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Prolidase-Independent Mechanism of Camptothecin-Induced Inhibition of Collagen Biosynthesis in Cultured Human Skin Fibroblasts

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The present study was undertaken to evaluate the mechanism of campthotecin (CPT)-induced deregulation of collagen metabolism in cultured human skin fibroblast. It has been found that CPT strongly induced inhibition of collagen biosynthesis. The mechanism of this phenomenon was found to be independent of prolidase activity, an enzyme that plays an important role in enhancement of collagen biosynthesis at post-translational level. In fact, the enzyme activity was found to be stimulated by CPT. Increase in the enzyme activity was accompanied by increase in the expression of β_1 integrin receptor and some β_1 integrin-dependent signalling proteins, Sos, MAPK (ERK_1, ERK_2) and transcription factor NF-κB. Since activation of β_1 integrin induces NF-κB that inhibits collagen gene transcription, therefore the mechanism of CPT-dependent inhibition of collagen biosynthesis may be related to β_1 integrin-dependent stimulation of NF-κB. Supporting evidence comes from experiments showing that specific MEK/ERK inhibitor (UO126) inhibited CPT-induced up-regulation of prolidase activity while it had no effect on CPT-induced inhibition of collagen biosynthesis and activation of NF-κB. The data suggest that CPT induces inhibition of collagen biosynthesis in cultured human skin fibroblasts by stimulation of NF-κB signalling.

Key words: camptothecin, collagen metabolism, integrin signalling, prolidase.

Abbreviations: BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate reagent; CPT, camptothecin; DMEM, Dulbecco's minimal essential medium; EDTA, ethylenediaminetetraacetic acid; ERK_1 and ERK_2, extracellular-signal-regulated kinase 1 and kinase 2; FAK, non-receptor focal adhesion kinase $pp125^{FAK}$; FBS, fetal bovine serum; Grb2, growth-factor receptor-bound protein 2; MAPK, mitogen activated protein kinases; MEK, mitogen/extracellular signal regulated kinase; NF-κB, nuclear transcription factor κB; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; Raf, protein kinase signaling proteins; Ras, GTP-binding protein; SDS, sodium dodecylsulfate; Shc, Src, non-receptor tyrosine kinases signaling proteins; Sos, son of sevenless protein; TBS-T, Tris buffered saline with Tween 20.

Collagen is the most abundant extracellular matrix (ECM) protein in mammals, responsible for maintenance of architecture and integrity of connective tissue. It also plays an important role in interaction with cell surface integrin receptors, through which it may participate in regulation of numerous physiological and pathological processes (*1*). The family of integrin receptors is responsible for ECM–cell as well as cell–cell interaction (*1*), cytoskeleton reorganization (*2*), intracellular ion transport, lipid metabolism, kinase activation, gene expression (*3*), cell cycle regulation (*4*), synthesis of collagen and metalloproteases (*5*) and cancer metastasis (*6*). Therefore any changes in collagen biosynthesis and degradation may potentially influence cellular metabolism.

It has been suggested that, collagen receptor, $\alpha_2\beta_1$ integrin is involved in signalling that regulates collagen production (*7*). We considered prolidase as a possible target for the β_1-integrin-dependent regulation of collagen biosynthesis. It is known that prolidase activity is regulated by β_1-integrin receptor (*8*).

Prolidase (EC.3.4.13.9) is a cytosolic imidodipeptidase, which specifically splits imidodipeptides with C-terminal proline or hydroxyproline (*9*). The enzyme plays an important role in the recycling of proline from imidodipeptides (derived from degradation products of collagen) for collagen resynthesis (*10*) and cell growth (*11*). The efficiency of proline recycling was found to be about 90% (*12*). Therefore, the regulation of the enzyme activity is of considerable interest.

Previously, we provided evidence that prolidase activity is regulated by β_1-integrin receptor (*8, 13*). Stimulated β_1-integrin receptor induces autophosphorylation of non-receptor protein kinase FAK (*14*), which is then capable of interacting with adaptor-proteins, such as Grb2, through Src and Shc proteins. This interaction allows activating further cascade of signalling pathway through Sos, Ras and Raf proteins (*15*) and subsequently, two MAP kinases: ERK_1 and ERK_2 (*16*). The end point of this cascade is induction of transcription factor(s) that

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regulate(s) gene expression of integrins, proteinases and many proteins involved in regulation of cell growth and differentiation (*17*).

Despite significant progress towards understanding the regulatory mechanisms of collagen metabolism, effective treatment of diseases that are accompanied by excessive collagen production (tissue fibrosis, scleroderma) is still lacking.

One of the agents that have been recently proposed as a selective inhibitor of collagen biosynthesis is camptothecin (CPT) (*18*). This is alkaloid from *Camptotheca* tree that has been proved as an anti-tumour agent in experimental models and as strong inhibitor of topoisomerase I (*19*). Some CPT derivatives such as topotecan and irinotecan are currently used in the treatment of various cancers (*19*). The mechanism of inhibitory action of CPT on collagen biosynthesis has not been fully explored.

It has been found that at least in some cell types activation of β_1-integrin receptor signalling induces activation of nuclear transcription factor NF-κB (*20*). It has been also suggested that CPT may inhibit collagen gene transcription by a mechanism involving activation of NF-κB (*21*).

The present study was undertaken to evaluate the effect of CPT on collagen biosynthesis, prolidase activity, as well as expressions of prolidase, β_1-integrin receptor, and some signalling proteins including SOS protein, MAP-kinases and nuclear transcription factor NF-κB in fibroblasts, the potent collagen-synthesizing cells.

MATERIALS AND METHODS

Materials—CPT, Anti–Goat IgG antibody, Anti–Mouse IgG antibody, aprotinin, bacterial collagenase, Fast BCIP/NBT reagent, L-glycyl-proline, L-proline, leupeptin, monoclonal (mouse) anti-phosphorylated MAPK antibody, Nonidet P-40, and phenylmethylsulfonyl fluoride were provided by Sigma Corp., USA., as were most other chemicals and buffers used. Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in cell culture were products of Gibco, USA. Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc., USA. Nitrocellulose membrane (0,2 μm), sodium dodecylsulphate (SDS), polyacrylamide, molecular weight standards were received from Bio-Rad Laboratories, USA. L-5[^{3}H] proline (28 Ci/mmol) was purchased from Amersham, UK. Monoclonal (mouse) anti-SOS and anti-FAK antibodies were obtained from Becton Dickinson Co., USA. Monoclonal (mouse) anti-β_1-integrin antibody was obtained from ICN Biomedicals Inc., USA. Polyclonal (rabbit) anti-NF-κB antibody and (goat) anti-β-actin antibody were the products of Santa Cruz Biotechnology Inc., USA. Polyclonal (rabbit) anti-human prolidase antibody was the gift from Dr James Phang (NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA). Anti-Rabbit IgG were obtained from Promega Corp., USA. NE-PER nuclear and cytoplasmic extraction reagents kit was from Pierce, USA.

Tissue Culture—All studies were performed on normal human skin fibroblasts (CRL-1474), purchased from American Type Culture Collection, Rockville, MD. The cells were maintained in DMEM supplemented with 5% (FBS), 2 mmol/l glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a 5% CO_2 incubator.

Determination of Prolidase Activity—The activity of prolidase was determined according to the method of Myara (*22*) which is based on measurement of proline by Chinard's reagent (*23*). Briefly, the monolayer was washed three times with 0.15 M of NaCl. Cells were harvested by scraping and suspended in 0.15 M NaCl, centrifuged at low speed ($200 \times g$) and the supernatant was discarded. The cell pellet was suspended in 0.3 ml of 0.05 M Tris-HCl, pH 7.8, and sonicated three times for 10 s at 0°C. Samples were then centrifuged ($16{,}000 \times g$, 30 min.) at 4°C. Supernatant was used for protein determination and then prolidase activity assay. Activation of prolidase required incubation with manganese; 100 μl of supernatant incubated with 100 μl of 0.05 M Tris-HCl, pH 7.8 containing 2 mM MnCl (II) for 2 h at 37°C. The prolidase reaction was initiated by adding 100 μl of the incubated mixture to 100 μl of 94 mM glycyl–proline (Gly–Pro) for a final concentration of 47 mM Gly–Pro. After additional incubation for 1 h at 37°C, the reaction was terminated with 1 ml of 0.45 M trichloroacetic acid. In parallel tubes reaction was terminated at time 'zero' (without incubation). The released proline was determined by adding of 0.5 ml of the trichloroacetic acid supernatant to 2 ml of a 1:1 mixture of glacial acetic acid: Chinard's reagent (25 g of ninhydrin dissolved at 70°C in 600 ml of glacial acetic acid and 400 ml of 6 M orthophosphoric acid) and incubated for 10 min at 90°C. The amount of proline released was determined colorimetrically by absorbance at 515 nm and calculated by using proline standards. Protein concentration measured by the method of Lowry (*24*). Enzyme activity was reported as nanomoles per minute per milligram of supernatant protein.

Collagen Production—Incorporation of radioactive precursor into proteins was measured after labelling of confluent cells in growth medium with CPT for 24 h with 5[^{3}H]proline (5 μCi/ml, 28 Ci/mM) as described previously (*25*).

Cytotoxicity Assay—Toxicity of CPT was determined by method of Plumb (*26*). Fibroblasts were maintained as described above. After 24 h incubation of the cells with CPT the medium was discarded and the cells were rinsed three times with phosphate buffered saline (PBS). Then the cells were incubated for 4 h in 2 ml of PBS with 50 μl of MTT (5 mg/ml). Medium was removed from the wells, and the cells were lyzed in 200 μl of DMSO with 20 μl of Sorensen's buffer (0.1 M glycine with 0.1 M NaCl, pH 10.5). The absorbance was measured at 570 nm.

Protein Extraction—Confluent human dermal fibroblasts were incubated in growth medium with various concentrations of CPT for 24 h or with 50 mM CPT for 0.5, 1 and 3 h or treated with various concentrations of CPT for 3 h in a 5% CO_2 incubator at 37°C. Then 3×10^6 cells were used for nuclear extracts isolation applying NE-PER nuclear and cytoplasmic extraction kit (Pierce, USA), according manufacturer's protocol.

SDS-PAGE—Slab SDS/PAGE was used, according to the method of Laemmli (*27*). Samples of cell supernatants (25–50 μg of protein) were incubated for 10 min at 100°C in 62.5 mmol/l Tris-HCl, pH 6.8, containing 2.0% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.001% bromophenol blue. Samples were electrophoresed on a Tris-Glycine gels at 100 V per gel for 30 min at room temperature.

Western Immunoblot Analysis—After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris, 0.2 mol/l glicine in 20% (v/v) methanol. The protein was transferred to 0.2 μm pore-sized nitrocellulose at 100 mA for 90 min using a BioRad electrophoresis unit according to the method described in the manual accompanying the unit. The membrane was blocked with 5% dried milk in TBS-T for 1 h in room temperature, slowly shaking. Then the nitrocellulose was incubated with monoclonal antibody against β_1-integrin, FAK, SOS or phosphorylated MAPK at concentration 1:5000 or polyclonal antibodies against prolidase, β-actin or NF-κB at concentration 1:1000 overnight. After the incubation, nitrocellulose was washed with TBS-T (1×15 min and 2×10 min) intensively shaking. In order to analyse the proteins second alkaline phosphatase labelled antibodies against mouse's Fc IgG or against rabbit's IgG or goat's IgG were added at concentration 1:2500 in TBS-T and incubated for 1 h slowly shaking. Then the membrane was extensively washed with TBS-T (5×10 min) and submitted to BCIP/NBT Western Immunoblotting detection system.

Statistical Analysis—In all experiments, the mean values for six independent experiments ± SD were calculated. The results were submitted to statistical analysis using Student's *t*-test, accepting $P < 0.05$ as significant.

RESULTS

Collagen biosynthesis and prolidase activity were measured in confluent human dermal fibroblasts that has been treated with 0.1, 5.0 and 50 μM of CPT. As can be seen on Fig. 1A, 24 h incubation of fibroblasts with CPT contributed to decrease in collagen biosynthesis in a dose-dependent manner. At 50 μM of CPT the process was inhibited to about 15% of control. The effect was unrelated to the toxicity of CPT as shown by MTT assay (Fig. 1C). An opposite effect was observed in the activity of fibroblast's prolidase (Fig. 1B). Increase in the concentration of CPT induced increase in the prolidase activity. The data suggest that CPT-induced collagen biosynthesis inhibition and CPT-induced prolidase activity stimulation may undergo through independent mechanisms.

Prolidase activity is regulated due to the signal induced by activated β_1-integrin receptor (*8*). In cells treated for 24 h with CPT slight increase in the receptor expression was noticed (Fig. 2A), while distinct increase in the expression of β_1-integrin-dependent signalling proteins was found, compared with control cells, as shown by Western Immunoblot analysis. As shown on Fig. 2, CPT at all studied concentrations induced distinct increase in the expression of FAK, Sos and MAPKs. However, inhibition of MAP-kinases (ERK1 and ERK2)

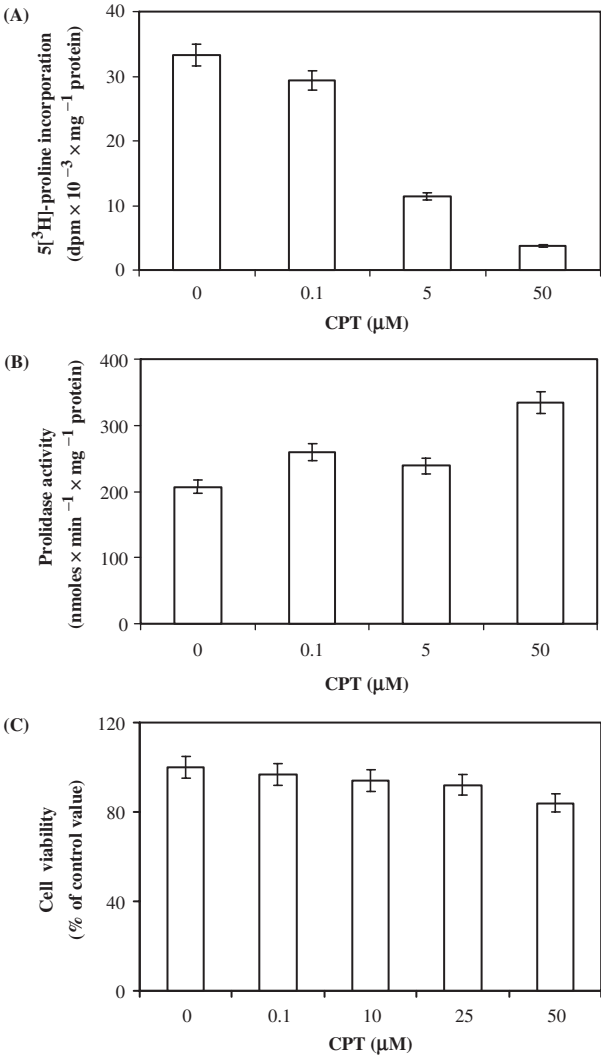

Fig. 1. **Collagen biosynthesis (A), prolidase activity (B) and cell viability (C) in human skin fibroblasts treated for 24 h with various concentrations of CPT.** The mean values of three independent experiment ± SD are presented.

by specific UO126 inhibitor did not affect collagen biosynthesis in fibroblasts as well as it did not counteract inhibitory effect of CPT on the biosynthesis of this protein (Fig. 3A). Moreover, in CPT-treated cells the UO126 inhibited expression of MAP-kinases (Fig. 3B) without effect on CPT-induced inhibition of collagen biosynthesis (Fig. 3A). It suggests that CPT-induced inhibition of collagen biosynthesis is independent of MAPK signalling cascade that stimulate prolidase activity. In fact, an addition of UO126 to CPT-treated fibroblasts did not affect significantly prolidase activity (Fig. 4). UO126 at 50 μM contributed to slight inhibition of prolidase activity while in the presence of CPT it restored the activity over the control value.

Since collagen biosynthesis inhibition induced by CPT is unrelated to prolidase activity we considered nuclear transcription factor NF-κB as a possible target for CPT-dependent inhibition of this protein. In fact it has been suggested that CPT may inhibit collagen gene

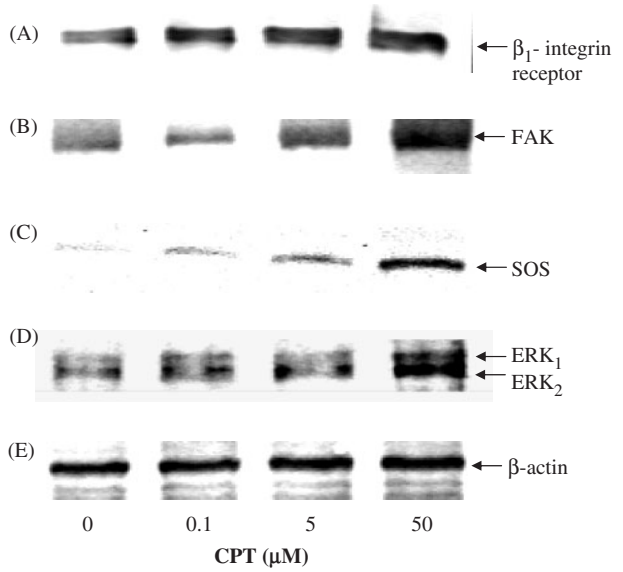

Fig. 2. **Western Immunoblot analysis of β_1-integrin receptor (A), FAK-kinase (B), SOS (C), phosphorylated MAPK (ERK_1, ERK_2) (D), β-actin (E) in cell extract of human skin fibroblasts treated for 24 h with various concentrations of camptotecin.**

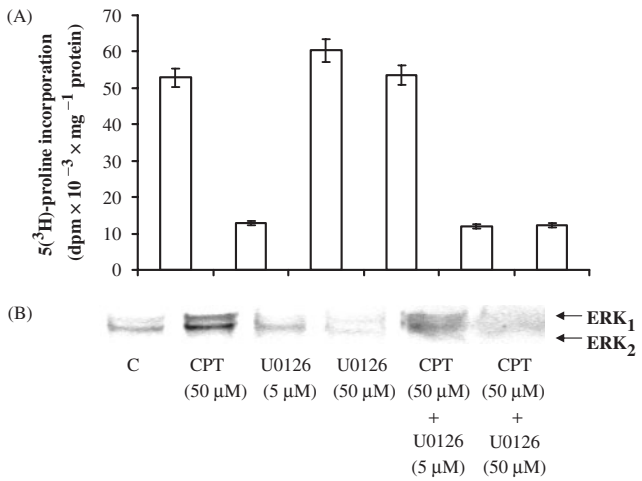

Fig. 3. **(A) Collagen biosynthesis in fibroblasts treated for 24 h with 50 μM camptothecin and various concentrations of MAPK inhibitor U0126.** The mean values of three independent experiment ± SD are presented. (B) Western Immunoblot analysis of phosphorylated MAPK (ERK_1, ERK_2) in cell extract of human skin fibroblasts treated for 24 h with 50 μM camptothecin and various concentrations of MAPK inhibitor U0126.

transcription by a mechanism involving activation of NF-κB (*21*). Moreover at least in some cell types activation of β_1-integrin receptor signalling induces activation of nuclear transcription factor NF-κB (*20*). Therefore, the expression of NF-κB was studied in CPT-treated fibroblasts.

Fibroblasts were incubated with 50 μM CPT for 0.5, 1 and 3 h, nuclear fractions were isolated and submitted for Western Immunoblot analysis using anti-NF-κB (p65) antibodies. As shown on Fig. 5A incubation of the

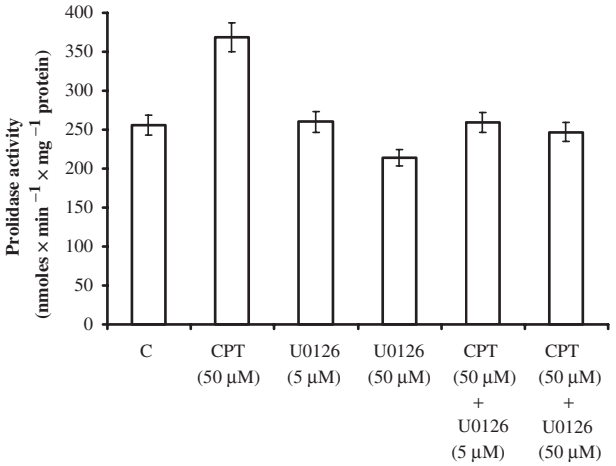

Fig. 4. **Prolidase activity in fibroblasts treated for 24 h with 50 μM and various concentrations of MAPK inhibitor U0126.** The mean values of three independent experiment ± SD. are presented.

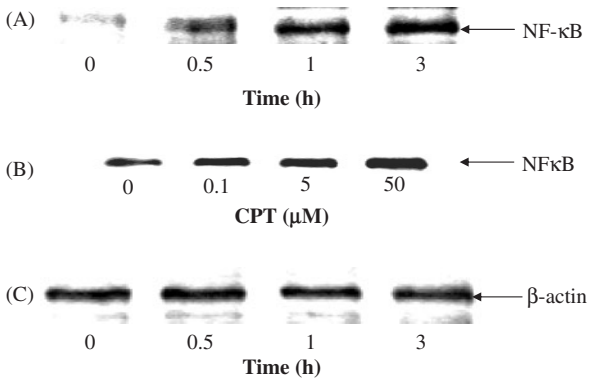

Fig. 5. **Time course experiment for fibroblasts treated with 50 μM CPT.** 15 mg of total proteins form nuclear extracts were submitted to Western Immunoblot analysis of NF-κB (A) Western Immunoblot analysis of NF-κB in cell extract of human skin fibroblasts treated for 3 h with various concentrations of CPT (B), 15 mg of total proteins from nuclear extracts were submitted to Western Immunoblot analysis for β-actin as a loading control (C).

cells with CPT lead to activation and translocation of NF-κB to nucleus after 0.5 h. No change of the β-actin expression was found in each group.

As can be seen on Fig. 5B human skin fibroblasts incubated with 50 μM CPT for 3 h expressed distinct increase in the amount of NF-κB protein compared with control cells, as evaluated by Western Immunoblotting. Increase in the NF-kB expression found in fibroblasts treated for 24 h with CPT was not affected by UO126 MAPK inhibitor (Fig. 6A). Conversely, CPT-induced increase in β1-integrin expression was counteracted by UO126 MAP-kinase inhibitor (Fig. 6B)

DISCUSSION

Integrins are family of adhesive receptors that are responsible for recognition and adhesion of cells to

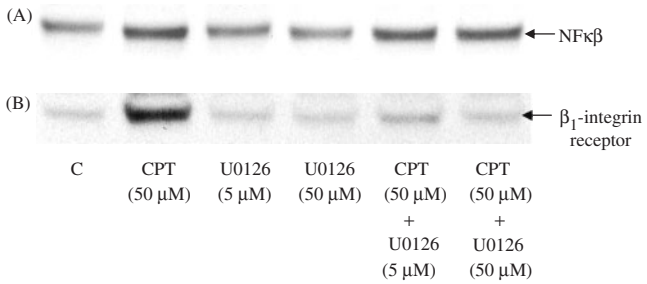

Fig. 6. **Western Immunoblot analysis of NF-κB (A) and β_1-integrin receptor (B) in cell extract of fibroblasts treated with 50 μM CPT and various concentration of U0126 for 24 h.**

ECM proteins (*1*). The interaction between integrin receptors and ECM proteins, e.g. collagen, can regulate cellular gene expression, differentiation, cell growth (*28, 29*) and can play an important role in tumorigenicity and invasiveness (*30, 31*). Therefore, modulation of integrin receptors signalling may play an important role in tissue metabolism and function.

Integrin receptor modulators have been recently extensively studied. Their ability to modulate cell–matrix and cell–cell interactions has been considered in many aspects. Some were found as a potent inhibitors of platelet aggregation (*32*), inhibitors osteoclast attachment and osteoclast-mediated bone resorption (*33*), modulators of human neutrophil chemotaxis (*34*), rat mesangial cell adhesion and proliferation (*35*) or myoblast fusion (*36*).

In this study we suggest, that CPT up-regulates β_1-integrin receptor expression and simultaneously down-regulates collagen biosynthesis in fibroblast. We found, that this phenomenon is accompanied by parallel increase in the expression of some signalling molecules induced by β_1-integrin receptor.

It is known, that in signalling pathway induced by stimulated β_1-integrin receptor several kinases and intracellular proteins are involved, including FAK, Grb-2, SOS, MAPK (ERK_1 and ERK_2). Stimulated β_1-integrin receptor induces autophosphorylation of kinase FAK (*14*). Although phosphorylated FAK-protein is incapable of direct phosphorylation of any substrates, it can interact with adaptor-protein Grb-2, containing SH_2-domains, which determine its ability to bind to proline-rich fragments of SOS-protein (*15*). Western Immunoblot analysis of selected signalling proteins in cells incubated in the presence of CPT showed significant increase in expression of SOS-protein as well as two phosphorylated MAP-kinases ERK_1 and ERK_2, compared with control.

Previously we have found that β_1-integrin-dependent signalling induces increase in prolidase activity that supports proline for collagen resynthesis. Lack of the enzyme impedes the efficient recycling of proline for collagen resynthesis (*12*) and cell growth (*11*). The efficiency of recycling of proline for collagen biosynthesis was found to be about 90% (*12*). Previously we found the link between collagen synthesis and prolidase activity in cultured skin fibroblasts treated with anti-inflammatory drugs (*37*), during experimental aging of the cells (*38*), fibroblasts chemotaxis (*39*) and cell surface integrin receptor ligation (*8*).

The discrepancy between increase in prolidase activity and decrease in collagen biosynthesis in fibroblasts treated with CPT can be explained at NF-κB level. In this study we provide evidence that CPT activate NF-κB in fibroblasts. The nuclear transcription factor is known to be involved in inhibition of collagen gene transcription (*21*). Recent study provided further evidence that NF-κB binds and inhibits both α1(I) and α2(I) collagen promoter (*21, 40*). On the other hand, NF-κB is induced by activated β_1-integrin receptor (*20*) that is known to stimulate prolidase activity (*8*). The result of present study suggests that CPT-induced up-regulation of prolidase activity may undergo through β1-integrin signalling cascade, since specific MEK/ERK inhibitor (UO126) inhibited this process. Conversely, the inhibitor had no effect on CPT-induced inhibition of collagen biosynthesis and activation of NF-kB. It seems that specific pathways of integrin signalling may play a dual role in the activation of the transcription factor and prolidase activity.

It is well established that binding of some types of cells to fibronectin results in the activation of NF-κB (*41, 42*). Moreover, stimulation of β_1 integrin in monocytic cells induces both NF-κB and MAPK activation (*43*). The authors of this study provided evidence that NF-κB and MAPK signalling pathway are separate and compete with each other (*43*). It cannot be excluded that different α subunits of integrin receptors may provide different moderating effects on β_1 integrin signalling. Good example is $\alpha_3\beta_1$ integrin signalling that preferentially activates MAPK pathway (*44*) which may compete with NF-κB pathway (*43*). Despite the high prolidase activity in CPT-treated fibroblast (that promote collagen biosynthesis at post-translational level) the inhibitory effect of NF-κB on collagen gene transcription may contribute to decrease in the protein synthesis. Therefore, it can be suggested that CPT or its derivatives may be considered as potential experimental agents in the therapy of collagen-related pathologies, like fibrosis.

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