Berk BC (1999) Agonist-stimulated cytoskeletal reorganization and signal transduction at focal adhesions in vascular smooth muscle cells require c-Src. J Clin Invest 103: 789–97
28. Rocic P, Lucchesi PA (2001) Downregulation by antisense oligonucleotides establishes a role for the proline-rich tyrosine kinase PYK2 in angiotensin II-induced signaling in vascular smooth muscle. J Biol Chem 21: 21
29. Rocic P, Govindarajan G, Sabri A, Lucchesi PA (2001) A role for PYK2 in regulation of ERK1/2 MAP kinases and PI 3-kinase by ANG II in vascular smooth muscle. Am J Physiol Cell Physiol 280: C90–9
30. Xi XP, Graf K, Goetze S, Fleck E, Hsueh WA, Law RE (1999) Central role of the MAPK pathway in ang II-mediated DNA synthesis and migration in rat vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 19: 73–82
31. Litvak V, Tian D, Shaul YD, Lev S (2000) Targeting of PYK2 to focal adhesions as a cellular mechanism for convergence between integrins and G protein-coupled receptor signaling cascades. J Biol Chem 275: 32736–46
32. Hauck CR, Klingbeil CK, Schlaepfer DD (2000) Focal adhesion kinase functions as a receptor-proximal signaling component required for directed cell migration. Immunol Res 21: 293–303
33. Sieg DJ, Ilic D, Jones KC, Damsky CH, Hunter T, Schlaepfer DD (1998) Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK-cell migration. Embo J 17: 5933–47

■