

# Mechanical Stretch Stimulates Protein Kinase B/Akt Phosphorylation in Epidermal Cells via Angiotensin II Type 1 Receptor and Epidermal Growth Factor Receptor\*

Received for publication, August 20, 2004, and in revised form, November 11, 2004  
Published, JBC Papers in Press, November 15, 2004, DOI 10.1074/jbc.M409590200

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**Mechanical stress is known to modulate fundamental events such as cell life and death. Mechanical stretch in particular has been identified as a positive regulator of proliferation in skin keratinocytes and other cell systems. In the present study it was investigated whether antiapoptotic signaling is also stimulated by mechanical stretch. It was demonstrated that mechanical stretch rapidly induced the phosphorylation of the proto-oncogene protein kinase B (PKB)/Akt at both phosphorylation sites (serine 473/threonine 308) in different epithelial cells (HaCaT, A-431, and human embryonic kidney-293). Blocking of phosphoinositide 3-OH kinase by selective inhibitors (LY-294002 and wortmannin) abrogated the stretch-induced PKB/Akt phosphorylation. Furthermore mechanical stretch stimulated phosphorylation of epidermal growth factor receptor (EGFR) and the formation of EGFR membrane clusters. Functional blocking of EGFR phosphorylation by either selective inhibitors (AG1478 and PD168393) or dominant-negative expression suppressed stretch-induced PKB/Akt phosphorylation. Finally, the angiotensin II type 1 receptor (AT1-R) was shown to induce positive transactivation of EGFR in response to cell stretch. These findings define a novel signaling pathway of mechanical stretch, namely the activation of PKB/Akt by transactivation of EGFR via angiotensin II type 1 receptor. Evidence is provided that stretch-induced activation of PKB/Akt protects cells against induced apoptosis.**

The application of mechanical forces is a ubiquitous challenge to skin cells. Both differentiation processes and proliferation can be induced by different qualities of mechanical stimulation. *In vitro* studies have demonstrated that mechanical pressure gives rise to differentiation processes (1, 2), while mechanical stretch supports proliferation in human epidermal cells (3–5). These findings fit the *in vivo* situation where mechanical pressure provokes horny skin formation, and mechanical stretch, as present in abdominal stretching during pregnancy, induces skin enlargement. Previous *in vitro* studies on

human skin keratinocytes have shown that mechanical stretch induces proliferation-associated signaling cascades of the mitogen-activated protein kinase pathway (4). Particularly the activation of extracellular signal-regulated kinase 1/2 may have functional aspects in stretch-mediated proliferation. As initial events in mechanotransduction, surface receptors of the integrin family are thought to recognize mechanical energy and transduce it into biological signals (6, 7). This assumption is supported by the finding that blocking of  $\beta_1$  integrins abrogates stretch-induced activation of extracellular signal-regulated kinase 1/2 (4).

In addition to increased proliferation, it could be that induction of antiapoptotic signaling pathways may contribute to the increase in cell number in response to mechanical stretch. In particular, the antiapoptotic kinase PKB<sup>1</sup>/Akt is a candidate signaling molecule shown to play a key role in suppression of apoptotic cell death (8, 9). Prototypically, PKB/Akt activation is transduced by cell surface receptors in response to insulin and mitogens such as epidermal growth factor (10–12). In addition, recent advances have shown that integrins, namely the  $\beta_1$  and  $\beta_4$  subsets, stimulate PKB/Akt phosphorylation and therefore may contribute to antiapoptosis (13, 14). Proximal from surface receptors, the phosphoinositide 3-OH kinase (PI3K) conveys activation of PKB/Akt via phosphoinositide-dependent kinases. It has been demonstrated that phosphoinositide-dependent kinase-1 phosphorylates PKB/Akt at threonine 308 (15), whereas the mechanism of the serine 473 phosphorylation is still under debate (16, 17). Studies regarding the impact of mechanical stimuli on PKB/Akt have been mainly carried out in endothelium-derived cells as hemodynamic changes caused by coronary circulation are known to contribute to pathophysiological processes of the blood vessel system (18–21). In this context, vascular smooth muscle cells may also respond to mechanical stretch by activation of PKB/Akt and therefore may contribute to the onset of proliferative disease of the vascular system (22). It is hypothesized that mechanical stress induced by therapeutic interventions such as stent implantation and balloon catheters contributes to the frequently observed restenosis (23).

In the present approach, it was found that PKB/Akt activation in response to mechanical stretch is also present in a keratinocyte model. Moreover the signaling events involved in the activation of PKB/Akt were identified. Transactivation of epidermal growth factor receptor (EGFR) by pertussis-sensi-

\* This work was supported by Volkswagenstiftung Grant I/77 731. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: PKB, protein kinase B; EGFR, epidermal growth factor receptor; PI3K, phosphoinositide 3-OH kinase; HEK, human embryonic kidney; AT1-R, angiotensin II type 1 receptor; GPCR, G protein-coupled receptor; PBS, phosphate-buffered saline; TGF $\alpha$ , transforming growth factor  $\alpha$ .

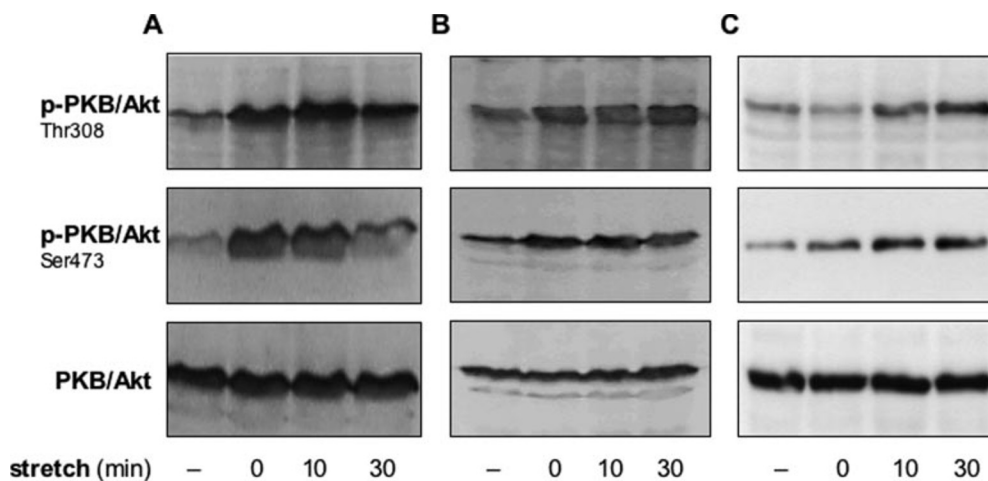


FIG. 1. **Mechanical stretch stimulates phosphorylation of PKB/Akt in epithelial cells.** The time course of PKB/Akt phosphorylation after a single 5-min stretch in serum-starved HaCaT (A), A-431 (B), and HEK-293 (C) cells is shown. The cells were incubated in flexible silicone chambers for 24 h in the absence of fetal calf serum, and then the silicone chambers were stretched to 10% over the initial length. Proteins were obtained as described under "Materials and Methods," and Western blotting was performed with phosphospecific antibodies directed toward threonine 308 and serine 473, respectively. Equal loading was monitored by using antibodies directed against total PKB/Akt. The blot shows representative results ( $n = 3$ ). *p*-, phospho-

tive GPCRs demonstrates a novel mechanism of PKB/Akt activation triggered by mechanical stretch.

#### MATERIALS AND METHODS

**Antibodies and Reagents**—The following antibodies were used. Phosphospecific antibodies raised against PKB/Akt (serine 473 and threonine 308) and an antibody against total PKB/Akt were from Cell Signaling Technology Inc. Antibodies raised against the activated form of the EGFR and total EGFR were purchased from BD Transduction Laboratories and Santa Cruz Biotechnology Inc. Detection of caspase 8 and 3 degradation was performed using antibodies from Cell Signaling Technology Inc. The PI3K inhibitors wortmannin and LY-294002 and the specific inhibitors of the epidermal growth factor receptor tyrosine kinase, AG1478 and PD168393, were purchased from Calbiochem-Novabiochem. Pertussis toxin from *Bordetella pertussis* to inactivate the  $\alpha$  subunits in the  $G_i$  and  $G_o$  subfamilies of heterotrimeric G proteins was purchased from Calbiochem-Novabiochem. Losartan to block the angiotensin II type 1 receptor (AT1-R) was a kind gift from Merck Sharp and Dohme. The endothelin receptor antagonists BQ-123 and BQ-788 were purchased from Calbiochem-Novabiochem. The apoptosis inducer staurosporine was purchased from Roche Applied Science.

**Cell Culture**—The spontaneously immortalized human keratinocyte cell line (HaCaT) (a generous gift from Professor Fusenig, German Cancer Research Institute, Heidelberg, Germany) was cultured in carbonate buffered Hanks' medium with 5% fetal calf serum and 1% penicillin-streptomycin at 37 °C in a 5% CO<sub>2</sub> atmosphere. The human epidermoid carcinoma cell line A-431 (American Type Culture Collection) and the human epithelial kidney line HEK-293 were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. All experiments were done in agreement with the local ethics commission.

**Mechanical Stimulation**—Stretching of *in vitro* cultured cells was performed as described previously (4, 5). Briefly, silicone elastomer MED-4011 (Armando Medizin Technik, Düsseldorf, Germany) was stirred, poured into preformed Teflon matrices, and allowed to polymerize. To allow cell attachment, silicone dishes were treated with 5.7% KOH in methanol for 5 min to neutralize the polymerization-derived HCl. After washing with double distilled water, silicone dishes were coated with 2% arginine for 2 h to facilitate cell attachment (24) and afterward rinsed with phosphate-buffered saline (PBS). Subsequently, the dishes were incubated with fetal calf serum for 2 h. After withdrawal of the serum, cells were plated in flexible silicone chambers and incubated for 24 h under regular conditions. Prior to the application of mechanical stretch, the cells were held for 24 h under serum-free conditions. Then the silicone chambers were extended to 10% for 5 min. After stretch stimulation, cells were either fixed for immunocytochemistry, or protein samples were prepared at the indicated time intervals.

**Transfection**—HEK-293 cells seeded in silicone dishes were transiently transfected with kinase-defective mutants of EGFR (K721A) using Lipofectamine 2000 reagent (Invitrogen). The construct was a

kind gift from Josef Yarden, Weizman Institute of Science, Rehovot, Israel (25). Because of a point mutation at position 721, Lys is replaced by Ala to inhibit the cytoplasmic kinase activity. After transfection, cells were allowed to synthesize proteins under regular conditions for 16 h. Before the application of mechanical stretch the cells were serum-starved for another 16 h.

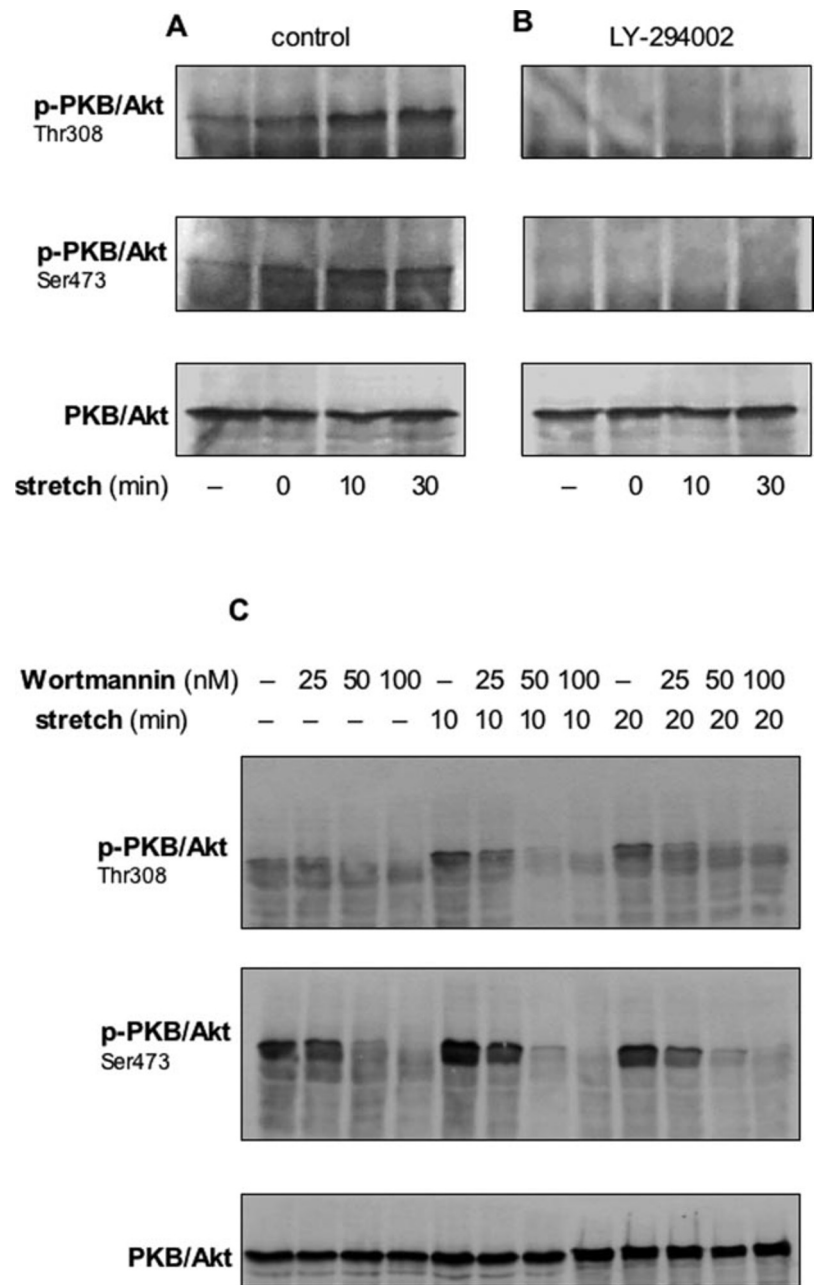
**Immunoblotting**—For detection of PKB/Akt and caspases 3 and 8, cells were lysed in 100  $\mu$ l of SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromphenol blue), sonicated, boiled for 5 min, and separated on SDS-polyacrylamide gels. For detection of EGFR, cells were scraped into lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerol phosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged. Protein concentration of the supernatant was determined (Bio-Rad DC protein assay kit) and standardized using bovine serum albumin. 20  $\mu$ g of protein were mixed with SDS sample buffer and run on SDS-polyacrylamide gels. Then proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked in blocking buffer (Tris-buffered saline (pH 7.6), 0.1% Tween 20, 5% nonfat dry milk) for at least 3 h at 4 °C followed by incubation with the primary antibody in Tris-buffered saline (pH 7.6), 0.05% Tween 20, and 5% bovine serum albumin. The bound primary antibodies were detected using anti-mouse IgG-horseradish peroxidase conjugate and visualized with the ECL detection system (Amersham Biosciences).

**Confocal Laser Scanning Microscopy**—Stretched HaCaT cells were fixed with 5% formaldehyde for 30 min at room temperature. The silicone dishes were cut into 1-cm<sup>2</sup> squares, placed in plastic vessels, and incubated with the EGFR antibody diluted in PBS with 1% bovine serum albumin for 60 min at 37 °C. After three washes with PBS, the silicone sheets were incubated with anti-goat-fluorescein isothiocyanate in PBS for 30 min at room temperature. Finally the silicone sheets were rinsed three times with PBS, placed on glass slides, and examined with a confocal laser scanning microscope (TCS, Leica).

#### RESULTS

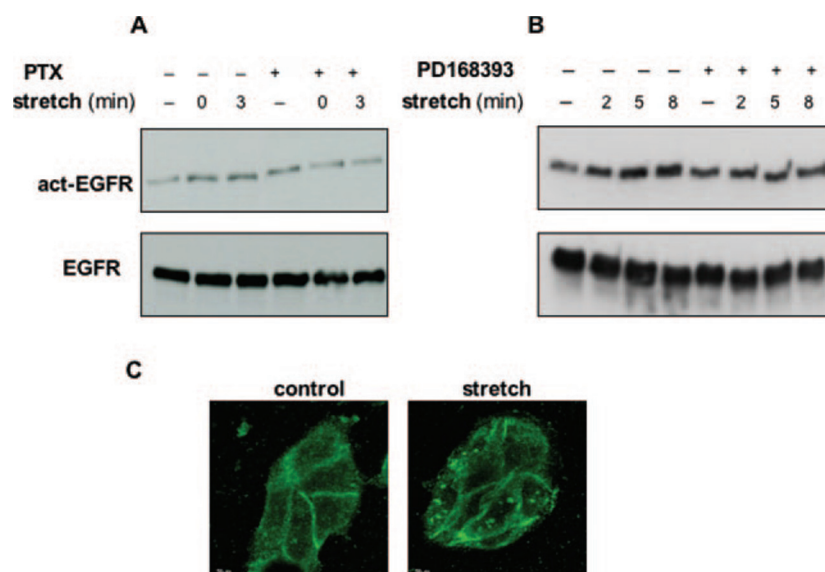
**Mechanical Stretch Induces Phosphorylation of PKB/Akt**—HaCaT cells were seeded into flexible silicon chambers and inserted into a stretching unit as described previously (4). Cells were unidirectionally stretched to 10% for 5 min and after relaxation were scraped into SDS sample buffer after 0, 10, and 30 min, respectively. Protein extracts were tested for PKB/Akt phosphorylation by immunoblotting. As shown in Fig. 1, mechanical stretch induced a rapid phosphorylation of PKB/Akt at both phosphorylation sites (threonine 308 and serine 473). The phosphorylation activity peaked between 10 and 30 min in the epithelial cell species tested.

**FIG. 2. Inhibitors of PI3K suppress stretch-mediated phosphorylation of PKB/Akt.** *A*, serum-starved HaCaT cells were stretched for 5 min, and total protein was prepared at the indicated time intervals. Protein extracts were subjected to Western blot and tested for phosphorylation of PKB/Akt. *B*, cells were pretreated with PI3K inhibitor LY-294002 (20  $\mu$ M) and then stretched as described in *A*. *C*, concentration-dependent effect of PI3K inhibitor wortmannin (25, 50, and 100 nM) on basal phosphorylation of PKB/Akt was tested in HaCaT cells (*lanes 1–4*). Additionally the effect of wortmannin on stretch-induced PKB/Akt phosphorylation was tested in cell proteins extracted after 10 min (*lanes 5–8*) and 20 min (*lanes 9–12*), respectively. The blot shows representative results ( $n = 3$ ). *p*-, phospho.

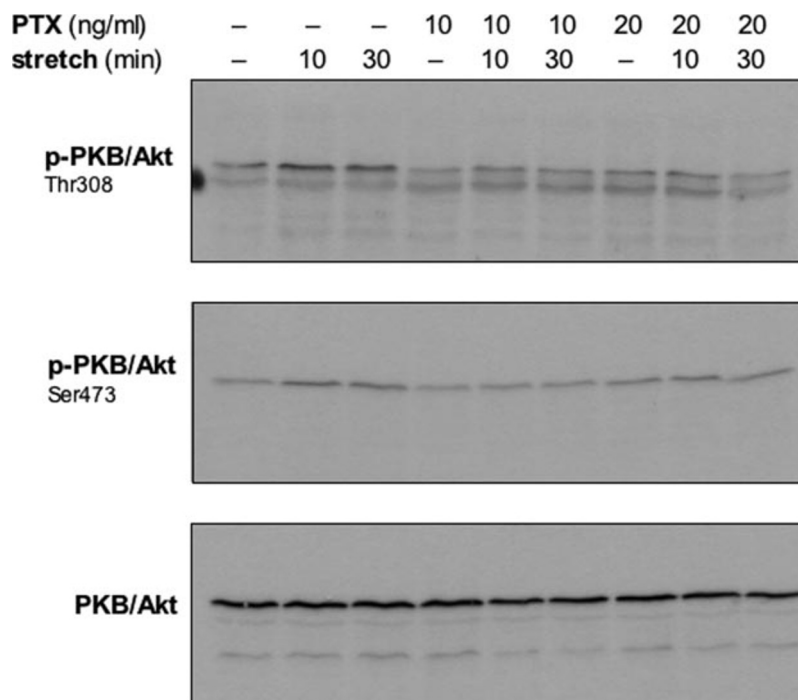


**Inhibitors of PI3K Block PKB/Akt Phosphorylation in Response to Stretch**—The role of PI3K was examined to elucidate the signal transduction pathways underlying the activation of PKB/Akt phosphorylation by mechanical stretch. There is a large body of evidence for the key role of PI3K in the activation of PKB/Akt in different cell systems (8, 26). In our study the function of PI3K was blocked by two highly selective inhibitors (wortmannin and LY-294002). It was found that a pretreatment for 1 h with 20  $\mu$ M LY-294002 completely blocked the stretch-induced phosphorylation of PKB/Akt (Fig. 2, *A* and *B*). To substantiate this finding, cells were treated with wortmannin at 25, 50, and 100 nM (Fig. 2*C*). In the presence of wortmannin, the basal phosphorylation of PKB/Akt was suppressed in a concentration-dependent manner (Fig. 2*C*, *lanes 1–4*). Likewise, the stretch-induced phosphorylation of PKB/Akt detected 10 and 20 min after stretch stimulation was reversed by increasing concentrations of wortmannin (Fig. 2*C*, *lanes 5–12*). These findings clearly demonstrate PI3K-dependent phosphorylation of PKB/Akt in response to mechanical stretch.

**Mechanical Stretch Induces Transactivation of EGFR; Pertussis Toxin and Losartan Abrogate EGFR Transactivation and Phosphorylation of PKB/Akt**—One of the best characterized pathways leading to activation of PKB/Akt is the binding of growth factors to respective receptor tyrosine kinases (27–29). In the present model, the relevance of this signaling cascade for mechanotransduction was tested. Immunoblots demonstrated a rapid phosphorylation of EGFR in response to mechanical stretch (Fig. 3, *A*, *lanes 1–3*, and *B*, *lanes 1–4*). These data were further substantiated by confocal laser scanning microscopy. The occurrence of EGFR clusters in response to mechanical stretch indicated functional activation (Fig. 3*C*). Furthermore it was studied whether the phosphorylation of EGFR in response to stretch is dependent on GPCR function. Therefore, cells were pretreated with pertussis toxin known to inactivate GPCR by catalyzing the ADP-ribosylation of  $\alpha$  subunits in the  $G_i$  and  $G_o$  subfamilies of heterotrimeric G proteins. In our model we could observe that pretreatment with pertussis toxin abolished the stretch-induced phosphorylation of EGFR (Fig. 3*A*). This finding gives



**FIG. 3. Mechanical stretch stimulates phosphorylation of the EGFR; GPCR inhibitor pertussis toxin and EGFR kinase inhibitor PD168393 abrogate transactivation.** *A*, serum-starved HaCaT cells were stretched for 5 min and extracted immediately (0 min) and 3 min after the stretching stimulus (lanes 1–3). Additionally cells were pretreated with 15 ng/ml GPCR inhibitor pertussis toxin (PTX, lanes 4–6) for 12 h and then stretched according to the aforementioned protocol. Protein extracts were subjected to Western blot and tested for EGFR phosphorylation using a phosphospecific antibody. Equal loading was monitored by using antibodies directed against total EGFR. *B*, cells were pretreated with 1  $\mu$ M EGFR kinase inhibitor PD168393 for 1 h and then stretched (lanes 5–8). Control activation of EGFR in response to mechanical stretch was confirmed (lanes 1–4). *C*, serum-starved HaCaT cells were stretched for 5 min, fixed, and hybridized with EGFR antibody. A fluorescein isothiocyanate-conjugated anti-mouse antibody served as a secondary antibody. Representative photographs for the basal cell section are shown. The blot shows representative results ( $n = 3$ ). *act-*, activated.



**FIG. 4. Inhibition of GPCR by pertussis toxin abrogates stretch-induced phosphorylation of PKB/Akt.** Serum-starved HaCaT cells were pretreated for 12 h with different concentrations of pertussis toxin (10 and 20 ng/ml) and then stretched for 5 min. Total protein was extracted after 10 and 30 min, respectively, and tested for PKB/Akt phosphorylation. Extracts derived from stretched cells in the absence of pertussis toxin served as a positive control (lanes 1–3). The blot shows representative results ( $n = 3$ ). PTX, pertussis toxin; *p-*, phospho-

evidence for a contribution of GPCRs to the transactivation of EGFR in response to mechanical stretch. Additionally, inhibition of EGFR tyrosine kinase by preincubation with 1  $\mu$ M PD168393 blocked EGFR transactivation by mechanical stretch. The functional link between GPCRs, EGFR, and PKB/Akt was further corroborated by showing an inhibition of PKB/Akt by pertussis toxin (Fig. 4). To characterize the GPCR-mediated effect, different inhibitors against GPCR subsets were tested. Findings derived from a vascular smooth muscle cell model suggested that angiotensin and endothelin receptors partake in the activation of extracellular signal-regulated kinase 1/2 in response to stretch (30). In our ex-

periments, losartan, an inhibitor of AT1-R, potently suppressed PKB/Akt phosphorylation in our model (Fig. 5A). Antagonists directed against the endothelin receptor subsets ET-AR and ET-BR (BQ-123 and BQ-788) showed no effect (Fig. 5B). These data give evidence that EGFR- and GPCR-signaling cascades cooperate in the phosphorylation of PKB/Akt in mechanotransduction.

**EGFR Inhibitors and Ectopic Expression of Kinase-deficient EGFR Abolish PKB/Akt Phosphorylation in Response to Stretch**—The data shown in Fig. 3 demonstrated phosphorylation of the EGFR in response to mechanical stretch. In the following experiments, it was investigated

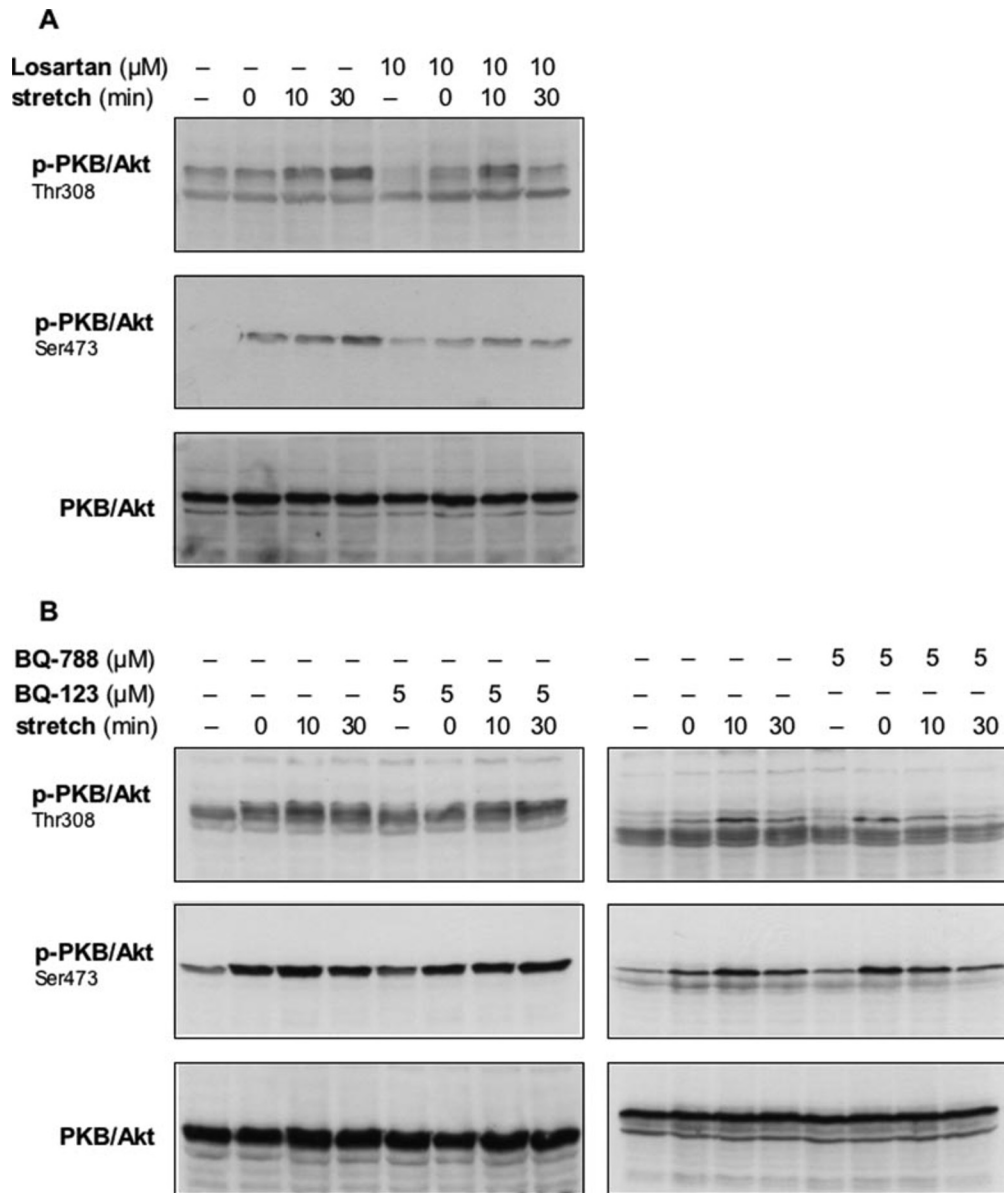


FIG. 5. **Inhibition of AT-1R abrogates stretch-mediated activation of PKB/Akt.** Serum-starved HaCaT cells were pretreated with 10  $\mu\text{M}$  losartan to block the AT1-R (A) or with 5  $\mu\text{M}$  BQ123 or BQ788 (B), both endothelin-1 receptor antagonists specific for the type A or type B subset, respectively. Cells were stretched for 5 min, and total protein was prepared at the indicated time intervals and tested for PKB/Akt phosphorylation. The blot shows representative results ( $n = 3$ ). p-, phospho-

whether phosphorylation of EGFR has functional implications for downstream PKB/Akt phosphorylation (Figs. 6–8). HaCaT cells were treated with AG1478, a specific inhibitor of the epidermal growth factor receptor tyrosine kinase, and then stretched (Fig. 6). The controls treated with solvent only showed the typical phosphorylation pattern of PKB/Akt (Fig. 6A). In contrast no phosphorylation of PKB/Akt was observed in cells treated with 50 nM AG1478 (Fig. 6B). To further confirm these findings, different concentrations of AG1478 were tested. In the absence of mechanical stretch AG1478 (10, 50, and 100 nM) had no effect on basal PKB/Akt phosphorylation at both phosphorylation sites (Fig. 6C). Only at 500 nM AG1478 was the basal phosphorylation of PKB/Akt attenuated. Fig. 6D shows the effect of increasing concentrations of AG1478 on stretch-induced phosphorylation of PKB/Akt. After stretching the cells for 5 min the protein was extracted and tested for PKB/Akt phosphorylation. Only the stretch control showed a clear phosphorylation of PKB/Akt at both phosphorylation sites (Fig. 6D, lane 2). It was found that

the stretch-induced phosphorylation of PKB/Akt was suppressed by AG1478 in a concentration-dependent manner. At 10 nM AG1478, the phosphorylation of PKB/Akt was distinctly inhibited. At higher concentrations, this effect was more pronounced. To substantiate these findings another specific EGFR inhibitor was tested. Phosphorylation of PKB/Akt in response to mechanical stretch was completely abrogated in the presence of 500 nM PD168393 (Fig. 8A). In addition to pharmacological inhibition, ectopic expression of kinase-deficient EGFR (K721A) was tested. It was found that overexpression of K721A in HEK-293 cells attenuated phosphorylation of PKB/Akt, corroborating the functional link between EGFR and PKB/Akt in the transmission of mechanical stretch (Fig. 8B).

**Functional Inhibition of EGFR by AG1478 and PD168393 Abolish TGF $\alpha$ -induced Phosphorylation of PKB/Akt**—Figs. 6 and 8A show that EGFR inhibitors AG1478 and PD168393 abrogated phosphorylation of PKB/Akt in response to mechanical stretch in HaCaT cells. To support these data, the functional

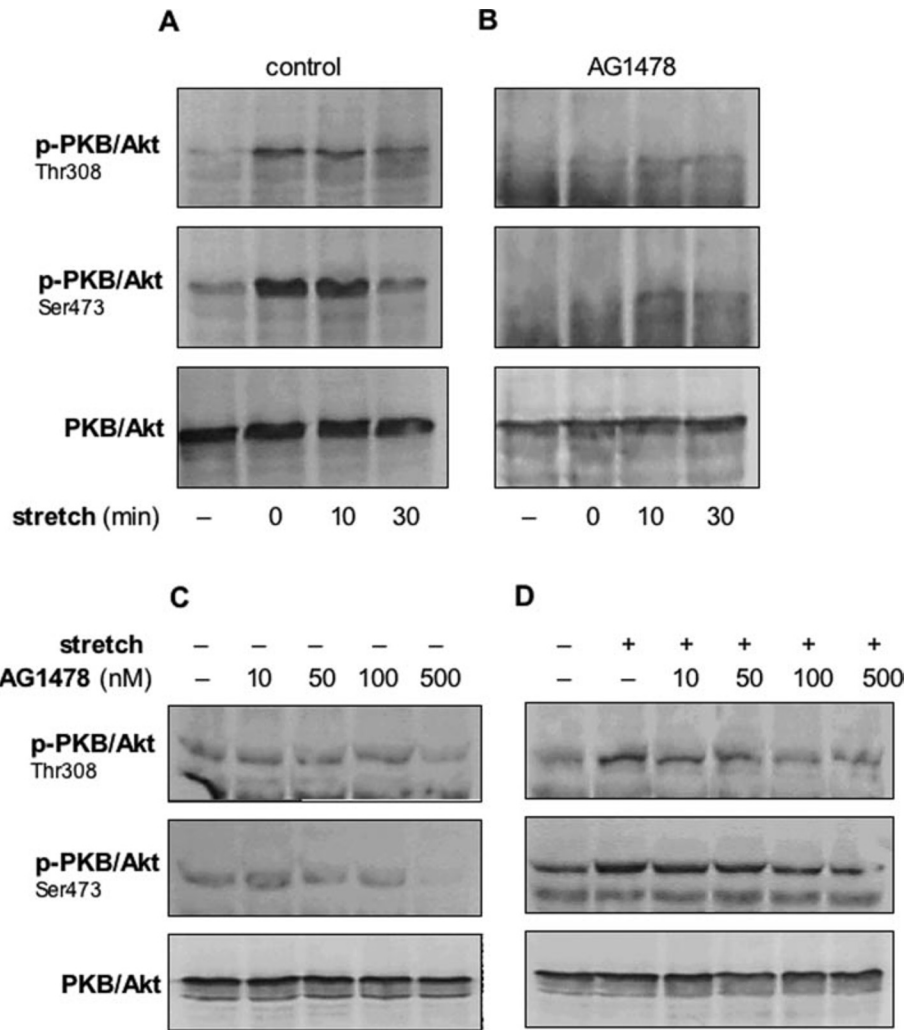


FIG. 6. Inhibition of the EGFR tyrosine kinase by AG1478 abrogates stretch-mediated activation of PKB/Akt. **A**, serum-starved HaCaT cells without additional treatment were used as a positive control for PKB/Akt phosphorylation in response to mechanical stretch. Cells were stretched for 5 min, and total protein was prepared at the indicated time intervals and subjected to Western blot. **B**, cells pretreated with 50 nM AG1478 were stretched and tested for PKB/Akt phosphorylation as described. **C**, the effect of different concentrations of AG1478 (10, 50, 100, and 500 nM) on the basal PKB/Akt phosphorylation was tested. **D**, cells were pretreated with different concentrations of AG1478 (10, 50, 100, and 500 nM) for 1 h, stretched for 5 min, and immediately lysed. Protein extracts were tested for PKB/Akt phosphorylation. The blot shows representative results ( $n = 3$ ). p-, phospho-

efficacy of both pharmacological inhibitors was validated. HaCaT cells were pretreated with increasing concentrations of AG1478 or PD168393 (10, 50, 100, and 500 nM) and then stimulated with 50 ng/ml TGF $\alpha$  serving as an EGFR ligand. Western blot analysis showed a concentration-dependent attenuation of PKB/Akt phosphorylation (Fig. 7A). Vice versa, in a second experiment, HaCaT cells were pretreated with a fixed concentration of both inhibitors (100 nM) and then stimulated with increasing concentrations of TGF $\alpha$  (10, 50, and 100 nM). Suppression of TGF $\alpha$ -induced PKB/Akt phosphorylation was detected (Fig. 7B) indicating the functional inhibition of EGFR by both pharmacological inhibitors tested.

**Mechanical Stretch Protects Staurosporine-induced Cleavage of Caspases 8 and 3**—In the present study we showed that mechanical stretch activates PKB/Akt, one of the key regulators in apoptosis. From this it could be construed that mechanical stretch is able to protect cells against apoptosis. This issue was addressed using a combination of mechanical stretch and staurosporine, a well known inducer of apoptosis. Data given in Fig. 9 show that staurosporine alone applied for 16 h led to a significant increase of cleaved caspase 8 and 3 fragments (lanes 2 and 3). When staurosporine and mechanical stretch were applied at the same time, no cleaved caspase 3 fragments were detected. Additionally, the amount of cleaved caspase 8 fragments was attenuated (lanes 5 and 6). Similarly, addition of staurosporine 1 and 4 h poststretching, respectively, led to a decrease in cleaved caspase fragments (lanes 7–10). These findings suggest that mechanical stretch protects cells against the onset of apoptosis.

#### DISCUSSION

Changes in cell morphology by the application of external and internal forces are considered to regulate gene expression and cell behavior. In particular, cell stretch is considered to be a stimulus supporting cell proliferation. Clinically, the use of so-called “skin expanders” represents an example for the application of mechanical stretch to increase skin material to generate skin grafts for cosmetic surgery (31). Furthermore there is evidence that mechanical stimuli contribute to tumorigenesis (32). Solid tumors especially exhibit interstitial hypertension caused by increased tumor vascularization and the absence of a functional lymphatic circulation (33). The increase of interstitial tumor hypertension gives rise to stretch stimulation of the tumor capsule. It seems likely that stretching of the tumor capsule triggers proliferation of capsule cells and therefore contributes to growth of tumor tissue.

The signaling pathways linking mechanical stretch to cell proliferation and survival are still not well described. In the present study we showed that mechanical stretch applied to human epithelial cells activates the antiapoptotic kinase PKB/Akt. This supports the assumption that the increase in cell number in response to mechanical stretch is, at least in part, due to a suppression of apoptosis. Activation of PKB/Akt in our model was sensitive to wortmannin and LY-294002 suggesting PI3K-dependent signaling. Others have shown that the necessity of PI3K for PKB/Akt activation is mediated by a direct interaction between phosphatidylinositol 3,4-bisphosphate and the pleckstrin homology domain of PKB/Akt (8).

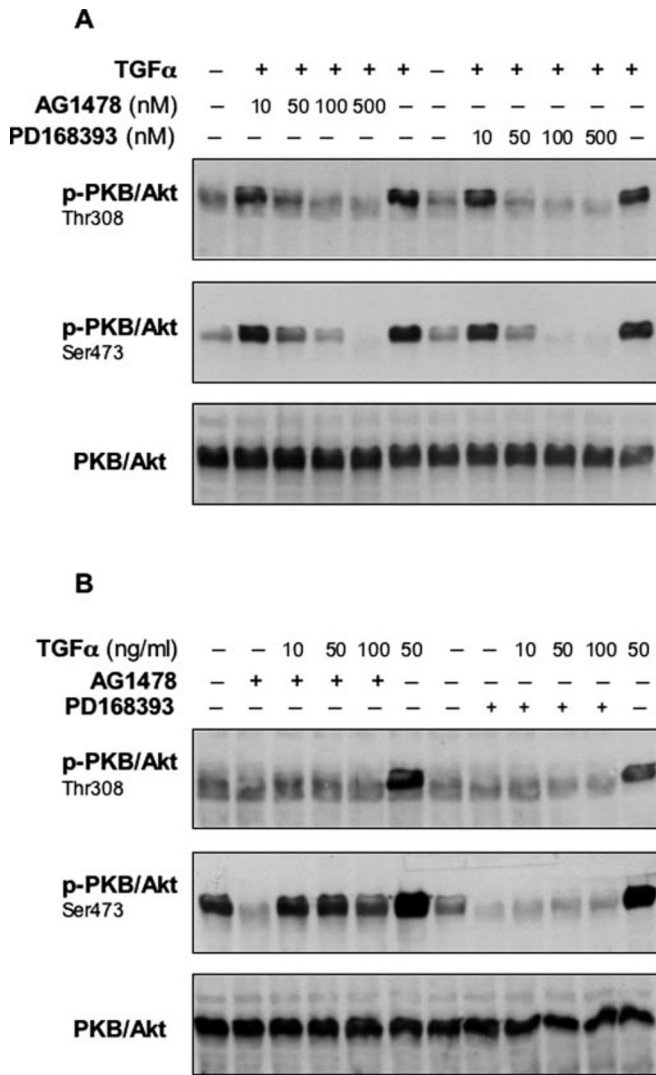


FIG. 7. Pharmacological inhibition of EGFR by AG1478 or PD168393 suppresses TGF $\alpha$ -mediated activation of PKB/Akt. Serum-starved HaCaT cells were pretreated for 1 h with the indicated inhibitors and then stimulated with TGF $\alpha$  for 5 min. *A*, different inhibitor concentrations (10, 50, 100, and 500 nM) were applied to the cells before stimulation with 50 ng/ml TGF $\alpha$ . *B*, different TGF $\alpha$  concentrations (10, 50, and 100 ng/ml) were applied to the cells after pretreatment with 100 nM inhibitor. The blot shows representative results ( $n = 3$ ). *p*-, phospho-.

Furthermore, in the present study a contribution of receptor tyrosine kinases, EGFR being the most prominent, to mechanosignaling was tested. It was demonstrated that mechanical stretch induces EGFR cluster formation and rapid phosphorylation of the receptor. Blocking of EGFR phosphorylation by inhibitors or dominant-negative expression abrogated the stretch-induced phosphorylation of PKB/Akt indicating a functional relevance. Since the fundamental findings of Rosette and Karin (34), who first discovered a non-classical mode of EGFR activation triggered by osmotic stress and UV light, the so-called transactivation of EGFR is now considered to play a key role in the pathophysiology of hyperproliferative diseases such as cancer (35). Several modes of EGFR transactivation have been described including via integrins (36), intracellular calcium (37), and GPCR (38). Although most investigations favor a ligand-independent transactivation mechanism, it has also been suggested that metalloproteases mediate an epidermal growth factor-like ligand shedding (39).

In our work it was demonstrated that stretch-mediated phos-

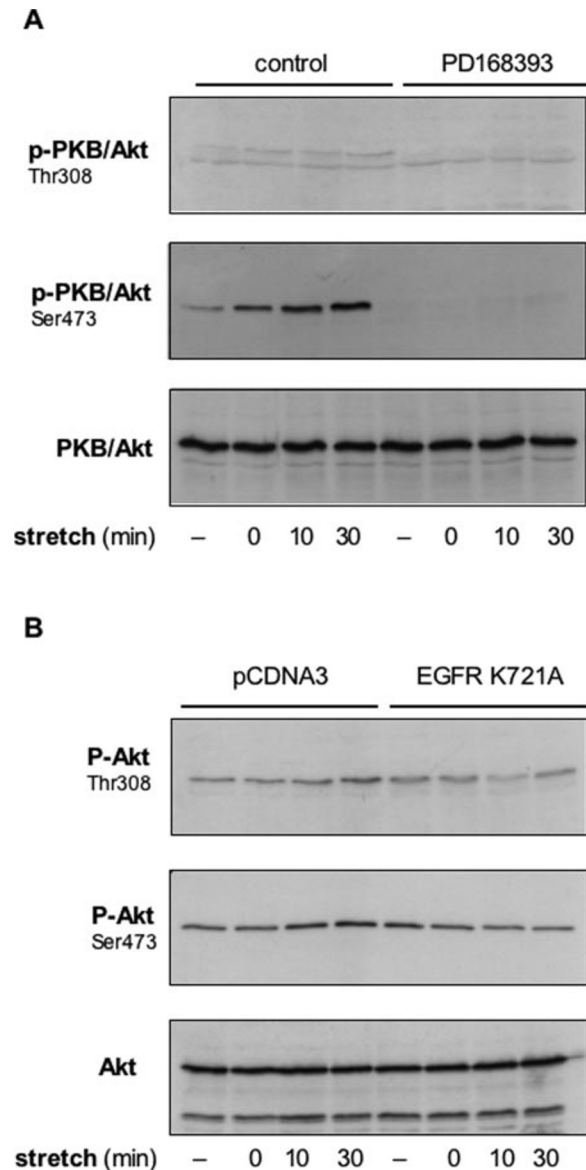


FIG. 8. Inhibition of the EGFR tyrosine kinase by PD168393 and dominant-negative expression abrogates stretch-mediated activation of PKB/Akt. *A*, serum-starved HaCaT cells were pretreated with 500 nM PD168393 for 1 h, stretched for 5 min, extracted after 0, 10, and 30 min, and tested for PKB/Akt phosphorylation. *B*, HEK-293 cells were transiently transfected with kinase-deficient mutants of EGFR (K721A) or pCDNA3 for control, respectively. After stretching, proteins were extracted after 0, 10, and 30 min and tested for PKB/Akt phosphorylation. The blot shows representative results ( $n = 3$ ). *p*-, phospho-.

phorylation of both EGFR and PKB/Akt is sensitive to pertussis toxin. These findings provide evidence that pertussis-sensitive GPCRs participate in this signaling cascade at an early stage. In an attempt to characterize the relevant GPCRs, we found a losartan-sensitive activation of PKB/Akt in response to stretch indicating a contribution of AT1-R in this signaling cascade. This finding is in agreement with recent published data suggesting a role for AT1-R in the transmission of mechanical signals (30, 40, 41). Although our work clearly demonstrated the EGFR as a key element in the downstream signaling to PKB/Akt, the measured phosphorylation signals were moderate compared with those of PKB/Akt. This implies a signal amplification as suggested by others (42, 43). According to this concept, the activity of EGFR, although very small, is sufficient for the induction of downstream elements.

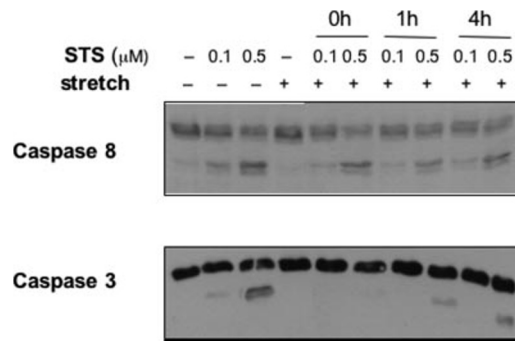


FIG. 9. **Stretching suppresses staurosporine-induced cleavage of caspase 8 and caspase 3.** Serum-starved HaCaT cells were treated with 0.1 or 0.5  $\mu\text{M}$  staurosporine with or without additional stretch application. Lanes 2 and 3 show the effect of staurosporine alone on the cleavage of caspases 8 and 3. In lanes 5 and 6 the addition of staurosporine and stretch application were at the same time (0 h). In lanes 7 and 8 staurosporine was added 1 h after stretch application, and in lanes 9 and 10 staurosporine was added 4 h after stretch application. Protein extracts were prepared after 16 h and tested for caspase 8 and 3 fragments. STS, staurosporine. The blot shows representative results ( $n = 3$ ).

Finally our studies provided initial evidence that activation of PKB/Akt signaling by mechanical stretch contributes to suppression of apoptosis. These findings contribute to the molecular understanding of the clinically well known hyperplastic effects induced by mechanical stretch.

**Acknowledgments**—We thank the late Dr. Robert Elez for inspiration and helpful discussions. His tremendous intellectual vision will always be sorely missed. We also thank Dr. Albrecht Piiper for insightful discussions. We are grateful to Dr. Adrian Sewell for critical reading of the manuscript.

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