# PDGF-induced Akt phosphorylation does not activate NF-κB in human vascular smooth muscle cells and fibroblasts

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Received 3 August 2000; accepted 8 August 2000

Edited by Marco Baggiolini

**Abstract A recent report suggested that platelet-derived growth factor (PDGF) activates nuclear factor-κB (NF-κB) by phosphorylation of the protein kinase Akt [Romashkova and Makarov, Nature 401 (1999) 86–90]. The present study investigates the role of Akt in the activation of NF-κB by tumor necrosis factor-α (TNFα, 10 ng/ml) and PDGF-BB (20 ng/ml) in human vascular smooth muscle cells (SMC), skin and foreskin fibroblasts. TNFα stimulated serine phosphorylation and degradation of the inhibitory protein IκBα and strongly induced nuclear NF-κB translocation and binding activity. PDGF did not induce serine phosphorylation or degradation of IκBα and did not enhance binding activity of NF-κB. In contrast, stimulation with PDGF resulted in a marked phosphorylation of Akt, but no Akt phosphorylation occurred after stimulation with TNFα. These data suggest that Akt phosphorylation is not involved in NF-κB activation in human SMC and fibroblasts.** 

*Key words:* Nuclear factor-κB; Akt phosphorylation; Cytokine; Growth factor; Vascular smooth muscle cell

## 1. Introduction

Nuclear factor-κB (NF-κB) is an important modulator of gene expression in immunity, inflammation [1], apoptosis [2], and atherosclerosis [3,4]. Accordingly, NF-κB is activated by multiple stimuli, including ultraviolet radiation, T-cell activation, viruses, bacterial lipopolysaccharide, oxidants and cytokines, such as tumor necrosis factor-α (TNFα) [1,5,6]. The effects of growth factors, such as platelet-derived growth factor (PDGF), on NF-κB activation are still not fully characterized. It has been reported that PDGF induces NF-κB activation in embryonic kidney and carcinoma cells [7], in fibroblasts [8,9], and in Jurkat T-cells [10]. The NF-κB signaling pathway in vascular smooth muscle cells (SMC) has been studied by Bourcier et al. [4]. In their investigation, stimulation of NF-κB binding by PDGF or serum was much weaker than stimulation by cytokines, such as interleukin-1β or TNFα, while Bond et al. [11] found no NF-κB activation at all by PDGF in SMC. In another recent study, stimulation of fibroblasts with PDGF did not cause NF-κB activation, while TNFα did [12]. The classical way of stimulating NF-κB is the activation of IκB kinase (IKK) [13], subsequent phosphorylation [5,14], ubiquitination and degradation of IκBα [13]. NF-κB then translocates to the nucleus and activates gene transcription.

In two recent studies, a new pathway for the activation of NF-κB by PDGF and TNFα has been described. Romashkova and Makarov [9] reported that PDGF stimulates the protein kinase Akt by activation of Ras and phosphatidylinositol 3-kinase (PI(3)K). Akt consecutively activates the IKK. This results in IκB phosphorylation and activation of NF-κB. Ozes et al. [7] have shown that TNF activates PI(3)K and its downstream target Akt and this is followed by induction of NF-κB binding activity.

In the present study, we investigated the involvement of Akt in TNFα- and PDGF-BB-induced NF-κB activation in three human cell types: vascular SMC, skin fibroblasts and foreskin fibroblasts. We found no activation of NF-κB by PDGF-BB, but a strong activation by TNFα. In contrast, a marked phosphorylation of Akt occurred after stimulation with PDGF-BB, but not after stimulation with TNFα. Thus, evidence is presented that Akt phosphorylation is not involved in the activation of NF-κB in cultured human vascular SMC and fibroblasts.

## 2. Materials and methods

### 2.1. Cell culture

Three types of human cells were used: SMC, skin fibroblasts and foreskin fibroblasts. SMC were isolated from human saphenous veins by the explant technique as previously described [15], skin fibroblasts were kindly provided by Dr. P. Rösen (Diabetes-Forschungsinstitut, Heinrich-Heine-Universität Düsseldorf, Germany), and foreskin fibroblasts were from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium containing 1 mM pyruvate, 2 mM L-glutamine, 1% (v/v) non-essential fatty acids, 50 U/ml penicillin, 50 μg/ml streptomycin and 15% fetal calf serum. Fibroblast medium contained 10% fetal calf serum, but no pyruvate, glutamine or fatty acids. Cells were kept in a humidified atmosphere of 5% $CO_2$ and 95% air at 37°C. Media were changed twice a week and the cells passaged using trypsin (0.05%)/ethylenediamine-tetraacetic acid (EDTA) (0.5 mM). Prior to stimulation with TNFα (10 ng/ml) and PDGF-BB (20 ng/ml), cells were serum-deprived for 72 h. Cell culture materials were purchased from Becton Dickinson (Heidelberg, Germany), fetal calf serum was from Life Technologies (Karlsruhe, Germany). SMC up to passage 12 and fibroblasts of passages 13–20 were used for the study.

### 2.2. Western immunoblot analysis

Cell extracts were prepared in sodium dodecyl sulfate (SDS) lysis

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*Abbreviations:* EDTA, ethylenediamine-tetraacetic acid; IκB, inhibitory protein κB; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; SMC, smooth muscle cells; TNFα, tumor necrosis factor-α

0014-5793/00/$20.00 
PII: S0014-5793(00)01957-8

buffer (4% w/v SDS, 20% glycerol, 0.0625 M sodium dihydrogen phosphate/disodium hydrogen phosphate, pH 7.0, 100 mM dithiothreitol, 0.1% bromophenol blue). Proteins were separated by SDS polyacrylamide gel electrophoresis (12%) and blotted onto polyvinylidene difluoride membranes (Immobilon™-P, Millipore, Bedford, MA, USA). Membranes were blocked in Blotto (Tris-buffered saline, 0.1% Tween®-20, 5% w/v non-fat dry milk) and then probed with primary antibodies (1:1000 in Blotto), followed by incubation with peroxidase-conjugated secondary antibodies (1:3000 in Blotto). Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany).

*2.3. Immunoprecipitation*

For immunoprecipitation, cells were washed with phosphate-buffered saline (PBS) and lysed for 30 min on ice with 1 ml RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.4% deoxycholate, 1% Nonidet P-40) supplemented with protease inhibitors (mammalian protease inhibitor cocktail, Sigma, Deisenhofen, Germany; 1% phenylmethylsulfonyl fluoride of a saturated solution in ethanol). After a brief sonication, samples were centrifuged at $12\,000\times g$ for 10 min at 4°C. Supernatants were pre-cleared with 10 μl of a 50/50 (v/v) slurry of protein A–Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany) in PBS for 2 h at 4°C. Protein A–Sepharose was pelleted by centrifugation at $12\,000\times g$ for 1 min and the supernatants were incubated with anti-IκBα antibodies (1 μg) on a rocking device overnight at 4°C. Then, 10 μl of a 50/50 (v/v) slurry of protein A–Sepharose in PBS was added and incubated on a rocking device for 1 h at 4°C. The protein A–Sepharose was pelleted and washed three times with RIPA buffer. Subsequently, proteins were lysed in SDS lysis buffer and subjected to immunoblot analysis as described above.

*2.4. Electrophoretic mobility shift assay (EMSA)*

Total cell extracts from TNFα- and PDGF-stimulated SMC were prepared as described by Müller et al. [16]. Nuclear extracts were prepared according to Dignam et al. [17] with minor modifications as previously described in detail [18]. A non-radioactive labeling technique with digoxigenin-11-ddUTP (Roche Diagnostics, Basel, Switzerland) was used. The sequence of the NF-κB binding specific oligonucleotide was 5′-AGT TGA GGG GAC TTT CCC AGG C-3′. For binding reaction, 6 μg protein was incubated with 0.155 pmol of 3′end-labeled probe and 3 μg poly(dI-dC) in binding buffer (Roche Diagnostics, Basel, Switzerland) in a final volume of 20 μl for 15 min at room temperature. A 25-fold excess of non-labeled probe was added to the reaction mixture for competition experiments. For supershift assays, antibodies (1 μg) were added to proteins 45 min before using the labeled probe. Electrophoresis was carried out on a 6% polyacrylamide gel at 4°C. Samples were transferred to positively charged nylon membranes (Roche Diagnostics, Basel, Switzerland), blocked in blocking buffer (1% blocking reagent, 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and then incubated with anti-digoxigenin-Fab fragments (1:10 000 in blocking buffer) conjugated with alkaline phosphatase. Finally, bands were visualized by chemiluminescence using the CSPD® system (Roche Diagnostics, Basel, Switzerland).

*2.5. Immunofluorescence microscopy*

For immunofluorescence microscopy, cells were washed with PBS, fixed for 5 min with cold (−20°C) methanol and air-dried. Then, cells were washed three times with PBS, blocked for 20 min with 10% horse serum (Vector Laboratories, Burlingame, CA, USA) in PBS, and incubated with anti-NF-κB (p65) antibodies (2 μg/ml in 1.5% horse serum in PBS) for 1 h. After washing with PBS, rhodamine-conjugated secondary antibodies (1:100 in 1.5% horse serum in PBS) were incubated for 45 min. Coverslips were mounted in SlowFade™ antifade reagent (Molecular Probes, Eugene, OR, USA) and samples were analyzed using a fluorescence microscope (IX-50, Olympus Optical, Hamburg, Germany).

*2.6. Antibodies*

Rabbit affinity-purified polyclonal antibodies against IκBα, IκBβ, NF-κB p50 and NF-κB p65 were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Phospho-specific ($Ser^{32}$) IκBα and ($Thr^{308}$) Akt antibodies were from New England Biolabs (Schwalbach, Germany). Peroxidase-conjugated anti-rabbit antibodies were from Dianova (Hamburg, Germany). Rhodamine-conjugated anti-rabbit antibodies were from Roche Diagnostics (Basel, Switzerland).

## 3. Results

### *3.1. TNFα but not PDGF stimulates IκBα phosphorylation and degradation*

The effects of PDGF (20 ng/ml) on IκBα in cultured human vascular SMC, skin and foreskin fibroblasts were compared to the effects of TNFα (10 ng/ml). Using phospho-specific ($Ser^{32}$) antibodies, a time-dependent phosphorylation of IκBα by TNFα was observed. IκBα phosphorylation was already detected 5 min after stimulation with TNFα, maximal effects were seen after 10 min, and phosphorylated bands disappeared after 30 min (Fig. 1A). Degradation of IκBα was detected 10 min after stimulation and reached its maximum at 15 min. Within 60–90 min, IκBα reached control levels (Fig. 1B). In contrast, PDGF (20 ng/ml) did not stimulate either

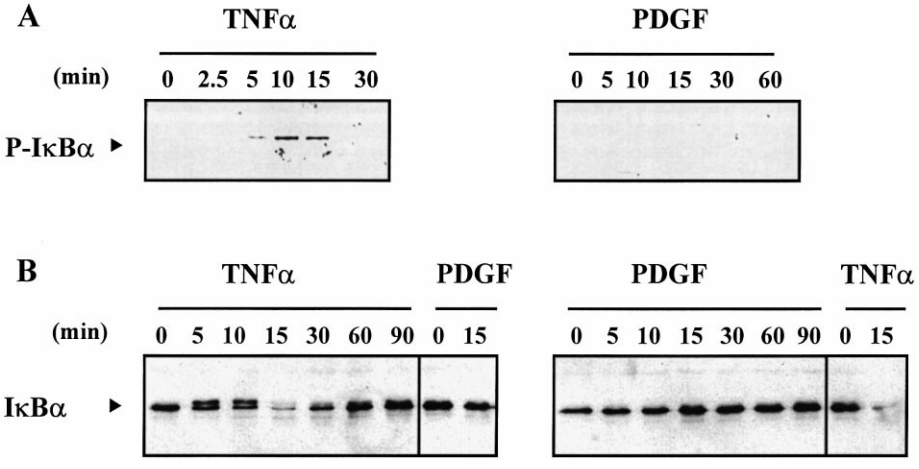


Fig. 1. Western blots demonstrating the effects of TNFα and PDGF on IκBα and IκBβ in SMC. Cells were serum-starved for 72 h and stimulated with TNFα (10 ng/ml) or PDGF (20 ng/ml) for the times indicated. Cell extracts were analyzed by Western blot. In contrast to PDGF, TNFα time-dependently stimulated serine phosphorylation (A) and degradation (B) of IκBα. Similar results were obtained in three to four independent experiments, using SMC and fibroblasts.

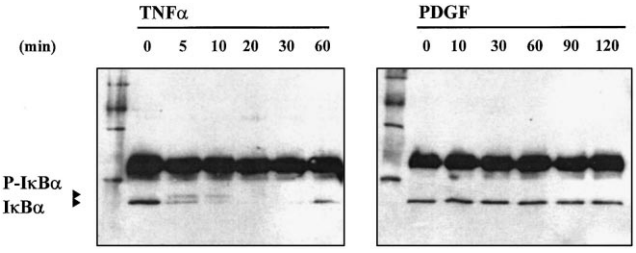


Fig. 2. Immunoprecipitations demonstrating the effects of TNFα and PDGF on IκBα in SMC. SMC were serum-starved for 72 h and stimulated with TNFα (10 ng/ml) or PDGF (20 ng/ml) for the times indicated. IκBα was immunoprecipitated from total cell extracts and immunoprecipitates were analyzed by Western blot using IκBα antibodies. After stimulation with TNFα a transient double band occurred, indicating IκBα phosphorylation. After 20 min stimulation with TNFα, IκBα could not be immunoprecipitated suggesting its degradation. In contrast, in PDGF-treated cells, IκBα was immunoprecipitated throughout the stimulation period. Similar results were obtained in three independent experiments.

phosphorylation (Fig. 1A) or degradation of IκBα (Fig. 1B). Even after longer stimulation periods (up to 5 h), PDGF did not induce IκBα degradation (not shown). IκBβ, which is also expressed in SMC and fibroblasts [4], was not affected by either TNFα or PDGF (not shown).

Consistent with these findings, we were able to immunoprecipitate IκBα from SMC stimulated with PDGF for different time periods (Fig. 2). In cells stimulated with TNFα for 5–10 min, a double band was immunoprecipitated with anti-IκBα antibodies (Fig. 2), indicating IκBα phosphorylation. After 20 min of stimulation with TNFα, no IκBα could be immunoprecipitated, indicating IκBα degradation. IκBα returned to immunoprecipitable levels at 60 min.

### 3.2. PDGF does not activate NF-κB

NF-κB binding activity was studied by EMSA. In all three cell lines, TNFα induced maximum NF-κB binding activity after 15–30 min (Fig. 3). In contrast, PDGF did not induce NF-κB binding activity within 75 min of stimulation (Fig. 3). Even longer stimulation with PDGF (up to 120 min) did not induce NF-κB binding activity (not shown).

In order to quantify the different potencies of TNFα and PDGF, nuclear translocation of NF-κB (p65) was studied at the single cell level by immunofluorescence microscopy. In unstimulated controls, nuclear localization of NF-κB (p65 subunit) was observed in $1 \pm 1\%$ of SMC (means ± S.E.M., $n = 3$). Stimulation of SMC with TNFα for 20 min resulted in a marked nuclear translocation of NF-κB (p65) in $97 \pm 3\%$ of TNFα-stimulated cells (means ± S.E.M., $n = 3$) (Fig. 4). In contrast, no significant nuclear NF-κB translocation was seen when cells were stimulated with PDGF for 30 min ($3 \pm 1\%$ of SMC, means ± S.E.M., $n = 3$) (Fig. 4). Similar results were found in fibroblasts. Even after longer stimulation (up to 4 h), PDGF-induced nuclear NF-κB translocation was not increased (not shown).

### 3.3. PDGF but not TNFα stimulates Akt phosphorylation

In order to assess whether Akt phosphorylation is involved in the activation of NF-κB, Western blot analyses were performed with phospho-specific ($Thr^{308}$) anti-Akt antibodies. After stimulation of cells with PDGF, Akt phosphorylation was strongly induced after 5 min, was maximal after 10 min and declined within 90 min (Fig. 5). In contrast, stimulation with TNFα did not result in phosphorylation of Akt (Fig. 5).

## 4. Discussion

NF-κB is involved in the expression of numerous cytokines, adhesion molecules, regulation of apoptosis, and is also believed to be involved in cell proliferation [7,15,19]. In addition, this transcription factor is regarded to be a key regulator of inflammatory responses [20]. Recently, the activation of NF-κB by Akt has been described [7,9]. The activity of the serine/threonine kinase Akt has been shown to be stimulated by growth factors acting through receptor tyrosine kinases such as PDGF, epidermal growth factor, and insulin [21,22]. Physiologically, Akt signaling seems to be involved in cell survival and suppression of apoptosis [23,24]. Among the nu-

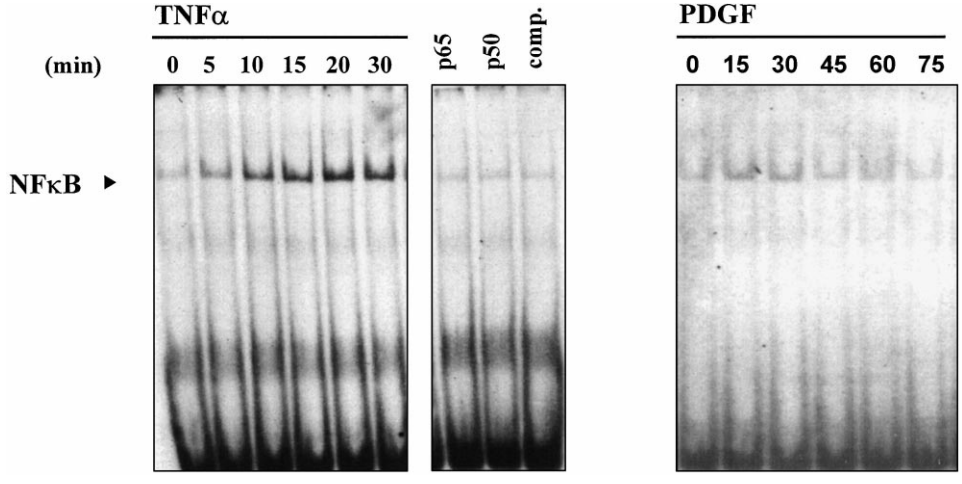


Fig. 3. EMSA demonstrating the induction of NF-κB binding activity by TNFα and PDGF in SMC. SMC were serum-starved for 72 h and stimulated with TNFα (10 ng/ml) or PDGF (20 ng/ml) for the times indicated. Total cell extracts were subjected to EMSA. Specificity of the retarded bands was determined using anti-p65 antibodies (p65) and anti-p50 antibodies (p50), as well as by competition with 25×excess of unlabelled probe (comp.) (samples from cells stimulated with TNFα for 15 min were used for specificity experiments). A marked, time-dependent induction of NF-κB binding activity was seen with TNFα. NF-κB binding activity with PDGF was not significantly induced. Similar results were obtained in three independent experiments.

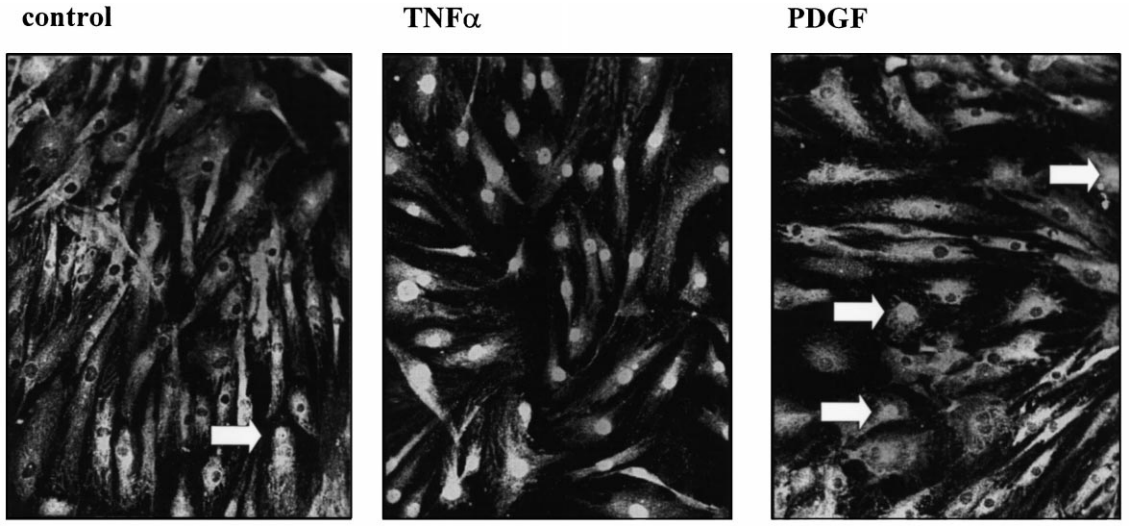


Fig. 4. Immunofluorescence staining demonstrating nuclear translocation of NF-κB (p65) in SMC. Cells were serum-starved for 72 h and stimulated with TNFα (10 ng/ml, 20 min) or PDGF (20 ng/ml, 30 min). The localization of p65 was determined by immunofluorescence microscopy. In unstimulated cells, p65 was localized in the cytoplasm. After stimulation with TNFα, p65 was translocated into the nuclei in almost 100% of the cells. Stimulation with PDGF resulted in a much weaker response. Similar results were obtained in three independent experiments, using SMC and fibroblasts. Arrows indicate NF-κB translocation in control cells and in PDGF-stimulated cells.

merous growth factors mediating migration and proliferation of SMC and fibroblasts, PDGF plays a central role [25]. For example, selective inhibition of the PDGFβ receptor with neutralizing antibodies significantly reduced neointima formation in baboons [26].

Because of the recently described new pathway of NF-κB activation by PDGF [9] and the involvement of Akt phosphorylation in NF-κB activation [7], we focused this study on the role of Akt in PDGF- and TNFα-induced NF-κB activation using cultured human vascular SMC and fibroblasts. As compared to TNFα, PDGF did not stimulate NF-κB in any of these cells. No significant nuclear translocation of NF-κB (p65) was observed after PDGF treatment. Consistent with these findings, NF-κB binding activity, measured by EMSA, was strongly induced by TNFα and not by PDGF. Although a strong activation of NF-κB occurred after stimulation with TNFα, no Akt phosphorylation was detected. Vice versa, PDGF, which did not activate NF-κB, caused a marked phosphorylation of Akt.

In a recent study with human skin fibroblasts and rat fibroblast-like synoviocytes, PDGF-induced NF-κB activation coincided with phosphorylation and degradation of IκBα. It was also shown that Akt is principally able to activate NF-κB [9]. Another study demonstrated that Akt phosphorylation is inducible by TNFα and coincides with NF-κB activation [7]. Thus, PDGF- and TNFα-induced activation of NF-κB is, at least in some cell systems, linked to the IκB kinase pathway.

Our findings appear to be at variance with the observation that Akt is necessary for NF-κB activation. A possible explanation could be that some of the studies reporting involvement of Akt in the NF-κB activation pathway were performed in cells with a high potential for proliferation, such as embryonic kidney cells, carcinoma cells [7], or T-cells [10]. Interestingly, in the study describing PDGF-induced activation of NF-κB by Akt, no Akt phosphorylation was found by TNFα [9], indicating that Akt is not essential for NF-κB activation. Thus, it is possible that Akt is principally linked to the NF-κB pathway, especially in embryonic or dedifferentiated cells. It might be speculated that a link between Akt, which provides a survival signal protecting cells from apoptosis [24], and the NF-κB pathway might contribute to the proliferation potency of some cell types. This hypothesis is supported by the observation that Akt is enhanced in many human tumors [24]. In other cell systems with a lower potential for cell division, this pathway might be deactivated or have lost its efficacy. Because the effects of Akt in the signaling cascade can vary widely in different cell types, it has been suggested that cultured cells can develop individual molecular mechanisms in cellular signaling [23].

Taken together, our findings demonstrate that stimulation

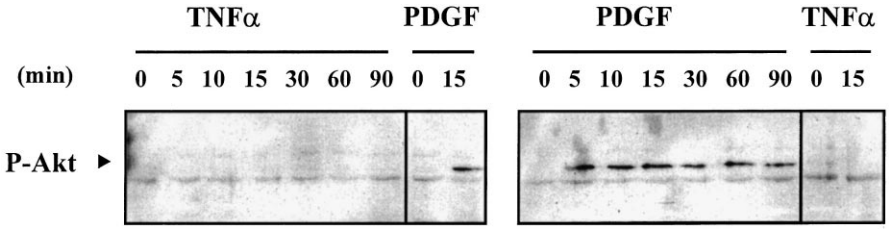


Fig. 5. Western blot demonstrating the effects of PDGF on Akt phosphorylation in SMC. Cells were serum-starved for 72 h and stimulated with TNFα (10 ng/ml) or PDGF (20 ng/ml) for the times indicated. Cell extracts were analyzed by Western immunoblotting. In contrast to TNFα, PDGF time-dependently stimulated Akt phosphorylation. Similar results were obtained in three independent experiments.

with PDGF induces Akt phosphorylation in cultured human vascular SMC, skin and foreskin fibroblasts, but does not result in NF-κB activation. In contrast, TNFα strongly activated NF-κB, but was not sufficient to stimulate Akt phosphorylation in all cell types used. It is concluded that Akt phosphorylation is not required for NF-κB activation in these cells.

*Acknowledgements:* We are grateful to Beate Weyrauther and Christine Machunsky for competent technical assistance and Erika Lohmann for expert secretarial assistance. This study was supported by the Deutsche Forschungsgemeinschaft (WE 2355/1-1, BR 1170/5-1).

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