Proteasome-dependent decrease in Akt by growth factors in vascular smooth muscle cells

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Abstract Akt is activated by growth factors to regulate various aspects of vascular smooth muscle cell function. Platelet-derived growth factor (PDGF) and insulin-like growth factor-1 activated Akt in vascular smooth muscle cells with a rapid reduction of total Akt protein that lasted for several hours. The downregulation of Akt required phosphatidylinositol 3-kinase activity, but not intrinsic Akt activity. The downregulation of Akt was abrogated by MG-132, a proteasome inhibitor, but not by inhibitors of calpain or cathepsins. Akt was found in ubiquitin immune complex after PDGF treatment. Proteasome-dependent degradation of Akt may provide a counter-regulatory mechanism against overactivation of Akt.

Key words: Akt; Vascular smooth muscle cell; Proteasome; Ubiquitin; Platelet-derived growth factor; Insulin-like growth factor-1; Phosphatidylinositol 3-kinase

1. Introduction

Vascular smooth muscle cells (VSMCs) are a principal cell type of blood vessels that are essential not only for physiological function of vasculature but are also involved in the pathophysiology of atherosclerosis and hypertension. In the last decade, extensive efforts have been made to elucidate the molecular mechanisms underlying pathophysiological responses in VSMCs to identify key signaling pathways including the mitogen-activated protein kinase (see [1] for review), NF-κB [2] and phosphatidylinositol 3-kinase (PI3-kinase)/Akt (also known as protein kinase B) pathways [3–7]. Among these, the PI3-kinase/Akt pathway participates in a wide range of cellular functions such as amino acid metabolism [3], cell survival [4], migration [5,6], proliferation [5,6] and differentiation [8] of VSMCs and other cell types (see [9,10] for recent review). Akt is known to be activated in VSMCs by various growth factors such as platelet-derived growth factor (PDGF) [11,12] and insulin-like growth factor-1 (IGF-1) [13] that are implicated in VSMC migration and proliferation in atherosclerosis (see [14] for review). While much is known of the activation of Akt in response to growth factor stimulation, the mechanisms to deactivate Akt are little understood. This prompted us to characterize the mechanism of Akt deactivation following PDGF stimulation in VSMCs. Unexpectedly, PDGF caused a rapid decrease in Akt protein levels concomitant with Akt activation. We provide evidence that PDGF causes regulated proteolytic downregulation of Akt that is dependent on PI3-kinase and proteasome activities. Proteasome-dependent downregulation of Akt may be a fundamental mechanism to regulate the activity and function of Akt in VSMCs.

2. Materials and methods

2.1. Cell culture

VSMCs were obtained from the aorta of 8-week-old male Wistar rats by enzyme dispersion as described previously [15]. Briefly, aortae were dissected under sterile conditions. After removing adventitia and endothelium, the aortic medial layer was minced with scissors and dispersed by incubating with 1 mg/ml collagenase (Sigma) and 10 U/ml elastase (Sigma) for 100 min at 37°C. Dispersed cells were plated and maintained in Dulbecco's modified Eagle medium (Invitrogen) containing 10% fetal bovine serum on culture plates coated with 20 μg/ml laminin. All experiments were performed using cells between passages 2 and 5. Confluent VSMCs were serum-starved for 24 h before experiments. The animal experimental protocol has been approved by the Yamaguchi University School of Medicine Animal Review Board. COS7, HEK293 and NIH3T3 cells were maintained in Dulbecco's modified Eagle medium containing 10% newborn calf serum (Invitrogen).

2.2. Cell culture experiments

For transient transfection, COS7 cells were transfected with pUSEamp-Myc-Akt(KM) expression plasmid encoding kinase-inactive Akt with a c-Myc epitope tag (Upstate Biotechnology) using FuGene6 (Roche). One plate of transfected cells was split into four plates and serum-starved for 24 h, followed by stimulation with (two plates) or without (two plates) 20 ng/ml IGF-1 (Sigma) for 10 min. Exogenous Akt(KM) was detected by Western blotting for the Myc epitope tag. For inhibition of PI3-kinase, VSMCs were treated with various concentrations of LY294002 (Sigma) 30 min before and during stimulation with 10 ng/ml PDGF (Invitrogen). For inhibition of protein synthesis, VSMCs were treated with 20 μM cycloheximide (Wako) for the indicated periods. For the inhibition of proteases, VSMCs were treated with various concentrations of MG-132 (Biomol), ALLN (Calbiochem), CA-074Me (Biomol), E-64-d (Peptide Institute) or vehicle (0.1% dimethyl sulfoxide) for 2 h before and during stimulation

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF-1, insulin-like growth factor-1; IRS, insulin receptor substrate; PDGF, platelet-derived growth factor; PI3-kinase, phosphatidylinositol 3-kinase; VSMC, vascular smooth muscle cell

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with PDGF. After stimulation with 10 ng/ml PDGF or 20 ng/ml IGF-1 for the indicated periods, cells were harvested and subjected to further analysis as described below.

2.3. Immunoprecipitation and Western blotting

For immunoprecipitation of ubiquitinated proteins, cells were harvested in lysis buffer containing 20 mM Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 100 μM leupeptin and 1 mM dithiothreitol. After clarifying the lysate by centrifugation (18 000×*g*, 15 min at 4°C), 500 μl of cell lysates containing equal amounts of proteins were subjected to immunoprecipitation by incubating with 5 μg each of two anti-ubiquitin monoclonal antibodies (clones #1B3 and #2C5, Medical and Biological Laboratories) overnight at 4°C, followed by incubation with protein G-Sepharose (Sigma) for 3 h at 4°C. After washing with lysis buffer, immunoprecipitated proteins were resolved on 10% or 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to polyvinylidene difluoride (PVDF) membrane, and subjected to Western blot analyses. For direct Western blotting analyses, cells were harvested in boiling SDS sample buffer containing 2% SDS, 50 mM Tris (pH 6.8), 10% glycerol and 5% 2-mercaptoethanol to obtain total cell lysates. Proteins were resolved on 9% SDS–PAGE and transferred to PVDF membranes. Total Akt was detected by anti-Akt polyclonal (Cell Signaling Technology) or monoclonal (Transduction Laboratories) antibodies. Active Akt was detected by anti-phospho-Akt (Ser473) or phospho-Akt (Thr308) polyclonal antibodies (Cell Signaling Technology). Ubiquitin was detected by anti-ubiquitin monoclonal antibodies (clones #1B3 and #2C5). Western blotting of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with a monoclonal antibody (Chemicon) served as a control for protein loading. Probed proteins were visualized by chemiluminescence (Super Signal, Pierce) and luminoimaging (LAS-1000, Fuji Film).

2.4. Statistical analysis

All data are expressed as means ± S.E.M. Statistical analyses were performed using analysis of variance. The post-test comparison was performed by the method of Bonferroni. P values of <0.05 were accepted to be significant.

3. Results and discussion

3.1. PDGF causes activation of Akt and a decrease in Akt protein

We characterized the time course of Akt activation by PDGF in VSMCs. Active Akt was detected by a phosphospecific antibody that recognizes phosphorylation of Akt at Ser473. As shown in Fig. 1A,B, Akt was rapidly activated (phosphorylated) by PDGF within 1 min, peaked at 10 min and remained active for at least 120 min (91% of the peak activation at 120 min). Western blotting with a phosphospecific antibody for Thr308 phosphorylation of Akt gave identical results (data not shown). Interestingly, the amount of total Akt, which was detected using polyclonal antibodies for C-terminus of Akt, rapidly decreased upon its activation. The amount of total Akt reached its lowest level, 23% of the initial level, at 30 min and then gradually recovered to 48% of the initial level at 120 min. Equal loading of proteins was confirmed by Western blotting for GAPDH (Fig. 1A). We also used a monoclonal antibody raised against the N-terminal half (Fig. 1C) or PH domain (data not shown) of Akt to confirm this result. As shown in Fig. 1C, both the C-terminus- and N-terminus-specific Akt antibodies detected the decrease in Akt protein upon stimulation with PDGF. Translocation of Akt in the cell is unlikely to account for these findings because the lysis conditions should disrupt the cytoarchitecture and solubilize the proteins. Therefore, we concluded that total

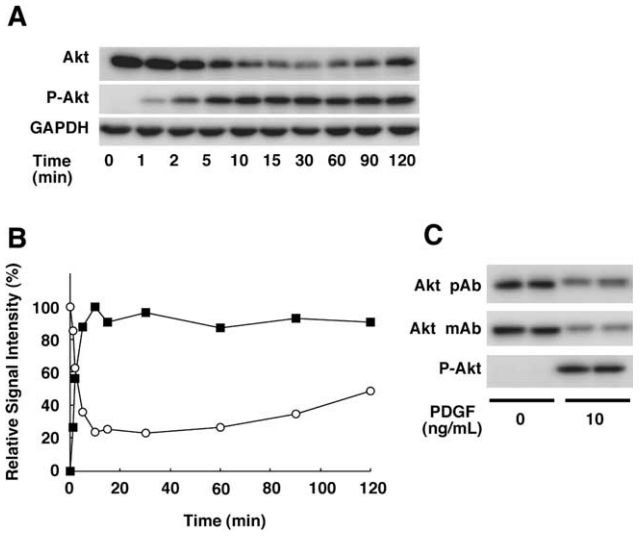

Fig. 1. PDGF activates Akt and decreases Akt protein levels. A: VSMCs were stimulated with 10 ng/ml PDGF for the indicated periods. Phosphorylated (active) Akt (P-Akt) and total Akt were detected by Western blotting. Western blotting for GAPDH is shown as a control for protein loading. B: Densitometric analysis of total Akt (open circles) and active Akt (closed squares) is shown. The relative signal intensities are shown where the peak signal is designated 100% for each Western analysis. Representative results of at least three independent observations are shown. C: Western analyses were performed after PDGF (10 ng/ml) stimulation for 10 min using polyclonal (pAb) and monoclonal (mAb) antibodies for total Akt, and phosphorylated (active) Akt (P-Akt) antibody. Representative results from at least four independent observations are shown.

Akt detected by Western blotting reflects the actual amount of Akt protein in the cell, which decreased following its activation by PDGF stimulation.

3.2. Signaling for the activation of and decrease in Akt

We next asked whether the decrease in Akt protein levels is specific for PDGF stimulation. IGF-1 is a well-characterized activator of the Akt pathway. As shown in Fig. 2A, IGF-1 activated Akt in VSMCs at 10 min, which was accompanied by the decrease in total Akt protein levels. Therefore, activation of Akt by both PDGF and IGF-1 is accompanied by a decrease in total Akt protein in VSMCs. Both PDGF and IGF-1 activate PI3-kinase, as well as other signaling pathways. We examined whether activation of PI3-kinase is necessary for the decrease in Akt protein. As shown in Fig. 2B,C, treatment of VSMCs with LY294002, a specific inhibitor of PI3-kinase, diminished both Akt activation and the decrease in Akt protein in a dose-dependent manner. Interestingly, 20 μM LY294002 completely abolished the decrease in Akt protein by PDGF, but only partially suppressed Akt activation (45 ± 3% of the activation without LY294002, $P<0.0001$ compared with PDGF alone, $n=4$). Akt protein decreased to 39 ± 4% of the initial level by PDGF stimulation without LY294002 ($P<0.0001$ compared with control, $n=4$) whereas it remained at control levels in the presence of 20 μM LY294002 (101 ± 22%; $P=0.95$ compared with control, $n=4$). Therefore, although both activation of Akt and the decrease in Akt protein depend on PI3-kinase activity, the latter is more sensitive to PI3-kinase inhibition. These results suggest that activation of Akt per se is not sufficient for the decrease in Akt protein and another PI3-kinase-depen-

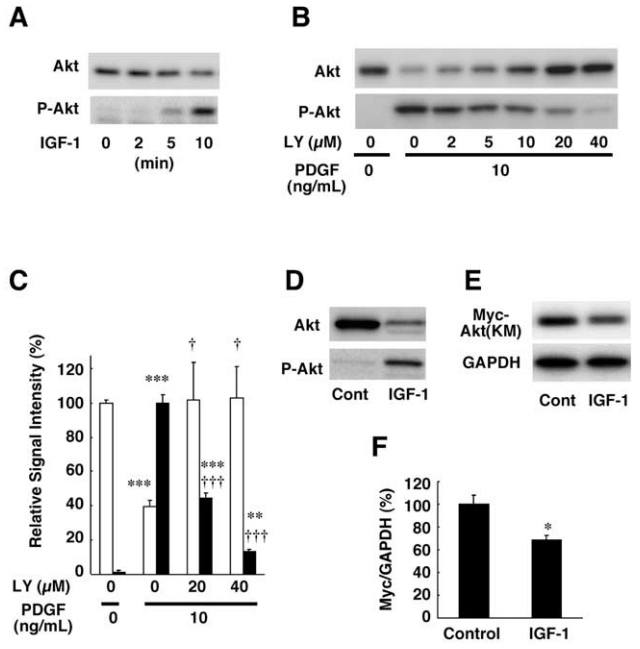

Fig. 2. PDGF-induced decrease in Akt requires PI3-kinase activity but not intrinsic Akt kinase activity. A: Total Akt and phosphorylated Akt (P-Akt) were probed with corresponding polyclonal antibodies after 20 ng/ml IGF-1 stimulation for the indicated periods. B: Total Akt and phosphorylated Akt (P-Akt) were detected by Western blotting before and after 10 ng/ml PDGF stimulation for 10 min in the presence of various concentrations of LY294002 (LY). C: Quantitative analyses of experiment B are shown. Total Akt levels are indicated by open columns where basal expression levels are designated 100%. Phosphorylated Akt is indicated by closed columns where signal intensity after PDGF stimulation without LY294002 is designated 100%. Data are means ± S.E.M. obtained from four independent observations. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control (without PDGF or LY294002), respectively. $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ compared with PDGF alone. D: COS7 cells were stimulated with 20 ng/ml IGF-1 for 10 min. Akt and phosphorylated Akt (P-Akt) were detected by Western blotting. E: COS7 cells were transiently transfected with an expression plasmid encoding kinase-inactive Akt with a c-Myc epitope tag (Myc-Akt(KM)). Experiments were performed as described in Section 2. Myc-Akt(KM) was detected by Myc Western blotting. GAPDH Western blotting served as a control for protein loading. F: Quantitative analysis of E is shown. Myc-Akt(KM) levels are indicated as relative to the basal expression level which was designated 100%. Data are means ± S.E.M. from three independent observations. $^{*}P < 0.05$ compared with control.

dent mechanism may participate in the decrease in Akt protein.

The activation-dependent decrease in Akt protein was not limited to VSMCs, because it was also observed in other cell types including COS7 (Fig. 2D), HEK293 and NIH3T3 cells (data not shown), suggesting that there may be universal molecular mechanisms that regulate total Akt protein levels following its activation regardless of cell type. We examined whether intrinsic kinase activity of Akt is necessary for its downregulation by utilizing a catalytically inactive Akt mutant with a Myc epitope tag (Akt(KM)). Akt(KM) was expressed in COS7 cells by transient transfection and protein levels were detected by Western blotting for the Myc epitope tag. As shown in Fig. 2E,F, exogenously expressed Akt(KM) protein also underwent downregulation in response to IGF-1 treatment. Therefore, intrinsic kinase activity of Akt is dispensable for its downregulation.

3.3. Proteasome-dependent downregulation of Akt

The rapid decrease in the amount of Akt suggested that Akt may be proteolytically degraded upon PDGF or IGF-1 stimulation rather than transcriptionally or translationally suppressed. To test this, we screened an array of protease inhibitors for an effect on PDGF-induced decrease in Akt. The protease inhibitors included MG-132 (proteasome), ALLN (calpain), CA-074Me (cathepsin B) and E-64-d (cathepsin B/H/L and calpain). Treatment of VSMCs with each of these protease inhibitors did not change basal levels of Akt protein nor Akt activation by PDGF. Among the inhibitors, MG-132, a proteasome inhibitor, caused a significant increase in Akt after PDGF stimulation (Fig. 3A). As shown in Fig. 3B, Akt levels after PDGF stimulation were 26 ± 3% ($n = 4$) of the basal level without MG-132 treatment, 36 ± 3% ($n = 4$) with 10 μM MG-132 and 56 ± 1% ($P < 0.001$ compared with control, $n = 4$) with 100 μM MG-132. Stability of Akt protein at the basal condition was assessed by treating VSMCs with cycloheximide to inhibit de novo protein synthesis. As shown in Fig. 3C, treatment of VSMCs with cycloheximide for 2 h minimally affected the basal level of Akt, indicating that Akt is relatively stable during this observation period. Treatment of VSMCs with cycloheximide and MG-132 also had little effect on Akt protein level. These results indicate that proteasome activity is responsible, at least in part, for the PDGF-induced decrease in Akt protein. In addition, a proteasome-dependent mechanism seems to be activated by PDGF to downregulate Akt, because MG-132 had little effect on the level of Akt protein in unstimulated cells in the absence or presence of cycloheximide.

We next examined whether PDGF promotes protein ubiquitination in VSMCs. Ubiquitinated proteins were immunoprecipitated by anti-ubiquitin monoclonal antibodies followed by Western blotting for ubiquitin or Akt. As shown in Fig. 3D, very few ubiquitinated proteins were detected in VSMCs with or without PDGF stimulation (lanes 1 and 2). However, several bands were detected in VSMCs treated with 100 μM MG-132, a proteasome inhibitor (lane 3, arrowheads). Interestingly, PDGF stimulation caused an increase in the intensity of multiple bands (lane 4, arrowheads), indicating that PDGF stimulates ubiquitination of multiple proteins in VSMCs. Akt was barely detectable in the ubiquitin immune complex in the absence of MG-132 (Fig. 3E, lanes 1 and 2). However, in the presence of MG-132, the ubiquitin immune complex contained Akt which increased in response to PDGF stimulation (Fig. 3E, lanes 3 and 4). Akt was not detected when immunoprecipitation was performed without primary antibody or with irrelevant anti-FLAG antibody (data not shown), confirming the specificity of the signal. However, anti-ubiquitin Western blot following Akt immunoprecipitation failed to demonstrate direct ubiquitination of Akt (data not shown). Therefore, Akt may make a multimolecular complex with unknown molecule(s) that is ubiquitinated in response to PDGF stimulation.

Our observations indicate that PDGF and IGF-1 cause activation of Akt concomitant with a decrease in Akt protein in VSMCs and other cell types. Expression levels of a number of proteins are regulated by proteolytic degradation in a PI3-kinase/Akt-dependent manner. These include insulin receptor substrate (IRS) 1 [16–18], IRS-2 [19], p27Kip1 [20], IκB [21], p53 [22], androgen receptor [23], FOXO3a and tuberin [24]. Our data indicate that Akt is another target of the regulated

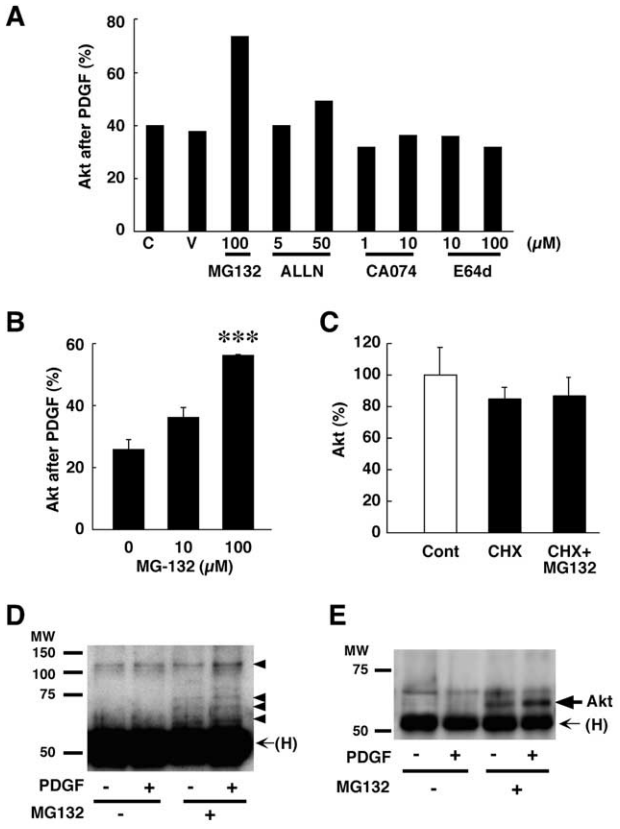

Fig. 3. Proteasome-dependent decrease in Akt protein. A: Western blotting for total Akt was performed before and after PDGF stimulation in the presence or absence (C, control) of dimethyl sulfoxide (V, vehicle), MG-132 (proteasome inhibitor), ALLN (calpain inhibitor), CA-074Me (cathepsin B inhibitor) and E-64-d (cathepsin B/H/L and calpain inhibitor). Densitometric analysis of the experiment is shown. Signal intensities of total Akt after PDGF stimulation are indicated as relative to those before PDGF stimulation, which is designated 100%. B: Quantitative analyses are shown for Akt level after PDGF stimulation in the presence of various concentrations of MG-132. Means ± S.E.M. from four independent observations are shown. ***$P < 0.001$ compared with control. C: Akt protein stability was assessed by treating VSMCs with 20 μM cycloheximide (CHX) with or without 100 μM MG-132 for 2 h. The Akt protein amount is shown relative to the control level without cycloheximide or MG-132 (Cont, open column), which is designated 100%. Data are means ± S.E.M. of four observations. D,E: Immunoprecipitation was performed with anti-ubiquitin antibodies in the presence or absence of PDGF and MG-132. Ubiquitinated proteins (D, arrowheads) or Akt (E, arrow) were detected with anti-ubiquitin or anti-Akt antibodies, respectively. (H) indicates IgG heavy chain. Another independent observation gave an identical result.

degradation in response to growth factor stimulation. In fact, Akt has been reported to be degraded in a proteasome-dependent manner when HSP90 was inhibited [25]. However, our results suggest that Akt may not be directly polyubiquitinated but may be bound to an unknown molecule that is ubiquitinated in response to PDGF stimulation. Since PDGF promoted ubiquitination of a number of molecules, Akt may be bound to one of these ubiquitinated molecules which may bring Akt to the proteasome degradation machinery.

Akt has been reported to participate in the control of cell survival, growth and differentiation of VSMCs [4–6,8]. Agonist-induced proteasome-dependent downregulation of Akt protein may provide an important counter-regulatory mechanism by which overactivation of Akt is blunted in growth factor-rich conditions such as vascular injury and atherosclerosis [11,13]. Further characterization of the regulated degradation of Akt will reveal the molecular mechanism of its proteasome-dependent downregulation and provide a fundamental understanding of this potential counter-regulatory mechanism induced by growth factors.

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