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Felodipine inhibits nuclear translocation of p42/44 mitogen-activated protein kinase and human smooth muscle cell growth

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Abstract

Objective: Smooth muscle cell (SMC) proliferation contributes to vascular structural changes in cardiovascular disease. Ca^{2+} antagonists exert antiproliferative effects and may also be clinically beneficial in the patients. However, the underlying mechanisms of action remain elusive. Activation of mitogen-activated protein kinases (MAPK), in particular p42/44mapk plays a central role in cell proliferation. We hypothesise that Ca^{2+} antagonists inhibit cell proliferation by interfering with the p42/44mapk pathway in human SMC. **Methods:** SMC were cultured from human aorta. Cell proliferation was analysed by [^{3}H]thymidine incorporation. Activation of p42/44mapk and the nuclear target protein Elk-1 was analysed by phosphorylation and p42/44mapk nuclear translocation by confocal microscope. **Results:** PDGF-BB (10 ng/ml) stimulated [^{3}H]thymidine incorporation, phosphorylated p42/44mapk, caused nuclear translocation of the enzymes and phosphorylated the nuclear target protein Elk-1. Felodipine (10^{-7} to 10^{-5} mol/l) inhibited [^{3}H]thymidine incorporation to PDGF-BB, had no effect on p42/44mapk phosphorylation. However, p42/44mapk nuclear translocation and Elk-1 activation stimulated by PDGF-BB were prevented by the Ca^{2+} antagonist. **Conclusion:** Activation of p42/44mapk, subsequent nuclear translocation and activation of Elk-1 are essentially associated with human SMC proliferation. The Ca^{2+} antagonist felodipine prevents p42/44mapk nuclear translocation (but not its activation) associated with inhibition of human SMC growth.

Keywords: Growth factors; Protein kinases; Protein phosphorylation; Smooth muscle

1. Introduction

Vascular smooth muscle cell (SMC) growth plays an important role in intimal thickening in atherosclerosis and restenosis as well as in venous bypass graft disease [1,2]. Furthermore, the process contributes to certain forms of vascular remodeling in hypertension [3]. Platelet-derived growth factor (PDGF) released mainly from activated platelets or produced by vascular cells is an important growth factor of SMC and involved in restenosis after angioplasty in animal models and in patients [4,5].

Ca^{2+} antagonists exert beneficial effects in patients with coronary artery disease which are thought at least in part to be due to inhibition of SMC proliferation. Previous studies demonstrated that expression of proto-oncogenes induced by growth factors are inhibited by Ca^{2+} antagonists [6]. However, the exact molecular mechanisms have not been elucidated, yet.

Mitogen-activated protein kinases (MAPKs) are important mediators of a variety of cellular responses to growth factors, hormones and cytokines [7]. The best studied p42/p44mapk cascade plays a pivotal role in proliferation of various cells including human SMC [8,9]. p42/p44mapk is activated by dual phosphorylation of threonine and tyrosine residues, achieved by the dual-specificity

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kinase MEK1/2. Whereas MEK1/2 remains in the cytoplasm, p42/p44mapk are translocated from the cytoplasm to the nucleus upon stimulation with growth factors [10–13]. This process allows activation of nuclear transcription factors such as c-Fos and Elk-1 and thereby induces downstream gene expression and cell cycle progression [14–16].

We hypothesised that the integrity of the p42/44mapk pathway (i.e. its activation and subcellular relocalisation) is essential for human SMC growth. Ca^{2+} antagonists such as felodipine may interfere with the p42/44mapk pathway and thereby arrest cell growth.

2. Methods

2.1. Chemicals and materials

Bovine serum albumin (BSA, 7.5%), a monoclonal antibody against α-smooth muscle actin and all chemicals for immunoblotting were from Sigma (Buchs, Switzerland); recombinant PDGF-BB was from R&D System GmbH (Wiesbaden-Nordenstadt, Germany); all tissue culture materials and media were from Gibco Life Technol (Basel, Switzerland); [^{3}H]methyl-thymidine was from Amersham Pharmacia Biotech Europe GmbH (Dübendorf, Switzerland); trichloroacetic acid was from Fluka (Buchs, Switzerland); rabbit anti-p42/44mapk (#9102) and anti-phospho-p42/44mapk antibodies (#9101), and rabbit anti-phospho-Elk-1 antibody (#9181) were from Cell Signaling Technol (Allschwil, Switzerland). Felodipine was kindly provided by AstraZeneca AG (Zug, Switzerland).

2.2. Cell culture

Human aortic SMCs were purchased from Clonetics Corp. The cells were cultured in DMEM containing 10% FCS supplemented with 20 mmol/l L-glutamine and HEPES buffer solution, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere (37°C; 95% air/5% CO_2) [17]. Cells were passaged by 0.01% EDTA-trypsin. The medium was changed every 2 to 3 days. All experiments were performed between passages 3 and 5.

2.3. DNA synthesis

SMC were seeded on 12-well plates at a density of 2×10^4/well and rendered quiescent in culture medium containing 0.2% BSA for 48 h. Cells were then stimulated with PDGF-BB (10 ng/ml) for 24 h in the presence or absence of felodipine (10^{-7} to 10^{-5} mol/l). DNA synthesis was measured by [^{3}H]thymidine incorporation (1 μCi/ml; 70–85 Ci/mol), as previously described [17].

2.4. Activation of p42/44mapk and Elk-1 activation

Activation of p42/44mapk and Elk-1 activation was analysed by phosphorylation of the enzymes and nuclear factor on Western blots using anti-phospho-p42/44 and anti-phospho-Elk-1 antibodies (1:1000). p42mapk activation was also analysed by the slower mobility of the phosphorylated form on Western blot using anti-p42mapk antibody. Staining of the membranes with Ponseau S was performed to make sure that the protein loading was the same. The cells were stimulated with PDGF-BB (10 ng/ml) for 15 min in the presence or absence of felodipine (10^{-5} mol/l) and then harvested in extraction buffer as described [17]. Twenty μg of the cell lysates were subjected to 10% SDS–PAGE for electrophoresis. Western blotting was performed as described and phosphorylation of p42/44mapk was visualised by ECL system (Amersham Pharmacia Biotech Europe GmbH).

2.5. p42/44mapk nuclear translocation

2.5.1. Immunofluorescence labeling

Quiescent SMC were cultured on coverslips and stimulated with PDGF-BB (10 ng/ml) for the time periods indicated. They were then washed twice with modified Ca^{2+}-free Hank's buffer (MHB) containing 2 mM EGTA and 5 mM MES (2-morpholino-ethanesulfonic acid, pH 6.2 to 6.4) and quickly substituted with 'permeabilization buffer', i.e. MHB containing 2% octyl-POE (*n*-octylpolyoxyethylene; Alexis, Switzerland) and 0.125% glutaraldehyde (Electron Microscopy Sciences, USA). After 5 min of permeabilization and prefixation, cells were fixed for 20 min with MHB containing 1% glutaraldehyde. The cells were then washed 3 to 4 times with MHB. Aldehyde groups were reduced by treating the cells twice for 10 min each with $NaHB_4$ (0.5 mg/ml) in MHB on ice. Immunofluorescence labeling was carried out as described [18] by incubating the cells with appropriate concentrations of primary (anti-p42/44mapk, 1:250) and fluorochrome-conjugated secondary antibodies (1:500) for 1 h each. Finally, the cells were washed with MHB, mounted bottom-up (i.e. inverted prior to mounting) in Mowiol 488 (Hoechst, Germany) containing 0.75% *n*-propyl-gallate as an anti-bleaching agent. Mounted slides were left to dry for 24 h at room temperature in the dark, and then stored at 4°C in the dark until viewed.

2.5.2. Confocal laser scanning microscopy

Micrographs were taken with a confocal microscope consisting of a Zeiss Axiovert fluorescence microscope with a Zeiss Plan Apo 63/1.4 oil objective lens and an Odyssey XL confocal laser-scanning unit (NORAN, USA), driven by the *Intervision* software package run on an INDY workstation (Silicon Graphics Inc., USA). The light source was an argon laser, tuned so that the excitation

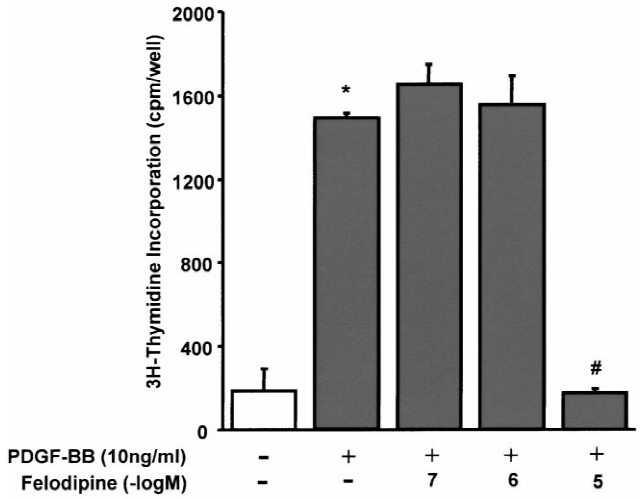

Fig. 1. Felodipine inhibited [^{3}H]thymidine incorporation in human SMC: stimulation of the cells with PDGF-BB (10 ng/ml) for 24 h markedly enhanced [^{3}H]thymidine incorporation in human VSMC, which was inhibited by felodipine (10^{-7} to 10^{-5} mol/l; $n=3$). *$P<0.05$ vs. control, $^{\#}P<0.005$ vs. PDGF.

wave length for Cy3 was 529 nm, and that for FITC and Cy2 488 nm. The confocal images were processed with the *Imaris* software from Bitplane AG (Zürich, Switzerland).

2.6. Statistics

All measurements are presented as means±S.E.M. Stimulation of [^{3}H]thymidine incorporation was expressed as mean±S.E.M. In all experiments, n equals the number of independent experiments. ANOVA followed by Scheffe's test for repeated measurements were used. A two-tailed P value ≤0.05 was considered statistically significant.

3. Results

Stimulation of human smooth muscle cells with PDGF-BB (10 ng/ml) for 24 h enhanced [^{3}H]thymidine incorporation from 186±110 to 1493±26 cpm/well ($n=3$, $P<0.05$; Fig. 1). This process was prevented in the presence of the Ca^{2+} antagonist felodipine (10^{-7} to 10^{-5} mol/l, 174±20 cpm/well; $n=3$, $P<0.005$ vs. PDGF alone, Fig. 1).

The stimulation of [^{3}H]thymidine incorporation by the growth factor PDGF-BB (10 ng/ml, 15 min) was associated with phosphorylation of p42/44mapk (Fig. 2A). Activation of p42/44mapk (also measured by slower mobility of activated p42mapk on the gel), however, was not influenced by felodipine (10^{-5} mol/l; Fig. 2A).

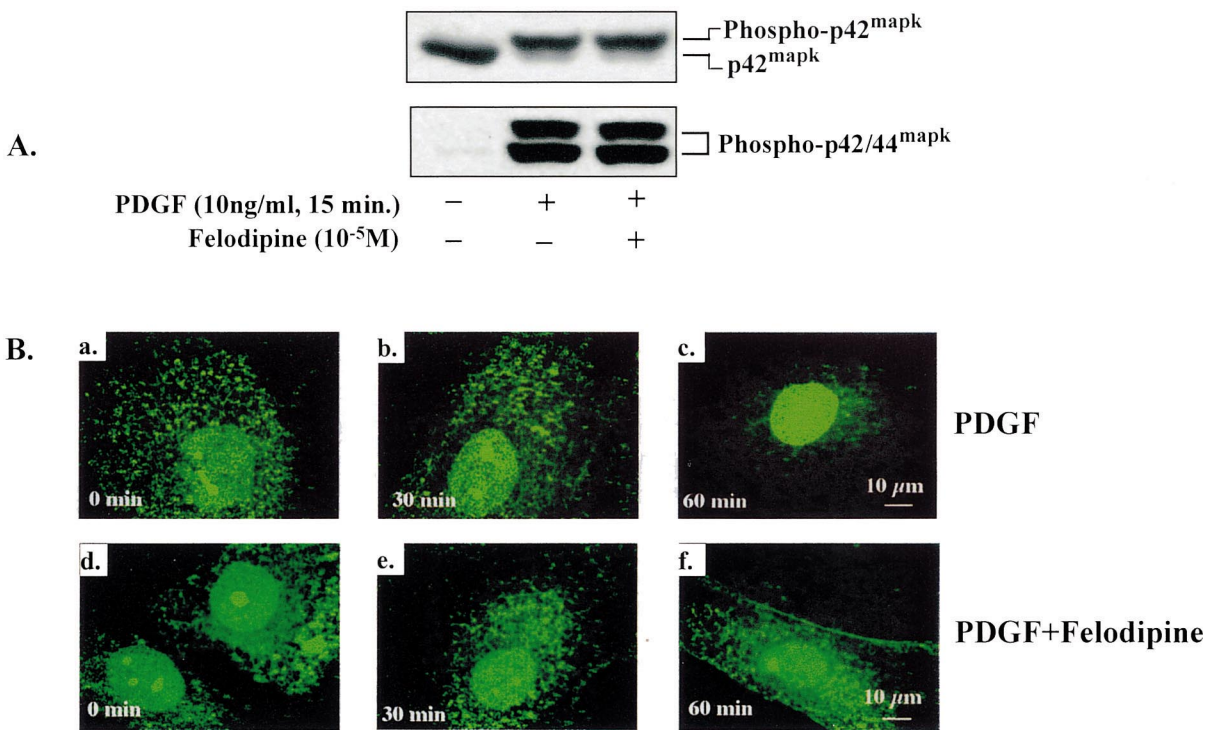

Fig. 2. Effects of felodipine on activation and nuclear translocation of p42/44mapk in human VSMC: (A) PDGF-BB (10 ng/ml, 15 min) phosphorylated p42/44mapk as measured directly by anti-phospho-p42/44mapk (lower panel) or slower mobility (upper panel) of p42mapk on 10% SDS–PAGE, which was not influenced by felodipine (10^{-5} mol/l). (B) PDGF-BB (10 ng/ml) induced nuclear translocation at 30 (inset b) and 60 min (inset c) which was prevented by felodipine (10^{-5} mol/l; inset e and f).

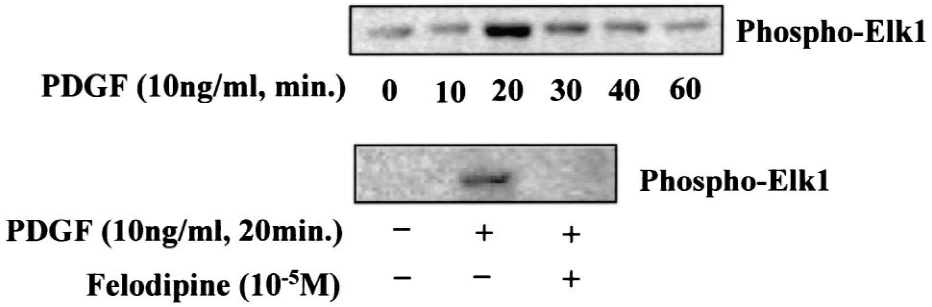

Fig. 3. Time course of PDGF-BB-induced Elk-1 phosphorylation (upper panel). The phosphorylation of Elk-1 induced by the growth factor was prevented by felodipine (10^{-5} mol/l, lower panel). The experiments were repeated three times.

PDGF-BB (10 ng/ml) induced translocation of p42/44mapk from the cytoplasm to the cell nucleus (Fig. 2B). This process could be documented by confocal microscopy 30 (inset b) and 60 min (inset c) after stimulation with the growth factor compared to baseline (inset a). Nuclear translocation was inhibited by felodipine (10^{-5} mol/l) at 30 and 60 min (insets e and f) as illustrated by a representative experiment ($n=3$).

Furthermore, one of the nuclear protein substrates of p42/44mapk, Elk-1, was phosphorylated by PDGF-BB (10 ng/ml; Fig. 3). Phosphorylation of Elk-1 reached its peak at 20 min and this process was inhibited by felodipine (10^{-5} mol/l; Fig. 3). This further confirmed that the entry of p42/44mapk into the nucleus was prevented by the Ca^{2+} antagonist felodipine.

4. Discussion

In this study we were able to further delineate the molecular mechanisms underlying human SMC proliferation and its inhibition by a Ca^{2+} antagonist. In particular, we could confirm the antiproliferative properties of this class of drugs and, for the first time, demonstrate that nuclear translocation of phosphorylated p42/44mapk and subsequent activation of Elk-1 are essentially associated with human SMC proliferation. The Ca^{2+} antagonist felodipine prevents p42/44mapk from entering the nucleus, while the drug does not interfere with the activation of the kinases. As a consequence, the nuclear protein Elk-1 cannot get phosphorylated. These effects of felodipine are associated with inhibition of human SMC proliferation.

Mitogen-activated protein kinases (MAPKs), in particular p42/44mapk, play a critical role in transmitting extracellular signals into the cell nucleus to initiate the expression of a number of genes responsible for cell proliferation [7–9]. Upon stimulation with growth factors p42/44mapk are phosphorylated at tyrosine and threonine residues by the dual specific upstream kinases MEK1/2. The phosphorylated or activated p42/44mapk then enter into the cell nucleus and phosphorylate a number of transcription factors such as TCF/Elk-1 resulting in c-Fos production, which then modulates transcription of other target genes via AP-1 complex formation [14,19].

Indeed, in the present study we demonstrated that in human SMC enhanced DNA synthesis induced by PDGF was associated with phosphorylation of p42/44mapk and subsequent translocation of the kinases from the cytoplasm into the nucleus. Moreover, this process also involved activation of the nuclear protein target Elk-1. The Ca^{2+} antagonist felodipine prevented DNA synthesis induced by PDGF as previously described for most compounds of this class [20], yet had no effect on p42/44mapk phosphorylation or activation. The fact that felodipine inhibited p42/44mapk entry into the cell nucleus and phosphorylation of the nuclear protein Elk-1 without inhibition of p42/44mapk activation as shown by confocal microscopy and Western blot using specific antibodies indicates that translocation of p42/44mapk from cytoplasm to the nucleus instead of activation of the enzymes was blocked by the Ca^{2+} antagonist. Thus, in human SMC, entry of activated p42/44mapk into the cell nucleus is essentially associated with cell proliferation. The necessity of p42/44mapk nucleus translocation in cell proliferation has also been observed in fibroblasts [8] and PC12 cells [21].

The mechanism of p42/44mapk translocation into the nucleus is not completely understood. It is proposed that phosphorylation of p42/44mapk is associated with and has been proposed as an essential step for the nuclear accumulation [22,23]. However, our present study showed that this association could be pharmacologically interrupted. The results also indicate that analysis of integrity of p42/44mapk pathway should include activation of the enzymes and nuclear translocation as well. It remains to be investigated which mechanisms are influenced by the Ca^{2+} antagonist and whether inhibition of p42/44mapk nuclear translocation is a general mechanism of Ca^{2+} antagonists requires testing of other members of this class of drugs.

Ca^{2+} antagonists are widely used in hypertension and angina and exhibit an array of biological effects in the cardiovascular system. In animal models and in patients, the drugs are able to prevent or reduce endothelial dysfunction in atherosclerosis and hypertensive remodel-

ling of resistance arteries [24–26]. The cellular mechanisms delineated in this study might be important for the ability of these drugs to prevent or reverse structural vascular changes in atherosclerosis, hypertension and restenosis. It may play an essential role in restenosis. The fact that clinical trials revealed inconsistent results in this condition [27] may relate to low local concentrations of the drug with systemic application. Indeed, the concentrations required to block p42/44mapk nuclear translocation might be higher than those commonly achieved in vivo to lower blood pressure or relieve angina. Thus, local drug delivery via specially designed balloons or drug-eluting stents might be a promising approach.

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