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ORIGINAL ARTICLE

The mechanism of hydralazine-induced collagen biosynthesis in cultured fibroblasts

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Abstract The finding that hydralazine (HYD) affects collagen metabolism led us to investigate the mechanism of its action on collagen biosynthesis, prolidase expression and activity, expression of $\alpha_2\beta_1$ integrin, insulin-like growth factor-I receptor (IGF-IR), focal adhesion kinase (FAK), mitogen-activated protein (MAP) kinases (ERK_1, ERK_2), and transcription factors hypoxia-inducible factor-1α (HIF-1α) and nuclear factor-κB p65 (NF-κB p65) in human dermal fibroblasts. Confluent fibroblasts were treated with micromolar concentrations (50–500 μM) of HYD for 24 h. HYD had no influence on cell viability. It was found that HYD-dependent increase in collagen biosynthesis was accompanied by a parallel increase in prolidase activity and expression, HIF-1α expression, and decrease in DNA biosynthesis, compared to untreated cells. Since collagen biosynthesis and prolidase activity are regulated by a signal induced by activated $\alpha_2\beta_1$ integrin receptor as well as IGF-IR, the expression of these receptors was measured by Western immunoblot analysis. The exposure of the cells to HYD contributed to the increase in IGF-IR expression without any effect on $\alpha_2\beta_1$ integrin receptor and FAK expressions. It was accompanied by a decrease in expression of MAP kinases and NF-κB p65, the known inhibitor of collagen gene expression. The data suggest that the HYD-dependent increase of collagen biosynthesis in cultured human skin fibroblasts results from activation of IGF-IR expression and prolidase activity and downregulation of NF-κB p65.

Keywords Collagen biosynthesis · Hydralazine · IGF-IR · NF-κB p65 · Prolidase

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Introduction

Hydralazine (HYD, 1-hydrazinophthalazine), a well-known medicine for hypertension, causes metabolic changes in the connective tissue, including increase in tissue collagen content, collagenolytic activity, and lysosomal exoglycosidases activity in rat tissues (Weglarz et al. 1990; Meilman et al. 1965). An increased activity of these enzymes suggest that HYD-induced connective tissue damage, probably is an inflammatory type (Olczyk et al. 1988). Fibroblasts treated with HYD synthesized procollagen which was severely deficient in hydroxyproline and hydroxylysine, indicating an inhibition of prolyl and lysyl hydroxylase reactions in the cells. Assays of prolyl and lysyl hydroxylase activities, however, revealed markedly increased levels in HYD-treated cells (Murad et al. 1985).

Collagen biosynthesis in human dermal fibroblasts may depend on the activity of prolidase (Surazynski et al. 2008a). Prolidase [E.C.3.4.13.9] is a cytosolic enzyme which catalyzes hydrolysis of imidodipeptides (mainly derived from collagen degradation), releasing proline, which is used for collagen resynthesis (Jackson and Heininger 1973; Jackson et al. 1975). Prolidase activity is stimulated through a signal mediated by collagen-β_1 integrin receptor interaction (Palka and Phang 1997). More interestingly, prolidase has been found to be involved in the regulation of transcription factor HIF-1α. The potential mechanism of this process was demonstrated in breast cancer cells (Surazynski et al. 2008b).

The activity of HIF-1α is controlled at the level of its degradation. The hydroxylation of specific proline residue in the oxygen-dependent degradation (ODD) domain of HIF-1α targets HIF-1α for ubiquitination and proteosomal degradation via Von Hippel–Lindau (VHL) tumor suppressor protein (Jaakkola et al. 2001). Proline and hydroxyproline, as products of prolidase function, inhibit the degradation of HIF-1α presumably by interference between

VHL and the hydroxyproline in ODD. As a result of overexpression of prolidase, HIF-1α is not degraded and function as transcription factor for the activation of some genes, as for instance vascular endothelial growth factor (VEGF) (Surazynski et al. 2008b). Therefore, stimulation of prolidase activity may prevent HIF-1α degradation and facilitate its role as an inducer of angiogenesis. It is known that hydralazine activates the HIF-1α pathway through the inhibition of prolyl hydroxylase domain activity and initiates a pro-angiogenic phenotype (Knowles et al. 2004).

Another factor that strongly stimulates collagen biosynthesis is insulin-like growth factor (IGF)-I, acting predominantly through the IGF-I receptor (Goldstein et al. 1989). The effects of IGF-I include the induction of collagen gene expression (Tanaka et al. 2002), upregulation of prolidase activity (Miltyk et al. 1998), stimulation of mitotic division, and prevention of apoptosis (Baserga 2005). Some of these activities are regulated through NF-κB, the known inhibitor of collagen gene expression (Kouba et al. 1999).

In this study, we examined the effect of HYD on collagen biosynthesis and prolidase activity and expression, DNA biosynthesis and cell viability, expression of $\alpha_2\beta_1$ integrin, IGF-I receptor, FAK, MAP kinases (ERK_1, ERK_2), and the transcription factors—NF-κB p65 and HIF-1α—in human dermal fibroblasts.

Materials and methods

Alkaline phosphatase-labeled anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG antibodies, aprotinin, bacterial collagenase, captopril, Fast BCIP/NBT reagent, hydralazine hydrochloride, L-glycyl-proline, L-proline, leupeptin, monoclonal (mouse) anti-IGF-IR antibody, monoclonal (mouse) anti-phosphorylated MAPK ERK1/2 antibodies, monoclonal (mouse) anti-phosphotyrosine antibody, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 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 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, and Coomassie Brilliant Blue R-250 were received from Bio-Rad Laboratories, USA. L-5[^{3}H] proline (28 Ci/mmol) was purchased from Amersham, UK. [^{3}H] Thymidine (6.7 Ci/mmol) was obtained from NEN (USA). Monoclonal (mouse) anti-β_1, polyclonal (rabbit) anti-α_2-integrin, polyclonal (rabbit) NF-κB p65, polyclonal (goat) anti-β-actin, and monoclonal (rabbit) anti-FAK antibodies were the products of Santa Cruz Biotechnology Inc., USA. Monoclonal (mouse) anti-hypoxia-inducible factor (HIF-1α) antibody was obtained from Becton, Dickinson Co., USA. Polyclonal anti-human prolidase antibody was donated by Dr. James Phang (NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA).

All studies were performed on normal human skin fibroblasts (CRL-1474) that were purchased from American Type Culture Collection, Manassas, VA, USA.

Collagen production

Incorporation of radioactive precursor into proteins was measured after labeling of confluent cells in growth medium, with HYD for the last 24 h with 5[^{3}H] proline (5 μCi/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.

Determination of prolidase activity and proline

The activity of prolidase was determined according to the method of Myara et al. (1982). Proline was measured by Chinard's reagent (Chinard 1952). Protein concentration was measured by the method of Lowry et al. (1951). Enzyme activity was reported as nanomoles of proline released from synthetic substrate, during 1 minmg^{-1} of supernatant protein of cell homogenate.

Immunoprecipitation

The cells at about 90 % of confluence were rinsed with phosphate-buffered saline (PBS), scraped out of the wells, and centrifuged at 1,000×*g* for 3 min. Then the cells (from six wells) were solubilized with lysis buffer containing 10 mM Tris–HCl, pH7.4, 250 mM NaCl, 0.5 % Nonidet P-40, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, at 4 °C for 10 min. The insoluble material was removed by centrifugation at 10,000×*g* for 5 min at 4 °C. The supernatant containing 100 μg of protein was added to 100 μg of Protein A-Sepharose that has been linked to the polyclonal anti-human prolidase antibody in the following manner: Protein A-Sepharose was washed three times with lysis buffer and 100 μl of suspension containing about 100 μg of beads was incubated for 1 h at 4 °C with either 20 μl of anti-prolidase antibody. Then, the conjugate was incubated for 1 h at 4 °C with shaking. Immunoprecipitate was washed four times with lysis buffer. Proteins were released from the beads by boiling in SDS sample buffer and loaded into a

10 % SDS–polyacrylamide gel (PAGE). The immunoprecipitates were analyzed by Western immunoblot.

SDS–PAGE and Western blot analysis

Slab SDS/PAGE was used, according to the method of Laemmli (1970), by using 10 % SDS-polyacrylamide gel. Western blot analysis was performed as described previously (Miltyk et al. 1998).

DNA biosynthesis assay

To examine the effect of hydralazine on fibroblast proliferation, the cells were plated in 24-well tissue culture dishes at 1×10^5 cells/well with 1 ml of growth medium. After 48 h ($1.6\pm0.1\times10^5$ cells/well), the plates were incubated with various concentrations of HYD and 0.5 μCi of [^{3}H] thymidine for 24 h at 37 °C. Cells were rinsed three times with PBS, solubilized with 1 ml of 0.1 M sodium hydroxide containing 1 % SDS, scintillation liquid (9 ml) was added, and radioactivity incorporated into DNA was measured in a scintillation counter.

Cell viability assay

The assay was performed according to the method of Carmichael et al. (1987) using 3-(4,5-di-methylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were cultured for 24 h with various concentrations of HYD in six-well plates, washed three times with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37 °C. The medium was removed, and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability in the presence of BA was calculated as a percent of control cells.

Statistical analysis

In all experiments, the mean values for three independent experiments done in duplicates±standard deviation (SD) were calculated. The results were submitted to statistical analysis using one-way ANOVA followed by Tukey test, accepting $P\leq0.05$ as significant versus control.

Results

Studies were performed on confluent fibroblasts, since collagen synthesis, prolidase activity, as well as IGF-IR expression depend on cell density (Myara et al. 1985; Makela et al. 1990).

Collagen biosynthesis was measured in confluent human dermal fibroblasts that have been treated with 50, 100, 250, and 500 μM of HYD or captopril (CAP). As can be seen on Fig. 1a, 24 h of incubation of confluent fibroblasts in the medium containing 10 % FBS and different concentrations of HYD contributed to the increase in collagen biosynthesis in a dose-dependent manner. At 500 μM, HYD induced a twofold increase in collagen biosynthesis compared to control.

The effect was not achieved in the cells treated with CAP, and it was not related to stimulation of DNA synthesis (Fig. 2a).

To explain the mechanism of this process, we considered prolidase as a target enzyme. Increase in collagen biosynthesis in HYD-treated cells was correlated to the increase in prolidase activity (Fig. 1b). It was accompanied by an increase in prolidase expression (Fig. 1c) and decrease in prolidase phosphorylation (Fig. 1d). Moreover, HYD-treated cells contained more free proline (about 15 μM) compared to control cells (about 11 μM) as detected by Chinard assay (data not shown).

Since prolidase has been found to be involved in the regulation of transcription factor HIF-1α, we determined its expression in fibroblasts treated with different concentrations of HYD for 24 h. A distinct increase in the expression of HIF-1α was observed compared to control cells (Fig. 1e).

The increase in collagen biosynthesis in HYD-treated fibroblasts was unrelated to cell proliferation. In fact, it was correlated to the slight decrease in DNA biosynthesis (Fig. 2a) and MAP kinases, ERK1/2 (Fig. 3e), compared to control. Moreover, cell viability was measured by the method of Carmichael et al. (1987) using tetrazolinum salt. The viability of cells incubated for 24 h with indicated concentrations of HYD is presented on Fig. 2b. HYD did not influence the viability of the cells. The stability of cells in respect to the rate of proliferation and cell viability may result from experimental model of confluent cells.

Collagen biosynthesis and prolidase activity were previously shown to be regulated due to the signal induced by the activated $\alpha_2\beta_1$ integrin receptor (Palka and Phang 1997) as well as insulin-like growth factor-I receptor (Goldstein et al. 1989; Miltyk et al. 1998). Therefore, the expression of $\alpha_2\beta_1$ integrin receptor (receptor for type I collagen) and IGF-IR were measured by Western immunoblot analysis. As can be seen in Fig. 3a, b, 24 h of treatment of the fibroblasts with HYD had very small effect on the expression of α_2 and β_1 integrin subunits. Similarly, very small differences were observed in the expressions of FAK (Fig. 3c). However, as shown on Fig. 3d, IGF-I receptor expression was increased in HYD-treated cells compared to the control cells. This suggests that the ability of HYD to induce increase of collagen biosynthesis may depend on the transcriptional

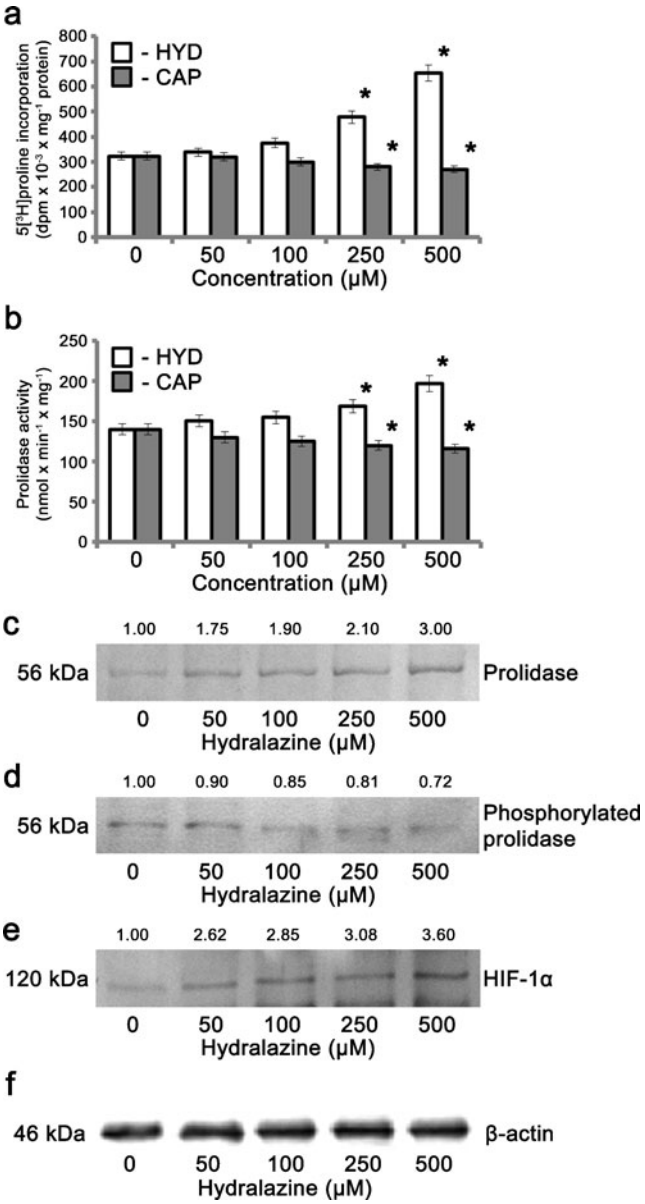

Fig. 1 Collagen biosynthesis (**a**) measured as 5[^{3}H] proline incorporation into proteins susceptible to the action of bacterial collagenase and prolidase activity (**b**) in confluent human skin fibroblasts incubated for 24 h in the medium containing 10 % FBS and different concentrations of HYD or CAP. The results present the mean values from six assays ± SD. The *asterisk* indicates $P \leq 0.05$. Western blot analysis for prolidase (**c**), phosphorylated prolidase (**d**), hypoxia-inducible factor (HIF-1α) (**e**), in control human skin fibroblasts (*lane 1*) and cultured in the medium containing 50 μM of HYD (*lane 2*), 100 μM of HYD (*lane 3*), 250 μM of HYD (*lane 4*), and 500 μM of HYD (*lane 5*). The mean values of six pooled cell homogenate extracts from six separate experiments are presented. The intensity of the bands was quantified by densitometric analysis. Densitometry was done with BioSpectrum Imaging System and presented as arbitrary units. Twenty micrograms of supernatant protein was run in each lane for prolidase and β-actin Western blot analysis. 60 μg of supernatant protein was run in each lane for HIF-1α Western blot analysis. The expression of β-actin served as a control for protein loading (**f**)

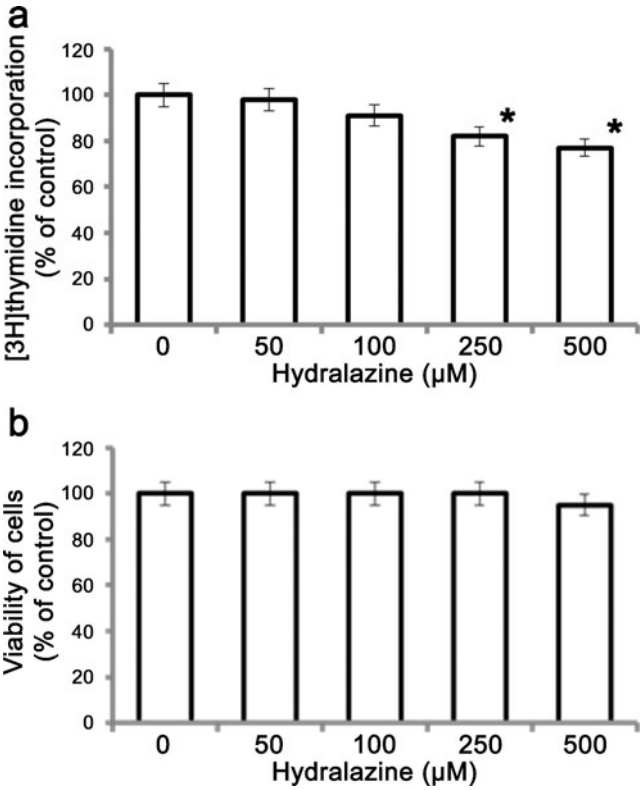

Fig. 2 DNA synthesis (**a**) and viability (**b**) of confluent human skin fibroblasts incubated for 24 h with different concentrations of hydralazine. The mean values±SD from three independent experiments done in duplicates are presented. The *asterisk* indicates $P \leq 0.05$

regulatory mechanism induced by signal mediated by IGF-I receptor. On the other hand, we have found that, in HYD-treated cells, there is decrease in the expression of NF-κB p65, the known inhibitor of collagen gene expression (Kouba et al. 1999) compared to control cells (Fig. 3f).

In view of this data, it seems that the increase of collagen biosynthesis caused by HYD may be a consequence of increase in IGF-I receptor signaling, prolidase activity and decrease in expression of NF-κB p65.

Discussion

The finding that HYD causes changes in collagen metabolism led us to investigate the mechanism of its action on collagen biosynthesis in fibroblasts—the main collagen-synthesizing cells (Makela et al. 1990). The drug *induces* hypertrophy in the myocardium after infarction in rats (Leite et al. 1995). Although several studies on animal models have shown that HYD treatment reduces fibrosis in some tissues, it cannot correspond to reduced collagen synthesis. Upregulation of collagen synthesis may reflect interstitial remodeling leading to increase or decrease of tissue collagen content depending on the rate of collagen degradation. It

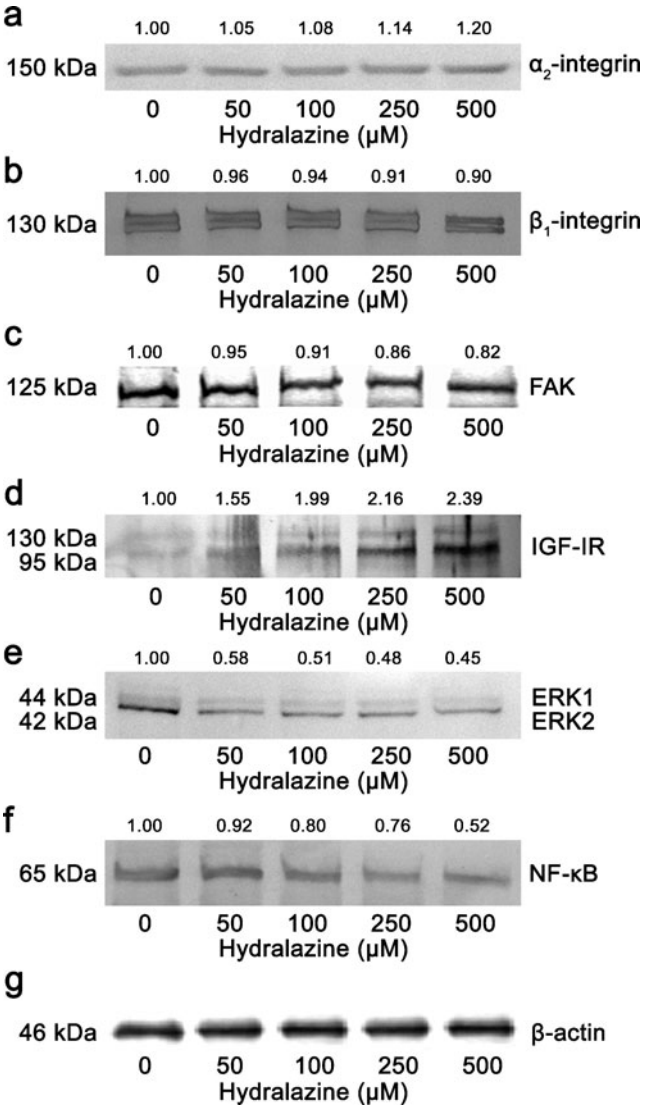

Fig. 3 Western blot analysis for α_2-integrin (**a**), β_1-integrin (**b**) receptor subunits, FAK (**c**), IGF-I receptor (**d**), ERK1/ERK2 (**e**), and NF-κB p65 (**f**) in control human skin fibroblasts (*lane 1*) and cultured in the medium containing 50 μM of HYD (*lane 2*), 100 μM of HYD (*lane 3*), 250 μM of HYD (*lane 4*), and 500 μM of HYD (*lane 5*). The mean values of six pooled cell homogenate extracts from six separate experiments are presented. The intensity of the bands was quantified by densitometric analysis. Densitometry was done with BioSpectrum Imaging System and presented as arbitrary units. The same amount of supernatant protein (20 μg) was run in each lane. The expression of β-actin served as a control for protein loading (**g**)

was supported by some studies showing HYD-dependent increase in type III collagen content in myocardium. An example is the paper of Tsotetsi et al. (2001). Conflicting results were obtained by Murad et al. (1985), however, in different experimental conditions, including prolonged time of incubation.

It seems that experimental concentrations of HYD, used in present study, are pharmacologically relevant. Maximal dose of hydralazine may contribute to the micromolar concentration in plasma. Several studies "in vitro" were performed with higher hydralazine concentration (Liu-Snyder et al. 2006). Hydralazine at 50 to 500 μmol/L rapidly induced HIF-1α protein in endothelial cells (HUVEC) (Knowles et al. 2004).

Our studies suggest potential mechanism for HYD-dependent effect on collagen biosynthesis. The specificity of HYD on collagen biosynthesis was corroborated by parallel experiments with CAP, another hypotensive drug that, in presented experimental conditions, had no effect on the process. It was confirmed in our previous studies (Karna et al. 2010).

Since collagen is regulated by IGF-IR (Goldstein et al. 1989), we postulated that the effects of HYD on collagen production may be related to alterations in intracellular signaling pathway generated by IGF-IR. In fact the data presented here show that HYD-induced collagen biosynthesis is accompanied by an increase in the expression of IGF-IR as well as prolidase activity. Previously, it has been shown that prolidase is stimulated by IGF-I (Miltyk et al. 1998). Moreover, prolidase is phosphoserine/threonine protein. Increase in the enzyme phosphorylation contributes to increase in the enzyme activity (Surazynski et al. 2001, 2005). Although a relatively slight increase (41 %) in prolidase activity in HYD-treated cells does not correspond to the high enzyme protein expression (threefold), it may be due to the low extent of prolidase phosphorylation. Nevertheless, much more (about 36 %) free proline content in HYD-treated cells was found compared to that of the control cells, suggesting upregulation of prolidase activity.

Prolidase catalyzes the final step in collagen degradation which completes the recycling of proline for collagen resynthesis (Jackson and Heininger 1973; Yaron and Naider 1993). The best and most abundant substrate for prolidase is glycyl-proline (Gly-Pro). Collagen represent polypeptide containing the highest amount of imido bonds compared to all known proteins. In α1 chains of type I collagen, Gly-Pro occurs 25 times (Jackson et al. 1975). Therefore, HYD-induced prolidase activity may represent one of the mechanisms of collagen biosynthesis regulation. The functional link between collagen and prolidase activity has been also found in cultured human skin fibroblasts treated with anti-inflammatory drugs (Miltyk et al. 1996), pyrroline-5-carboxylate (Miltyk and Palka 2000), during experimental aging of these cells (Palka et al. 1996), fibroblast chemotaxis (Palka et al. 1997), and cell surface integrin receptor ligation (Palka and Phang 1997).

Prolidase plays an important role in HIF-1α signaling. The mechanism of this process involves products of prolidase activity, proline, or hydroxyproline that prevent hydroxylation of specific proline residue in the ODD domain of HIF-1α and prevent targeting HIF-1α for ubiquitination

and proteosomal degradation via Von Hippel–Lindau (VHL) tumor suppressor protein (Jaakkola et al. 2001). Therefore, proline and hydroxyproline may contribute to activation of HIF-1α and activation of some proneoplastic genes, as for instance VEGF (Surazynski et al. 2008b). Therefore, stimulation of prolidase activity (e.g., by HYD) may prevent HIF-1α degradation and facilitate its role as an inducer of angiogenesis. The mechanism was proved in breast and colon cancer cell lines (MCF-7, MDA-MB 231, and DLD-1), showing prolidase-dependent differences in HIF-1α activation (Surazynski et al. 2008b; Karna et al. 2012). Although in present studies we found increased proline concentration in hydralazine-treated cells, it is uncertain whether it is a product of degradation of proline-containing dipeptides or proline biosynthesis. It cannot be excluded as another possibility. HYD has been shown to inhibit prolyl hydroxylase activity in cell-free assay and prevented hydroxylation (and subsequent degradation) of HIF-1α in cultured cells (Knowles et al. 2004; Bhatnagar et al. 1972). This process prevents also posttranslational modification of collagen prolyl residues essential for the formation of stable collagen fibers (Murad et al. 1985).

Nevertheless, the constellation of changes induced by HYD, found in this study suggest important role of prolidase in upregulation of collagen synthesis. It supports our previous studies on butyrate on the regulation of collagen biosynthesis (Karna et al. 2006). Several other studies suggest that prolidase-dependent regulation of collagen biosynthesis may take place at the transcriptional level. The transfection of colorectal cancer cells with prolidase vector inhibited NF-κB expression (Surazynski et al. 2008a), a well-recognized inhibitor of expression of α1 and α2 subunits of type I collagen (Kouba et al. 1999; Rippe et al. 1999; Miltyk et al. 2007). Another evidence for the role of prolidase in the regulation of NF-κB expression provides an experiment showing that inhibition of prolidase activity by Cbz-Pro contributed to upregulation of NF-κB expression in fibroblasts (Surazynski et al. 2008a). In fact, our data showed that the HYD-dependent increase in collagen biosynthesis is accompanied by a decrease in the expression of NF-κB.

The mechanism of HYD-dependent stimulation of prolidase activity may undergo indirectly and may involve IGF-IR. In patients, HYD is known to upregulate 964 genes. Some of them produce proteins of MAPK signaling (De la Cruz-Hernández et al. 2011). However, increase in the expression of IGF-IR was independent of FAK and MAP kinase (ERK_1 and ERK_2) expression. Therefore, we suggest that the HYD-dependent increase of collagen biosynthesis in cultured human skin fibroblasts results from the activation of IGF-IR expression and prolidase activity and down-regulation of NF-κB p65.

Conclusion

The data suggest that HYD may exert its effect on collagen biosynthesis through stimulation of prolidase activity, expressions of IGF-IR, HIF-1α, and inhibition of NF-κB p65.

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Conflict of interest The authors declare that they have no conflict of interest.

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