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Prolidase Dependent Inhibition of Collagen Biosynthesis in Chinese Hamster Ovary Cells

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Collagen is responsible for maintenance of connective tissue integrity, and through interaction with integrin receptors may participate in regulation of numerous physiological and pathological processes. An important role in collagen biosynthesis plays prolidase. It was previously found that nickel chloride inhibited prolidase activity in Chinese hamster ovary cells (CHO-C9). The cells lack any detectable ornithine aminotransferase and P5C synthase activities, and therefore require addition of free proline or glicyl-proline (converted to glycine and proline) for growth. We have found that Ni(II) contributed to decrease in collagen and hydroxyproline content in CHO cells incubated with Gly-Pro, whereas it had no effect on hydroxyproline content in the cells incubated with proline. Decrease in collagen content was not related to decrease in type I collagen mRNA level suggesting regulation of this process at post-transcriptional level. However decrease in expression of Sos and phosphorylated MAP-kinases were found in the cells growing in the presence of Gly-Pro and Ni(II). Decrease in the expression of these proteins was not related to inhibition of signalling induced by growth factors, since no changes were observed in expression of AKT in CHO cells incubated with Ni(II). The results presented provide evidence for important role of prolidase in collagen biosynthesis.

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

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 and growth. Several line of evidence suggest that collagen metabolism is regulated by prolidase.

Prolidase [EC.3.4.13.9] is a manganese-dependent cytosolic imidodipeptidase, which specifically splits imidodipeptides with C-terminal proline or hydroxyproline (*7*). Prolidase catalyses the final step of collagen degradation. The enzyme plays an important role in the recycling of proline from imidodipeptides (derived from degradation products of collagen) for collagen resynthesis (*8*) and cell growth (*9*). The efficiency of proline recycling was found to be about 90% (*10*). Lack of the enzyme impedes the efficient recycling of proline for collagen resynthesis (*10*) and cell growth (*9*). Previously we found the link between collagen synthesis and prolidase activity in cultured skin fibroblasts treated with antiinflammatory drugs (*11*), during experimental ageing of the cells (*12*), fibroblasts chemotaxis (*13*) and cell surface integrin receptor ligation (*14*). Therefore, the regulation of the enzyme activity is also of considerable interest.

Previously, we found that prolidase activity is regulated by β_1-integrin receptor (*15*). Stimulated β_1-integrin receptor induces autophosphorylation of nonreceptor protein kinase FAK (*16*), which is then capable of interacting with adaptor-proteins, such as Grb2, through Src and Shc proteins. This interaction allows activation of further cascade of signalling pathway through Sos, Ras and Raf proteins (*17*) and subsequently, two MAP kinases: ERK_1 and ERK_2 (*18*). The end point of this cascade is induction of transcription factor(s) that regulate(s) gene expression of integrins, proteinases and many proteins involved in regulation of cell growth and differentiation (*19*).

Our previous findings revealed that nickel chloride inhibited prolidase activity and CHO cells growth in a dose-dependent manner (*20*). We have shown that Ni(II) is a competitive inhibitor of prolidase with respect to Mn(II), with Gly-Pro as the substrate (*20*). Therefore, we decided to evaluate collagen biosynthesis and expression of MAP kinases in CHO cells treated with Ni(II).

MATERIALS AND METHODS

Materials—L-Proline, L-glicyl-L-proline, prolidase purified from hog kidney and other chemicals were purchased from Sigma Chemical (St Louis, MO). Polyclonal antibody anti-α1 chain of collagen type I was from

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Santa Cruz. Dullbecco's modified Eagle's medium and fetal bovine serum were from Gibco (Rockville, MD).

Tissue Culture—The proline auxotroph CHO-C9 cell line was cloned from CHO-K1 cells originally obtained from the American Type Culture Collection. CHO-C9 cells lack any detectable ornithine amino transferase and P5C synthase activities. The cells were maintained at 37°C in 5% CO_2 incubator in 72 cm^2 tissue culture flasks, in Dullbecco's modified Eagle's medium supplemented with 5% foetal bovine serum, 2 mM glutamine and 0.5 mM proline. For growth experiments cells were counted in a hemocytometer and inoculated at a concentration of 5×10^5 in a 60 mm Falcon dish. The cells were incubated for two days in medium as described above, then the medium was exchanged to Dullbecco's modified Eagle's medium supplemented with 0.5% foetal bovine serum-contained addenda as described in the legends for specific experiments.

Determination of Prolidase Activity—The activity of prolidase was determined according to the method of Myara *et al*. (*21*) which is based on colourimetric determination of proline using Chinard's reagent (*22*). Briefly, the monolayer was washed three times with 0.15 mM of NaCl. Cells were trypsinized and centrifuged at 200*g* and the supernatant was discarded. The cell pellet was suspended in 1 ml of 0.05 mM HEPES, pH 7.8 and sonicated 3 times for 10 S at 0°C. Samples were then centrifuged (18,000*g*, 30 min) at 4°C and the supernatant was used for protein determination (Bradford method) and prolidase activity assays. Activation of prolidase requires incubation with manganese(II); 100 µl of supernatant was therefore preincubated with 100 µl of 0.05 mM HEPES, pH 7.8 containing 2 mM $MnCl_2$ for 24 h at 37°C. After activation, the prolidase reaction was initiated by addition of 100 µl of the activated mixture to 100 µl of 94 mM glicyl-proline (Gly-Pro) for a final concentration of 47 mM. After additional incubation for 1 h at 37°C, the reaction was terminated with 1 ml of 0.45 mM trichloroacetic acid. To parallel blank tubes, trichloroacetic acid was added at time 'zero'. The released proline was determined by addition 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 released proline was determined colourimetrically by absorbance at 515 nm and calculated using proline standards. Enzyme activity was reported in nanomoles of proline released per minute per unit of the enzyme or milligram of protein.

Hydroxyproline Assay—Hydroxyproline content in lysates of cell layers was determined by the method of Woessner (*23*).

Quantification of Type I Collagen mRNA—The relative level of type I collagen mRNA was examined using semi-quantitative RT-PCR method. Total RNA was extracted from chondrocytes using Rneasy kit (Qiuagen, Hilden, Germany) according to manufacturer's procedure. RNA was quantified spectrophotometrically at 260 nm. Total RNA (1 µg) was used to prepare cDNA. cDNA synthesis was performed in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP mix, 2.5 µM oligo dT_{15}, 20 U RNasin Ribonuclease Inhibitor, 100 U M-MLV Reverse Transcriptase (Promega, MD, USA) in final volume 50 µl. The reactions were performed using MJ Research Thermal Cycler (Waterstown, MA, USA). The mixture was incubated at 42°C for 1 h and then heated in 95°C for 5 min. PCR was carried in total reaction volume of 20 µl, contained 2 µl 10-fold PCR buffer, 2 µl cDNA, 40 µM each dNTP, 1.5 unit HotStarTagDNA polymerase (Qiuagen, Hilden, Germany) and 200 nm of each primers. Specific primers were used as follows: sense 5′-TTCCCC CAGCCACAAAGAGTC-3′, antisense 5′-CGTCATCGCA CAACACCT-3′ for type I collagen, sense 5′-CCAGATC ATGTTTGAGACCT-3′, antisense 5′-GCACAGCTTCTCC TTAATGT-3′ for β-actin. PCR was carried out under following conditions: 5 min at 95°C to activate polymerase, 1 min denaturation at 95°C, 45 s annealing at 62°C, 1 min extension at 72°C for 30 cycles, with additional 7 min extension for last cycle. Controls with water replacing template were included in all experiments. Amplification products and 100-bp ladder (Amersham, United Kingdom) were separated on 2% agarose gel, stained with ethidium bromide, visualized under UV light (UVP, Inc.) and submitted to density analysis using One Dscan/Zero Dscan software (Scanalytics Inc., USA).

SDS–PAGE—Slab SDS/PAGE was used according to the method of Laemmli (*24*). 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 glycine 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 1:500 or polyclonal antibodies against prolidase, collagen type I and β-actin at concentration 1:1,000, 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 1:1,000 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-blotting detection system.

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

RESULTS

Collagen content was measured by hydroxyproline determination in CHO cells treated with proline,

glicyl-proline with or without nickel chloride. Evaluation of collagen content was conducted colourimetrically according to the method of Woessner. As presented on Fig. 1A an addition of 0.5 mM proline or 0.15 mM Gly-Pro caused increase in hydroxyproline content in CHO cells in the similar manner. Addition of 0.01 mM Ni(II) had no significant effect on Hyp content in CHO cells incubated with Pro or Gly-Pro. Increase in Ni(II) concentration to 0.05 and 0.15 mM decreased Hyp content to 79 and 59% of control value in the cells incubated with Gly-Pro, respectively. In the cells incubated with Pro, nickel had no significant effect on hydroxyproline content in CHO cells. Addition of 0.5 mM of proline to the cells incubated with 0.15 mM glicyl-proline and 0.15 mM Ni prevented the cells from decrease in Hyp content. Western-immunoblot analysis for type I collagen in the cell extract of CHO cells proved changes in the protein expression similar to Hyp content (Fig. 1B).

Decrease in collagen content may result from decrease in collagen gene expression. To determine whether nickel affects the expression of type I collagen RNA, RT-PCT was conducted. CHO cells were incubated with various concentrations of nickel chloride for 24 h, RNA was harvested and analysed. Our results indicated that treatment of the cells with Ni(II) has no effect on type I collagen mRNA level (Fig. 2).

Collagen is a ligand for β_1-integrin receptor. Decreased amount of collagen could alter signalling pathway induced by this protein. To test this hypothesis expression of Sos (Fig. 3), and phosphorylated MAP-kinases (Fig. 4) was measured by Western-immunoblot analysis. We found decreased expression of both proteins only in the cells growing in the presence of glicyl-proline and nickel(II). No differences in the expression of both proteins were found in CHO cells growing in medium with proline and nickel.

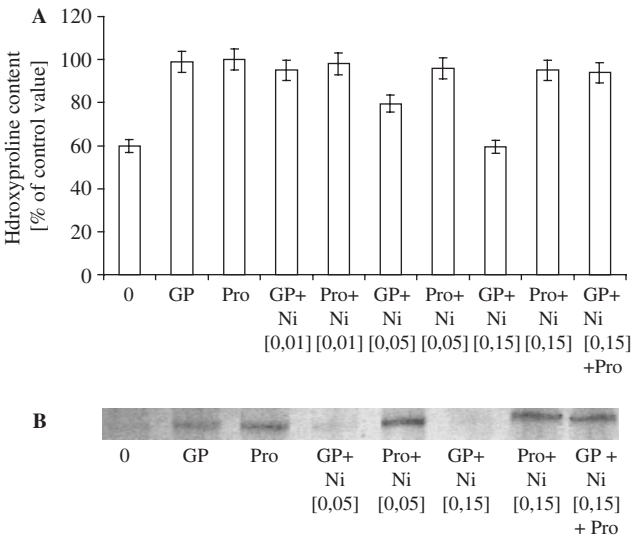

Fig. 1. **(A) Hydroxyproline content in CHO cells incubated in medium with glicyl-proline (GP) or proline (Pro) with various concentrations of Ni(II) for 24 h.** '0' bar represent hydroxyproline content in the cells grown in cell-culture medium without proline or glicyl-proline. (B) Western-immunoblot analysis of type I collagen in CHO cells treated with cell-culture medium with glicyl-proline (GP) or proline (Pro) with various concentrations of Ni(II) for 24 h.

Akt/PKB plays important roles in the signalling pathways in response to growth factors and other extracellular stimuli that regulate several cellular functions, including cell growth. To exclude potential influence of nickel on signal induced by growth factors, expression of AKT was evaluated by Western-immunoblot analysis. As seem on Fig. 5, no changes were observed in expression of this protein in the CHO cells incubated with Ni(II).

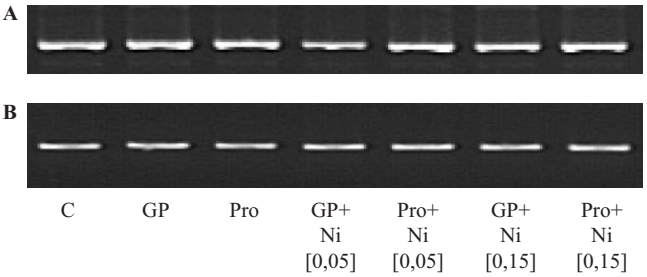

Fig. 2. **(A) RT–PCR: the expression of mRNA type I collagen in CHO cells cultured for 24 h in medium with 0.15 mM glicyl-proline or 0.5 mM proline with various concentrations of nickel chloride.** (B) The expression of β-actin served as a control.

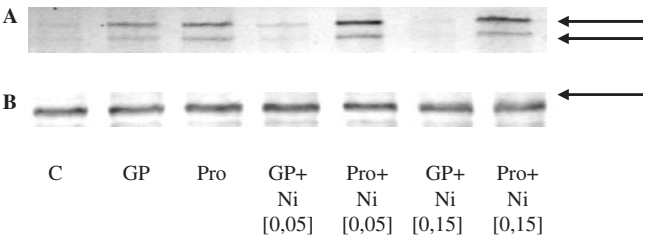

Fig. 3. **(A) Western-immunoblot analysis of phosphorylated MAP-kinases (ERK_1/ERK_2) in CHO cells incubated in medium with glicyl-proline (GP) or proline (Pro) with various concentrations of Ni(II).** (B) Western-immunoblot analysis of β-actin as a loading control.

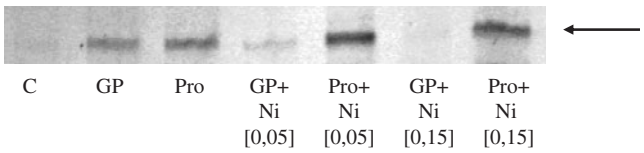

Fig. 4. **Western-immunoblot analysis of SOS protein in CHO cells incubated in medium with glicyl-proline (GP) or proline (Pro) with various concentrations of Ni(II).**

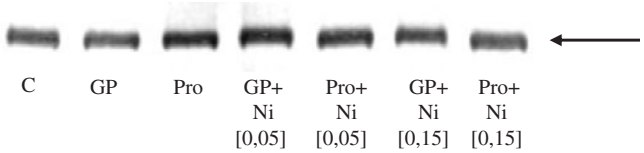

Fig. 5. **Western-immunoblot analysis of AKT in CHO cells incubated in medium with glicyl-proline (GP) or proline (Pro) with various concentrations of Ni(II).**

DISCUSSION

In the present study we provide further evidence for the important role of prolidase in collagen biosynthesis.

Our previous findings revealed that nickel chloride inhibited prolidase activity in a dose-dependent manner in CHO cells (*20*). We have shown that Ni(II) is a competitive inhibitor of prolidase in respect to Mn(II), in a cell-free system, but also in cells having Mn(II) physiologically bound to the cellular enzyme (*20*). We used the proline-auxotrophs CHO-C9 cells exhibiting a rate of growth in Gly-Pro medium indistinguishable from those with proline medium. At 0.15 mM Ni(II), the inhibition of prolidase activity reduced the growth rate of CHO-C9 cells in Gly-Pro medium by 50%. Importantly, Ni(II) at this concentration had no effect on the rate of growth on Pro. It suggested that the mechanism of the cells growth inhibition by Ni(II) is due to inhibition of prolidase.

Exposure of cells to nickel(II) causes inactivation of prolyl hydroxylases (*25*). Prolyl hydroxylation is important for collagen biosynthesis through stabilization of the triple-helical conformation of collagen molecule. Decrease in collagen content in CHO cells treated with Gly-Pro and Ni(II) was not related to inhibition of prolyl hydroxylase, since there was no inhibitory effect on collagen biosynthesis in CHO cells incubated with Pro and nickel (II).

Inhibition of prolidase activity by nickel may impair several important cellular functions. Collagen, which accounts for about one-third of total body proteins is not only essential for maintenance of connective tissue architecture but also is important in signalling. We evaluated collagen biosynthesis in the cells treated with Ni(II). We were unable to determine collagen content with the radiometric method of Peterkofsky because addition of free proline to conditioned medium containing 5[^{3}H]-proline would lead to dilution of marker and inaccurate results. We have found that nickel inhibited collagen biosynthesis only in CHO cells cultured in the medium with Gly-Pro and had no effect on biosynthesis of this protein in cells incubated with Pro. This suggested that inhibition of prolidase catalyzing the final step in the degradation of collagen (*26*) by nickel altered turnover of collagen.

Collagen is known ligand for cell surface receptors α_2 β_1 integrin. This family of signalling molecules modulates gene expression, genomic stability, cellular differentiation and cell proliferation (*27–29*). Recently it has been suggested the possible role of adhesion molecules in the initiation of cancer (*30*) and the metastatic potential of tumour cells. Inhibition of collagen biosynthesis by nickel probably down-regulated signal induced by interaction of collagen with integrin receptor. In CHO cells incubated with Gly-Pro and nickel(II) expression of Sos and MAP-kinases proteins participating in signal transduction induced by integrin receptor was decreased, as compared to the cells incubated with Pro and Ni(II). Decrease in the expression of these proteins was not related to inhibition of signalling induced by growth factors, since no changes were observed in expression of AKT in the CHO cells incubated with Ni(II). This data support hypothesis that inhibition of collagen biosynthesis by nickel is due to decrease in proline availability for this process.

The critical function of prolidase is to provide free proline from the complete degradation of proteins from both nutritional and endogenous sources. Free proline is not only a substrate for protein synthesis, but also has several regulatory functions. A number of these functions involve proline's oxidation (*31*) via proline oxidase, a p53-regulated gene with a strong role in apoptosis (*32*, *33*). The inhibition of proline production from prolyl dipeptides could thus alter the cellular response to apoptotic stimuli. A second function of proline is to be a source of P5C, which is known to mediate redox-dependent regulatory functions (*31*, *34*). Additionally, P5C can be a source of both glutamate and ornithine. The latter has been emphasized as a source of arginine which is the precursor for the cell signalling molecule, nitric oxide.

Inhibition of prolidase by nickel may cause the accumulation of prolyl dipeptides degraded from both exogenous and endogenous proteins. It has been found that imidodipeptides stimulated intracellular collagen degradation. In fibroblast cultures from prolidase dependent patients, an increase in rapidly degraded collagen and decrease in proline pool has been found in comparison to control cells (*35*). Collagen which accounts for about one-third of total body proteins represent polypeptide containing the highest amount of imino-bonds compared to all known proteins. In α1 subunit (1464 amino acids) of type I procollagen, proline is coupled to glycine 119 times and in α2 subunit (1366 amino acids) 106 times. In fully hydroxylated collagen, proline may occur either as hydroxylated amino acid in doublet of glicyl-hydroxyproline (Gly-Hyp) and unhydroxylated amino acid, glicyl-proline (Gly-Pro). In α1 or α2 subunit of matured hydroxylated collagen, Gly-Pro may occur at least 25 times (*10*). Therefore, degradation of collagen produce large quantities of Gly-Pro, that may be utilized with different rate, depending on prolidase activity.

There are a number of bioactive peptides with the X-Pro sequence at the N-terminus, *e.g*. erythropoietin, IL-1, IL-10, IL-13, TNF-β, IGF-1 and bradykinin, to name but a few (*36*). The enzymes converting these peptides from their inactive propeptides must recognize the X-Pro N-terminus and thus may be inhibited by X-Pro imidodipeptides. It can not be excluded that inhibition of prolidase by nickel *in vivo*, with the resulting accumulation of prolyl dipeptides, may inhibit the activation of these cytokine peptides and produce variations in cellular phenotype. However the role of imidodipeptides in regulation of cellular metabolism requires to be explored. The above data suggest that regulation of prolidase activity is of great importance. The mechanism of prolidase activity regulation may originate at both β_1-integrin (*14*) and IGF-I (*37*) receptors signalling. Increase in expression of β_1-integrin receptor and its signalling through FAK leads to increase in the expression of NFκB (*38*). The transcription factor is well known as an inhibitor of type I (*39*, *40*) and type II (*41*) collagen gene expression as well as stimulator of MMPs production (*42*). Therefore suppression of these pathways may represent novel strategy for protection of ECM destruction during connective tissue diseases. Nevertheless, the complex of regulatory mechanisms may affect collagen metabolism at the level of prolidase activity. The data presented in this study provide evidence for the important role of prolidase in regulation of collagen metabolism. Moreover, it was

found that toxicity of nickel(II) is due to inhibition of prolidase and prolidase-dependent function, including inhibition of collagen biosynthesis.

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