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## Captopril-dependent inhibition of collagen biosynthesis in cultured fibroblasts

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The mechanism underlying the dermatological manifestations that accompany captopril therapy is not known. The facts that prolydase plays an important role in collagen biosynthesis and that captopril directly inhibits prolydase activity led us to evaluate its effect on collagen biosynthesis in cultured human skin fibroblasts. Confluent fibroblasts were treated with millimolar concentrations (0.2–1 mM) of captopril (CAP) for 48 h. It was found that CAP-dependent decrease in prolydase activity was accompanied by parallel decrease in collagen biosynthesis. Since insulin-like growth factor receptor (IGF-IR) is the most potent regulator of both collagen biosynthesis and prolydase activity, and prolydase is regulated by  $\beta_1$  integrin signaling, the effect of CAP on IGF-IR and  $\beta_1$  integrin receptor expressions was evaluated. It was found that exposure of the cells to 0.3 mM CAP contributed to a decrease in IGF-IR,  $\alpha_2\beta_1$  integrin receptor and MAPK/ ERK1/2 expressions. The data suggest that CAP-dependent decrease of collagen biosynthesis in cultured human skin fibroblasts results from inhibition of prolydase activity that may occur through inhibition of  $\alpha_2\beta_1$  integrin and IGF-IR signaling.

### 1. Introduction

Captopril (*N*-[(*S*)-3-mercapto-2-methylpropionyl]-L-proline), used as a competitive inhibitor of angiotensin-converting enzyme in the control of hypertension and congestive heart failure, affects skin metabolism (Peng et al. 2007). Structurally captopril is similar to dipeptides having L-proline as the C-terminal residue. Dipeptides of the x-pro type are substrates exclusively for prolydase. This dipeptidase [Prolydase EC.3.4.13.9] is present in all tissues and plays an important role in the recycling of proline from imidodipeptides (derived from degradation products of collagen) for collagen re-synthesis (Yaron and Naider 1993) and cell growth (Emmerson and Phang 1993). The efficiency of recycling of proline was found to be about 90% (Jackson et al. 1975). It is evident that an absence of prolydase severely impedes the recycling of collagen proline. Some clinical symptoms related to collagen deficit, such as skin lesions, can be attributed to prolydase deficiency (Freij et al. 1984). On the other hand, increased activity of liver prolydase was found during the fibrotic process (Myara et al. 1987). This suggests that the enzyme activity (despite collagen gene expression) may be a step-limiting factor in the regulation of collagen biosynthesis (Surażyński et al. 2008). Although it is known that prolydase is inhibited by captopril and that captopril therapy interferes with collagen metabolism (Ganapathy et al. 1985; Radzicka and Wolfenden 1991), the molecular mechanism of this process is not known. Collagen, which accounts for about one third of total body protein, is not only essential for the maintenance of connective tissue architecture. The interaction between cells and extracellular matrix (ECM) proteins, e.g., collagen, can regulate cellular gene expression, differentiation and growth (Bissel 1981; Carey 1991). The interaction is mediated by specific cell surface recep-

tors of the integrin family. The  $\alpha_2\beta_1$  integrin is known to be one of the main collagen receptors. Activation of this receptor by collagen ligation initiates the cascade of a signaling pathway including FAK, Src, Shc, Grb2, Sos, Ras, Raf and MAP kinases, ERK1 and ERK2 (Boudreau and Jones 1999). Decrease in collagen availability for integrin receptor interaction may therefore potentially alter cellular metabolism. Prolydase activity is stimulated through a signal mediated by collagen -  $\beta_1$  integrin receptor interaction (Palka and Phang 1997, 1998). This pathway is known to be involved in phosphorylation of several intracellular proteins.

Another important aspect of collagen biosynthesis regulation is at the level of insulin-like growth factor-I receptor (IGF-IR). IGF-I is one of the potent collagen-stimulating growth factors in collagen-synthesizing cells (Goldstein et al. 1989). Stimulation of IGF-I receptors induces activation of several signaling proteins, such as Grb2, Src and Shc, Sos, Ras, and Raf proteins, and subsequently, two MAP kinases: ERK1 and ERK2 (Werner and Le Roith 2000). The end point of this signaling is induction of transcription factors that regulate collagen gene expression. The way in which CAP may affect the processes is not known. The aim of this study is therefore to identify the cellular mechanisms of the effect of CAP on collagen biosynthesis in cultured human dermal fibroblasts.

### 2. Investigations and results

Collagen biosynthesis and prolydase activity were measured in confluent human dermal fibroblasts treated with 0.2 mM, 0.3 mM, 0.5 mM and 1 mM captopril (CAP). As can be seen in Fig. 1, 48 h incubation of confluent fibroblasts in medium

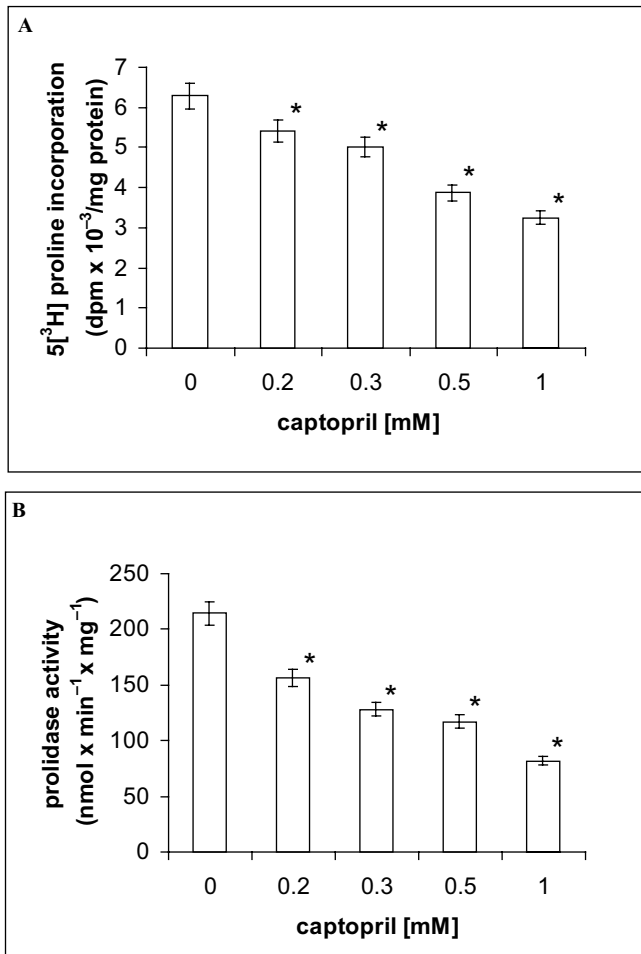


Fig. 1: Collagen biosynthesis (A) measured as  $5[^3\text{H}]$  proline incorporation into proteins susceptible to the action of bacterial collagenase and prolidase activity (B) in confluent human skin fibroblasts incubated for 48 h in medium containing 10% FBS and different concentrations of captopril (CAP). Results present mean values from 6 assays  $\pm$  S.D. \*  $P < 0.01$

containing 10% FBS and different concentrations of CAP contributed to decreased collagen biosynthesis (Fig. 1A) and prolidase activity (Fig. 1B) in a dose-dependent manner. At 0.3 mM CAP induced a decrease in collagen biosynthesis to about 80% and in prolidase activity to about 60% of control. The effect was not achieved in cells treated with CAP for 24 h (data not shown). The decrease in prolidase activity was not accompanied by a decrease in the expression of the enzyme as shown by Western immunoblot analysis (Fig. 2A).

The data show that CAP inhibits collagen biosynthesis in skin fibroblasts and suggest that the effect may result from inhibition of prolidase activity, but not prolidase expression.

Collagen biosynthesis and prolidase activity have been shown previously to be regulated by the signal induced by activated  $\alpha_2\beta_1$  integrin receptors (Palka and Phang 1997; Ivaska et al. 1999a) as well as by insulin-like growth factor-I receptors (IGF-IR) (Goldstein et al. 1989). Therefore, the expression of  $\alpha_2\beta_1$  integrin receptor (receptor for type I collagen) and of IGF-IR were measured by Western immunoblot analysis. As can be seen in Fig. 2 B and C, 48 h treatment of fibroblasts with 0.3 mM CAP contributed to a distinct decrease in the expression of  $\alpha_2$  and  $\beta_1$  integrin subunits, compared to the control cells. In addition, as shown in Fig. 3 A, a distinct decrease in IGF-I receptor expression was found in CAP-treated cells, compared to control cells. Since phosphorylation of MAP kinases ERK1 and ERK2 results from  $\alpha_2\beta_1$  integrin and IGF-I receptor signaling (Boudreau and Jones 1999; Valentini and Baserga

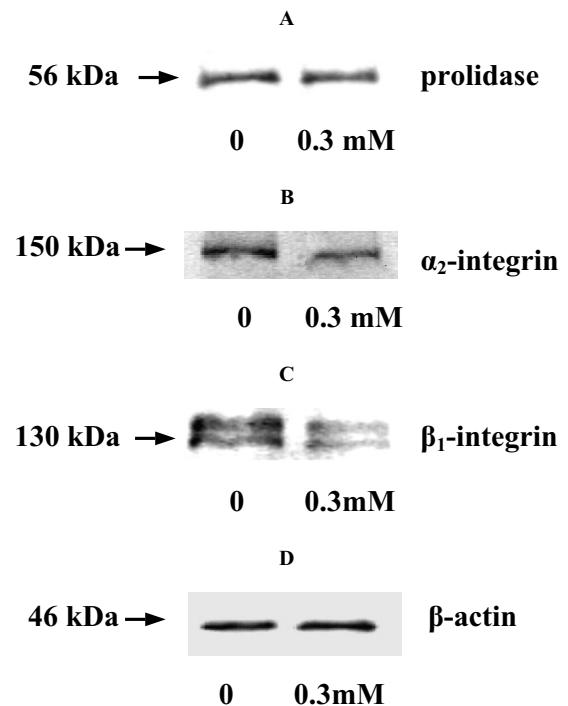


Fig. 2: Western blot analysis for prolidase (A),  $\alpha_2$ -integrin receptor (B) and  $\beta_1$ -integrin receptor (C) in control human skin fibroblasts cultured in the medium containing 10% FBS (lane 1) and in addition incubated with 0.3 mM of CAP (lane 2). Mean values of 6 pooled cell homogenate extracts are presented. Band intensity quantified by densitometric analysis. Same amount of supernatant protein (20  $\mu\text{g}$ ) run in each lane.  $\beta$ -actin expression served as a control for protein loading (D)

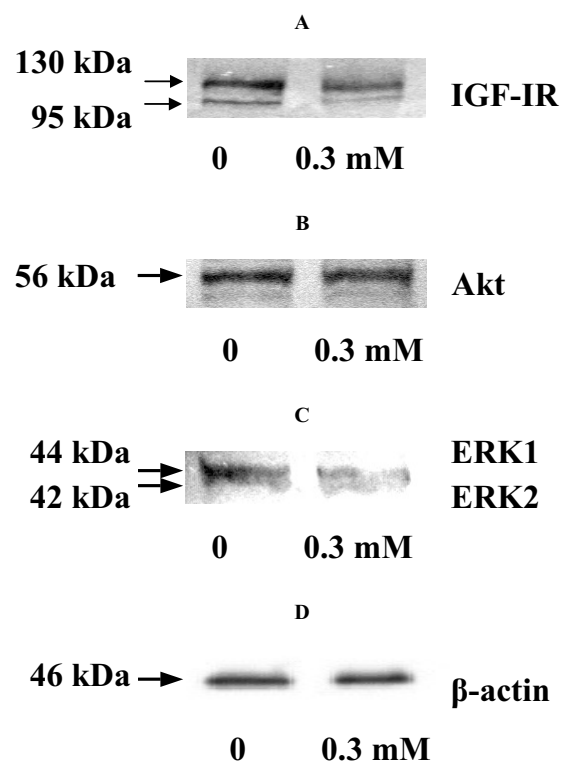


Fig. 3: Western blot analysis of IGF receptor (A), Akt (B) and MAP kinases ERK1 and ERK2 (C) in control human skin fibroblasts cultured in medium containing 10% FBS (lane 1) and in addition incubated with 0.3 mM of CAP (lane 2). Mean values of 6 pooled cell homogenate extracts are presented. Band intensity quantified by densitometric analysis. Same amount of supernatant protein (20  $\mu\text{g}$ ) run in each lane.  $\beta$ -actin expression served as control for protein loading (D)

2001), expression of MAP kinases was measured in cells treated for 48 h with CAP. CAP affected the expression of MAP kinases (ERK1, ERK2) but not Akt protein. As can be seen in Fig. 3 B, 48 h treatment of fibroblasts with 0.3 mM CAP had no effect on the expression of Akt protein, although it contributed to a distinct decrease in the expression of phosphorylated ERK1 and ERK2 (Fig. 3C), compared to the control cells.

This suggests that the ability of CAP to induce a decrease in collagen biosynthesis may involve inhibition of both integrin  $\alpha_2\beta_1$  and IGF-I receptor expression and signaling that down-regulates prolidase activity.

### 3. Discussion

The finding that captopril is a competitive inhibitor of prolidase led us to investigate its role in collagen biosynthesis in fibroblasts – the main collagen synthesizing cells (Makela et al. 1990). It is well established that prolidase, providing proline for collagen biosynthesis, is a rate-limiting factor in the regulation of this process (Karna et al. 2000, 2001; Galicka et al. 2001; Surazyński et al. 2008). Therefore, the mechanism of prolidase activity regulation is of considerable interest.

Captopril (CAP) is an inhibitor of rat, pig and human prolidase.  $K_i$  for the inhibition of rat kidney and liver prolidase is in the range of  $2.5\text{--}3.5 \times 10^{-5}$  M, while the value is considerably higher in humans (Ganapathy et al. 1985). In pig kidney prolidase the  $K_i$  for CAP was found to be  $2.7 \times 10^{-4}$  M (Radzicka and Wolfenden 1991). The effect of CAP on the activity of other enzymes has also been observed. However, “*in vitro*” assays on tissue or cell homogenate extracts do not reflect the situation *in vivo* or in living cells. In fact, our present and previous studies show that the activity of prolidase was not affected in fibroblasts treated with CAP for 24 hours. A significant decrease in prolidase activity due to CAP treatment was observed after 48 h incubation. This suggests that the effect of CAP on prolidase activity is indirect. Some studies suggest that the antifibrotic effect of CAP on the heart and kidney is partially mediated by *N*-acetyl-seryl-aspartyl-lysyl-proline (Peng et al. 2007). Moreover, angiotensin II-induced MAPK activation and TGF- $\beta$  secretion is suppressed by CAP. Angiotensin-converting enzyme inhibition confers renoprotection in adriamycin nephropathy by reducing intrarenal angiotensin II and augmenting *N*-acetyl-seryl-aspartyl-lysyl-proline expression that together attenuate MAPK signaling and its downstream proinflammatory and fibrogenic effects (Tang et al. 2008). There is some evidence that CAP has a preventive effect against the increase in aorta collagen content (Wojakowski et al. 1999) and that it can play a role in the distribution of collagen during cardiac hypertrophy (Gagnon et al. 2004). It has been found that CAP is able to inhibit the conversion of procollagen to collagen, by inhibiting the specific procollagen protease (Männistö et al. 2001). However, the signaling mechanism of these phenomena has not been established.

Collagen is as a ligand for  $\alpha_2\beta_1$  integrin. It has previously been shown that the  $\alpha_2\beta_1$ -integrin receptor is involved in signaling, regulating collagen biosynthesis (Ivaska et al. 1999a) and prolidase activity (Palka and Phang 1994, 1997). Another important aspect of collagen biosynthesis regulation is at the level of insulin-like growth factor-I receptor (IGF-IR). IGF-I is one of the most potent collagen-stimulating factors in collagen synthesizing cells (Goldstein et al. 1989). Therefore, we regarded  $\alpha_2\beta_1$  integrin and IGF-IR as a potential target in CAP-induced decrease of the above processes.

Our observations suggest that in fibroblasts CAP reduces collagen biosynthesis primarily through inhibition of  $\alpha_2\beta_1$  integrin receptor and IGF-I-IR expression. Presumably, inhibition of pro-

lidase activity in fibroblasts due to CAP action is a result of suppression of signaling by  $\beta_1$  integrin and IGF-IR. Both,  $\beta_1$  integrin (Palka and Phang 1997) and IGF-IR (Miltyk et al. 1998) signaling have been found to play an important role in prolidase activity regulation.

It is known that, integrin  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  are the major cellular receptors for collagen, and that collagens bind to these integrins at the alpha subunit (Ivaska et al. 1999b). We demonstrated that CAP-dependent decrease in collagen production was accompanied by a decrease in  $\alpha_2$  integrin expression, as well as activation of MAP-kinases. It cannot be ruled out that the CAP-dependent decrease in prolidase activity, but not expression, is a consequence of a decrease in phosphorylation of the enzyme, due to a decrease in MAP-kinase activity. Previously it has been found that prolidase is a phosphoprotein and phosphorylation increases the enzyme activity (Surazyński et al. 2001). Therefore, despite the lack of differences in prolidase expression in CAP-treated fibroblasts, the activity could be decreased due to down-regulation of enzyme phosphorylation.

The results of the present study suggest that in fibroblasts CAP may exert its effect on collagen biosynthesis by inhibiting prolidase activity through down-regulation of the expression of IGF-IR,  $\alpha_2\beta_1$  integrin and phosphorylated MAP-kinases (ERK1, ERK2).

### 4. Experimental

#### 4.1. Materials

Alkaline phosphatase-labeled anti-mouse IgG, anti-rabbit IgG and anti-goat IgG antibodies, bacterial collagenase, Fast BCIP/NBT reagent, L-glycyl-proline, L-proline, monoclonal (mouse) anti-IGF-IR antibody, and monoclonal (mouse) anti-phosphorylated MAPK antibody 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  $\mu$ m), sodium dodecylsulphate (SDS), polyacrylamide, molecular weight standards and Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories, USA. L-5[ $^3$ H] proline (28 Ci/mmol) was purchased from Amersham, UK. Monoclonal (mouse) anti- $\beta_1$ , polyclonal (rabbit) anti- $\alpha_2$ -integrin antibodies, and polyclonal (goat) anti- $\beta$ -actin antibody were products of Santa Cruz Biotechnology Inc., USA. Monoclonal phospho-Akt (rabbit) antibody was purchased from Cell Signaling Technology, Inc., USA. Polyclonal anti-human prolidase antibody was donated by Dr. James Phang (NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA).

#### 4.2. Biochemical studies

##### 4.2.1. Tissue culture

All studies were performed on normal human skin fibroblasts (CRL-1474), that were purchased from the American Type Culture Collection, Manassas, VA, USA. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/l glutamine, 50 U/ml penicillin, and 50  $\mu$ g/ml streptomycin at 37 °C in a 5% CO<sub>2</sub> incubator. Cells were counted in a hemocytometer and cultured at  $1 \times 10^5$  cells per well in 2 ml of growth medium in 6 well plates (Costar). Cells reached confluence at day 6 and in most cases such cells were used for assays. Cells were used in the 8th to 14th passages.

##### 4.2.2. Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara et al. (1982). Protein concentration was measured by the method of Lowry et al. (1951). Enzyme activity was reported as nanomoles of proline released from the synthetic substrate, during one minute per milligram of supernatant protein of cell homogenate.

##### 4.2.3. Collagen production

Incorporation of radioactive precursor into proteins was measured after labeling of confluent cells in growth medium, with CAP for the last 48 h with 5[ $^3$ H] proline (5  $\mu$ Ci/ml, 28 Ci/mM) as described previously (Oyamada et al. 1990). Incorporation of tracer into collagen was determined by

digesting proteins with purified *Clostridium histolyticum* collagenase, according to the method of Peterkofsky et al. (1982). Results are shown as combined values for cell plus medium fractions.

#### 4.2.4. SDS-PAGE

Slab SDS/PAGE was used, according to the method of Laemmli (1970), using 10% SDS-polyacrylamide gel.

#### 4.2.5. Western Immunoblot Analysis

After SDS-PAGE, the gels were allowed to equilibrate for 5 min. in 25 mmol/l Tris and 0.2 mol/l glycine in 20% (v/v) methanol. The protein was transferred to 0.2 µm pore-size nitrocellulose at 100 mA for 1 h using a LKB 2117 Multiphor II electrophoresis unit. The nitrocellulose was incubated with: monoclonal anti-β<sub>1</sub> and polyclonal anti-α<sub>2</sub>-integrin antibodies at a concentration of 1:1000; polyclonal antibody against β-actin at 1:3,000; polyclonal antibody against prolidase at 1:5,000; monoclonal antibodies against IGF-IR and phospho-Akt at 1:1,000, monoclonal antibody against phosphorylated-MAPK protein at 1:5,000 in 5% dried milk in TBS-T (20 mmol/l Tris-HCl buffer, pH 7.4, containing 150 mmol/l NaCl and 0.05% Tween 20) for 1 h. In order to analyze β<sub>1</sub> integrin subunit, IGF-IR and phosphorylated MAP kinases, second antibody-alkaline phosphatase conjugated, anti-mouse IgG (whole molecule) was added at a concentration of 1:7,500 in TBS-T, in order to analyze prolidase, α<sub>2</sub>-integrin subunit and phospho-Akt second antibody alkaline phosphatase conjugated, anti-rabbit IgG (whole molecule) was added at 1:5,000, and in order to analyze β-actin second antibody-alkaline phosphatase conjugated, anti-goat IgG (whole molecule) was added at 1:5,000 in TBS-T and incubated for 30 min slowly shaking. Then nitrocellulose was washed with TBS-T (5 × 5 min) and submitted to Sigma-Fast BCIP/NBT reagent. The intensity of the bands was quantified by densitometric analysis.

#### 4.2.6. Statistical analysis

In all experiments, the mean values for three independent experiments done in duplicate ± standard deviation (S.D.) were calculated. The results were submitted to statistical analysis using Student's t-test, accepting P < 0.01 as significant.

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