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SHORT REPORT

Extracellular matrix and HIF-1 signaling: The role of prolidase

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Hypoxia-inducible factor-1 (HIF-1) plays an important role in stress-responsive gene expression. Although primarily sensitive to hypoxia, HIF-1 signaling can be regulated by a number of stress factors including metabolic stress, growth factors and molecules present in the extracellular matrix (ECM). Degradation of ECM by metalloproteinases (MMP) is important for tumor progression, invasion and metastasis. ECM is predominantly collagen, and the imino acids (Pro and HyPro) comprise 25% of collagen residues. The final step in collagen degradation is catalyzed by prolidase, the obligate peptidase for imidodipeptides with Pro and HyPro in the carboxyl terminus. Defective wound healing in patients with inherited prolidase deficiency is associated with histologic features of angiopathy suggesting that prolidase may play a role in angiogenesis. Because HIF-1α is central to angiogenesis, we considered that prolidase may modulate this pathway. To test this hypothesis, we made expression constructs of human prolidase and obtained stable transfectants in colorectal cancer cells (RKO). Overexpression of prolidase resulted in increased nuclear hypoxia inducible factor (HIF-1α) levels and elevated expression of HIF-1−dependent gene products, vascular endothelial growth factor (VEGF) and glucose transporter-1 (Glut-1). The activation of HIF-1-dependent transcription was shown by prolidase-dependent activation of hypoxia response element (HRE)-luciferase expression. We used an oxygen-dependent degradation domain (ODD)-luciferase reporter construct as a surrogate for HIF-1α as an *in situ* prolyl-hydroxylase assay. Since this reporter is degraded by VHL-dependent mechanisms, the increased levels of luciferase observed with prolidase expression reflected the decreased HIF-1α prolyl hydroxylase activity. Additionally, the differential expression of prolidase in 2 breast cancer cell lines showed prolidase-dependent differences in HIF-1α levels. These findings show that metabolism of imidodipeptides by prolidase plays a previously unrecognized role in angiogenic signaling.

Key words: proline; hypoxia; invasion; extracellular matrix; matrix metalloproteinase

The transcription factor, hypoxia-inducible factor-1 (HIF-1), plays an important role in stress-responsive gene expression.[1,2] HIF-1 is composed of 2 subunits, HIF-1α and HIF-1β.[1] Its activity is controlled primarily at the level of HIF-1α degradation.[2] The hydroxylation of specific proline residues in the oxygen-dependent degradation domain targets HIF-1α for ubiquitination and proteasomal degradation. In the context of cancer biology, target genes of HIF-1 are upregulated with hypoxic and nutrient stress and mediate angiogenesis, glycolysis, metastasis and survival.[3]

HIF-1 signaling responds to decreased oxygen tension, because oxygen is necessary for prolyl hydroxylation and degradation of HIF-1α;[1–3] hypoxia, therefore, stabilizes HIF-1α. However, HIF-1 signaling[1,2] also can be regulated under normoxic conditions by other mechanisms including hormones, growth factors and other stress-related factors.[3] Recent work has shown that metabolic intermediates in the TCA cycle can affect HIF-1α.[4] In the context of cellular interaction with its microenvironment, the activation of matrix metalloproteinases (MMP) and the degradation of extracellular matrix (ECM) also may contribute to angiogenic signaling.[5,6]

The major component of ECM is collagen, the most abundant protein in the body constituting more than a quarter of total body proteins.[7] Collagen is essential not only for musculoskeletal structure, but also for the integrity of tissue architecture. The various collagens have a triple-stranded helical structure contributed by the high content of proline and hydroxyproline which together make up 25–30% of collagen residues. During inflammation and cancer invasion, as much as 20% of tissue collagen in the microenvironment is degraded, thereby releasing a large quantity of peptides containing proline and hydroxyproline.[8] The contribution of these special substrates to cellular metabolism and regulation has only recently been appreciated.[9,10]

Prolidase (E.C.3.4.13.9) is the obligate enzyme for releasing proline and hydroxyproline from the carboxyl terminus of imidodipeptides.[11] Thus, prolidase plays an important role not only in nutrition but also in the degrading of collagen from ECM, recycling proline for protein synthesis and providing a substrate for bioenergetics.[12] Hydroxyproline, though not used for protein synthesis, may also play a special role in bioenergetics. The clinical findings in humans with inherited prolidase deficiency emphasize its importance in human physiology. These individuals exhibit defective wound healing resulting in extensive skin ulcerations.[13] Of special interest, histologic features on postmortem examination included marked angiopathy not only in skin ulcerations[14] but also in internal tissues,[15] suggesting that the deficiency in prolidase results in defective angiogenesis. Although these associations are of considerable interest, the mechanisms underlying them are not understood. Importantly, the role of prolidase linking matrix degradation with angiogenic signaling mechanisms has not been previously described.

We propose that prolidase is involved in the angiogenic signaling central to the hypoxia/inflammation response by modulating the processing of HIF-1α. Using expression constructs of human prolidase and isolating stable transfectants of colorectal cancer cells (RKO), we characterized the responses to overexpression of prolidase. We now report that prolidase activity mediates an important signal to activate the angiogenic pathway; this finding may

Abbreviations: ECM, extracellular matrix; HIF, hypoxia inducible factor; MMP, matrix metalloproteinase; ODD, oxygen-dependent degradation domain; Pro, proline; HyPro, hydroxyproline; VEGF, vascular endothelial growth factor.

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have important implications in therapeutic strategies especially those targeting MMP.

Material and methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM), glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc. (Gaithersburg, MD). Zeomycin, Lipofectamine 2000 and pcDNA vector were purchased from Invitrogen, (Carlsbad, CA). Glycyl–Proline (Gly–Pro), Glycyl–Hydroxyproline (Gly–HyPro) and *N*-benzyloxycarbonyl-L-proline (Cbz-Pro) were products of Bachem (King of Prussia, PA). Proline and hydroxyproline were obtained from Sigma (St Louis, MO). Because of the poor aqueous solubility of Cbz-Pro, this compound was dissolved in DMSO, and a similar quantity of DMSO was added to the control preparations.

Cell culture

RKO, a human colorectal cancer cell line, MCF-7 and MDA-MB 231, 2 breast cancer cell lines, were from the American Type Culture Collection, (Rockville, MD). We chose RKO cells for their ease of transfection and relatively low levels of prolidase. The cells were maintained in DMEM (high glucose) supplemented with 10% FBS (HyClone Laboratories Logan, UT), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Cells were generally maintained in 75 cm^2 flasks (Corning) but for the experiments, cells were plated onto 100-mm dishes (Falcon) with 10 ml of medium.

Determination of prolidase activity

The activity of prolidase was determined as previously described according to the method of Myara *et al.*[11] Activation of prolidase requires incubation with Mn (II) for 24 hr at 37°C. The final concentration of substrate glycyl-proline (Gly-Pro) was 47 mM and the duration of incubation was 1 hr at 37°C. Enzyme activity was reported in nanomoles of proline released per minute per milligram of protein.

Western blot analysis

Protein preparations were as follows: for VEGF analysis, serum-free media was exposed to cells for 2 days followed by 20-fold concentration through a 5 kDa cut-off Amicon Ultra filter (Millipore, Bedford, MA). Other protein preparations, both nuclear and cytoplasmic, were prepared using the method described with the NE-PER kit from Pierce (Rockford, IL). Equal amounts of protein (9–25 μg) were mixed with 4X LDS sample buffer (Invitrogen) with DTT reducing agent (Invitrogen) and electrophoresed on NuPage 4–12% Bis-Tris gradient gels using NuPage MES SDS running buffer (Invitrogen). Gel proteins were electro-blotted onto nitrocellulose membranes (Bio Rad, Hercules, CA) using 2X NuPage transfer buffer (Invitrogen) and the Trans Blot SD semi-dry transfer cell (Bio Rad, Hercules, CA). Subsequently, blots were blocked for 1 hr at room temperature with 3% nonfat milk in tris-buffered saline (TBS/NFDM), pH 8.0 (Sigma, St. Louis, MO). The primary antibodies used were: rabbit anti-prolidase[16] (1:1000), mouse anti-HIF-1α (1:500) from BD Transduction Laboratories (San Diego, CA); Mouse anti-VEGF (C-1), Rabbit anti-GAPDH (1:1000), Mouse anti-Actin (C-2), (1:1000) and rabbit anti-Glut1 (1:500) from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were goat anti-mouse or anti-rabbit HRP-conjugated antibodies (Bio Rad, Hercules, CA). All antibodies were diluted in TBS/NFDM, and incubated with the blots for the indicated time: primary antibodies, overnight at 4°C and secondary antibodies, 1 hr at room temperature. After incubation, blots were washed according to standard procedure using TBS with Tween (Sigma, St. Louis, MO), and developed using the enhanced chemiluminescence procedure (Amersham Biosciences, Piscataway, NJ).

ELISA assay for VEGF

Conditioned serum-free medium collected as described above was assayed for the presence of VEGF using the method described in the ELISA kit for VEGF from ALPCO Diagnostics (Windham, NH).

Plasmid constructs and stable transfectants

The constructions of HRE-Luc plasmid and HIF-1α ODD plasmid are described elsewhere.[17,18] The former monitors HIF-dependent transcription and the latter is an indicator of *in situ* prolyl-hydroxylation. cDNA amplified from RT-PCR encoding the full sequence of prolidase was inserted into pcDNA3.1 vector (Invitrogen) to generate the prolidase plasmid. Transfection was carried out using lipofectamine 2000 (Invitrogen) using the manufacturer's protocol. Briefly, culture medium was removed from wells and the adherent cells washed twice with PBS. 0.5 ml of transfection complex in Opti-MEM I medium containing lipofectamine 2000 (2 μl) and plasmid (1 μg), was then added to each well. Control cells were transfected with empty vector. Cells with the appropriate vector stably integrated were selected in the presence of Zeomycin (400 μg/ml for 1 week) and are composed of pooled clones.

Reporter assays

About 2×10^5 RKO cells per well were plated into 6-well plates in 2 ml media. Next day cells were transfected with 50 ng/well CMV-Luc-ODD and 200 ng/well pRL-tk Renilla luciferase (RL-tk) or 100 ng/well HRE-Luc and 200 ng/well RL-tk plasmids with 2 μl of the transfection reagent, Lipofectamine 2000. After transfection, plasmid expression was allowed for 16–24 hr. The reporter expression was detected using the Dual-Glo luciferase system (Promega, Madison, WI) and the MicroLumat LB 96V Luminometer (Bad Wildbad, Germany). The data are presented as mean values ± SEM of normalized relative luciferase units (RLU), which represent the ratio of luminescence produced by CMV-Luc-ODD or HRE-Luc plasmid to the luminescence of the RL-tk reporter in the same well (Promega).

Statistical analysis

In all experiments, the mean values for at least 3 assays ± SEM were calculated unless otherwise indicated. The results were submitted to statistical analysis using the Student's *t*-test, accepting * or +, $p < 0.05$ and ** or ++, $p < 0.01$, as significant.

Results and discussion

Prolidase stable transfectants

Our previously published studies showed that prolidase participates not only in regulating collagen synthesis but also is involved in many physiological and pathological processes.[16] To test our hypothesis that prolidase may play a role in angiogenesis, we obtained a gain-of-function experimental model by transfecting RKO cells with a prolidase cDNA expression plasmid and obtained stable transfectants. These cells showed markedly increased prolidase expression (Fig. 1*a*) and increased prolidase enzyme activity (Fig. 1*b*) compared to vector transfected controls.

Effect of prolidase expression on hypoxia/inflammation signals

The aforementioned link of prolidase with inflammation and wound healing led us to monitor mediators of the hypoxia/inflammation response pathway.[1] We considered the effect of prolidase on the expression of vascular endothelial growth factor (VEGF), a potent angiogenic factor, glucose transporter-1 (Glut-1), a mediator of a limiting step in glucose metabolism and transforming growth factor beta (TGFβ), a stimulator of collagen synthesis, and monitored their expression in cells stably transfected with prolidase. In the medium of cells stably transfected with prolidase, the level of VEGF was markedly higher than in control cells (Figs. 1*c*–1*d*). Glut-1, which participates in the adaptive hypoxia/inflam-

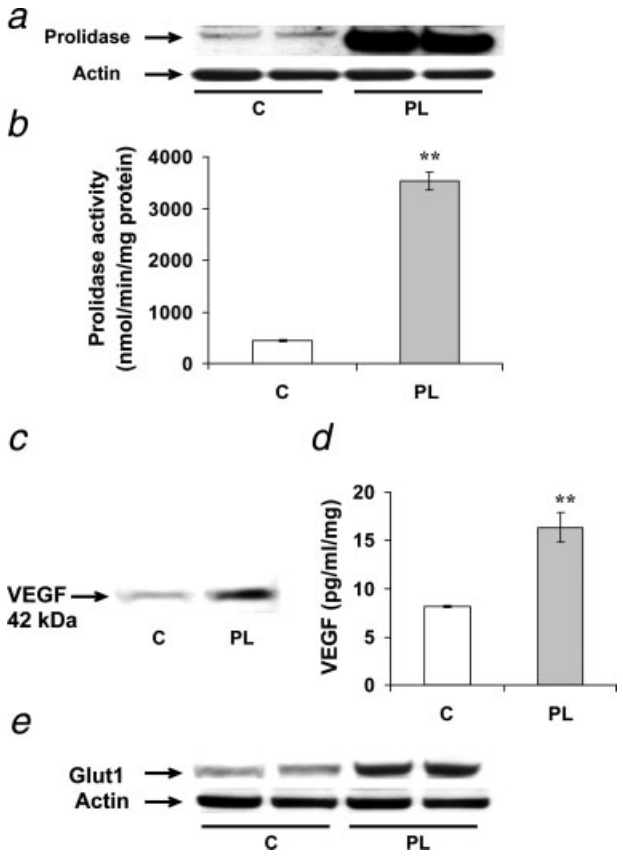

FIGURE 1 – Prolidase expression in RKO cells stably transfected with empty vector as control (C) or with prolidase (PL) as monitored by (*a*) western immunoblot or by (*b*) measurement of prolidase enzymatic activity. Data shown represent mean ± SEM of at least 3 determinations and the difference is statistically different by student's "*t*" test (**, $p < 0.01$). (*c*) VEGF expression was determined by western immunoblot in the conditioned medium of RKO cells stably transfected with empty vector (C) or with prolidase (PL) and is a typical blot from multiple experiments. (*d*) VEGF expression was determined by ELISA assay in the conditioned medium of RKO cells stably transfected with empty vector (C) or with prolidase (PL). Data shown represent the mean ± SEM of at least 3 determinations. The difference is significant by student's "*t*" test (**$p < 0.01$). (*e*) Glut-1 expression was determined in cell extracts by western immunoblot. For both *a* and *e*, typical blots are shown, and replicates represent results from separate cell harvests.

mation response, was also upregulated with overexpression of prolidase (Fig. 1*e*). In contrast to VEGF and Glut-1, the expression of TGFβ (isoforms 1 and 3) showed no difference between prolidase transfected and control cells (data not shown).

The effect of prolidase on HIF-dependent transcription and HIF-1α

Since VEGF and Glut-1, downstream targets of hypoxia inducible factor (HIF-1),[1] were found to be upregulated in prolidase-overexpressing cells, we chose to investigate the effect of prolidase on the HIF-1 axis. We first transfected an HRE-luciferase construct into cells stably overexpressing prolidase. The activation of the HRE promoter site was 2.5-fold that of cells transfected with empty vector (Fig. 2*a*), and as expected, levels of HIF-1α were markedly higher in the nuclear fraction of stably transfected prolidase cells than in vector controls (Fig. 2*b*). Thus, we concluded that the increase in VEGF and Glut-1 with prolidase overexpression was accompanied by the activation of HIF-1-dependent transcription.

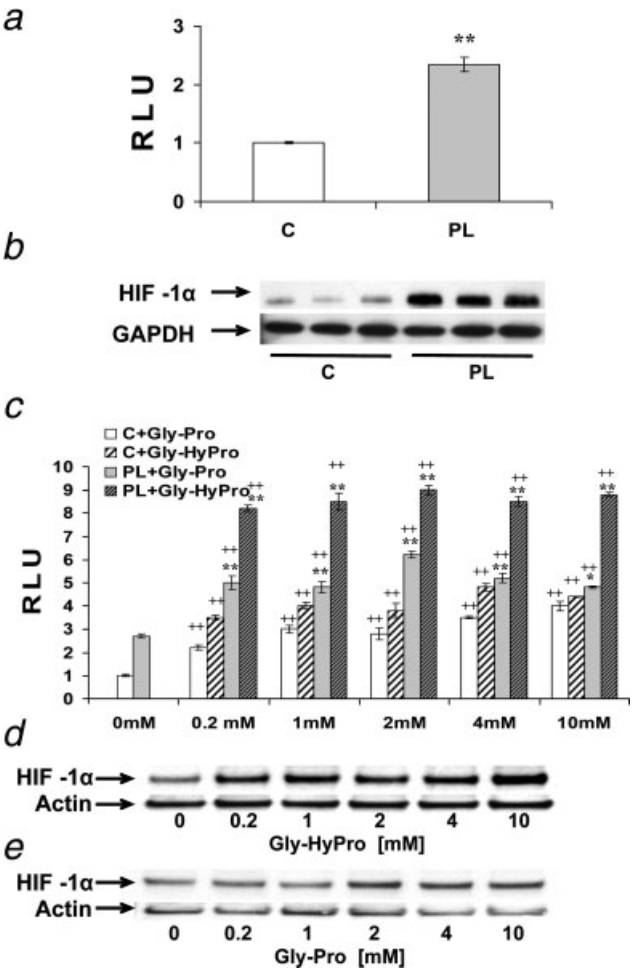

FIGURE 2 – In *a* and *c*, HIF-dependent expression of luciferase activity. RKO cells, stably transfected with prolidase (PL) or empty vector (C), were transiently transfected with the luciferase-expressing constructs HRE-Luc and pRL-tk. Expression data are given as relative luciferase units (RLU) and represent mean ± SEM of at least 3 determinations. In *c*, cells were exposed to dipeptides at the indicated concentrations for 24 hr. The differences as compared to the level in cells without dipeptides (plus signs), or the differences between C cells and PL cells at the corresponding dipeptide concentrations within each group (asterisks) are statistically significant by student's "*t*" test (*, $p < 0.05$; ** or ++, $p < 0.01$). In *b*, stably transfected C and PL cells were plated at 6×10^6 cells per 10-cm plate in complete DMEM containing 1 g glucose per liter and incubated for 48 hr before nuclear lysates were prepared for HIF-1α immunoblot analysis. The duplicates represent separate cell harvests. In *d* and *e*, immunoblots were prepared from PL cells exposed to the dipeptides at various concentrations for 24 hr before harvest.

Effects of prolidase substrates on HIF-dependent transcription and HIF-1α

Prolidase is an enzyme that catalyses the hydrolysis of imidodipeptides with C-terminal proline or hydroxyproline such as Gly-Pro or Gly-HyPro. We considered it likely that the observed effects on HIF-1 transcription and HIF-1α levels were due to the catalytic activity of prolidase (see Prolidase in breast cancer cells). However, we exposed cells to the presence in the medium of prolidase substrates, *i.e.* Gly-Pro or Gly-HyPro, and monitored expression of HRE-luciferase as well as levels of HIF-1α. In Figure 2*c* we see that the activity of the HRE reporter increased with overexpression of prolidase even without added prolidase substrates as in Figure 2*b*. However, with addition of Gly-Pro or Gly-HyPro,

the expression of the reporter increased further at all concentrations tested. Two points are noteworthy in these results. First, Gly-HyPro seemed to be more effective than Gly-Pro. Second, the effects of these substrates were evident in cells transfected with vector alone (Fig. 2*c*). Presumably the latter finding was due to basal expression of prolidase in RKO cells (Figs. 1*a* and 1*b*). We then examined HIF-1α levels in nuclear extracts from cells exposed to increasing concentrations of either Gly-Pro or Gly-HyPro. In cells stably transfected with prolidase, increasing concentrations of Gly-HyPro yielded increasing HIF-1α levels (Fig. 2*d*). Although increased levels also were seen with increasing Gly-Pro concentrations, the effect was not apparent until higher Gly-Pro concentrations (Fig. 2*e*). These results clearly show that the effects of prolidase overexpression are related to its enzymatic function since substrates augmented the effects on both activation of the HRE-reporter and the levels of HIF-1α in nuclear extracts.

The effect of prolidase on HIF-1α degradation

Increased levels of HIF-1α may result from increased production or decreased degradation.[2] Since the half-life of HIF-1α is short, marked changes in nuclear levels can be seen with alterations of degradation. It is well established that degradation is dependent on hydroxylation of Pro-402/564 located in the oxygen-dependent degradation domain (ODD) of HIF-1α.[2] Hydroxylation of ODD is required for HIF-1α interaction with the Von Hippel-Lindau (VHL) tumor suppressor protein, critical for ubiquitinylation and proteasomal degradation. To test whether prolidase affects HIF-1α degradation dependent on ODD-VHL interaction, we used a reporter plasmid expressing a protein, which is the fusion product of the ODD fragment of HIF-1α with luciferase (CMV-Luc-ODD). In a previous publication, it was established that the level of this fusion protein is determined by the rate of ODD-VHL dependent degradation.[17,18] The CMV-Luc-ODD reporter was transfected into RKO cells stably transfected with prolidase or with vector. We found a marked increase in Luc-ODD levels in cells transfected with prolidase signifying that the degradation of the fusion protein was decreased (Fig. 3*a*). In addition, when these cells were exposed to Gly-Pro or Gly-HyPro, we found a further increase in the levels of Luc-ODD. Again, Gly-HyPro was more potent than Gly-Pro at least in cells overexpressing prolidase.

Products of prolidase activity and HIF-1α degradation

The aforementioned results with added prolidase substrates suggested that the effects on HRE and HIF-1α are due to the products of prolidase catalytic activity. Therefore, we examined ODD-Luc degradation in RKO cells expressing control levels of prolidase but with increasing medium concentrations of product proline, hydroxyproline or glycine. As shown in Figure 3*b*, hydroxyproline and proline markedly decreased ODD-Luc degradation. However, hydroxyproline appeared more potent, producing an effect at lower concentrations and also producing a greater maximal effect. Glycine had no effect on HIF-1α levels (data not shown). These data indicate that proline and hydroxyproline inhibit the degradation of HIF-1α via the VHL-dependent proteosomal pathway. However, hydroxyproline was considerably more potent than proline. In control cells, an increasing effect was seen with hydroxyproline, whereas with Gly-HyPro, the effect plateaued at 1 mM, and the magnitude with the imidodipeptide did not attain the same level as with the free imino acid. Thus, we demonstrated that forced prolidase expression increased angiogenic signaling by decreasing degradation of HIF-1α. Furthermore, we showed that the products of prolidase, particularly hydroxyproline, can mimic this effect even when prolidase is expressed at levels in control cells. The finding that hydroxyproline is more potent than proline suggested that the effect may involve interference with the recognition process between VHL and the hydroxyproline in ODD. Alternatively, reactive oxygen species (ROS) or Δ^1-pyrroline-5-carboxylate (P5C), produced from proline by proline oxidase, may inhibit prolyl hydroxylation of ODD. Elucidation of the mechanism mediating the observed effect are currently underway in our laboratory.

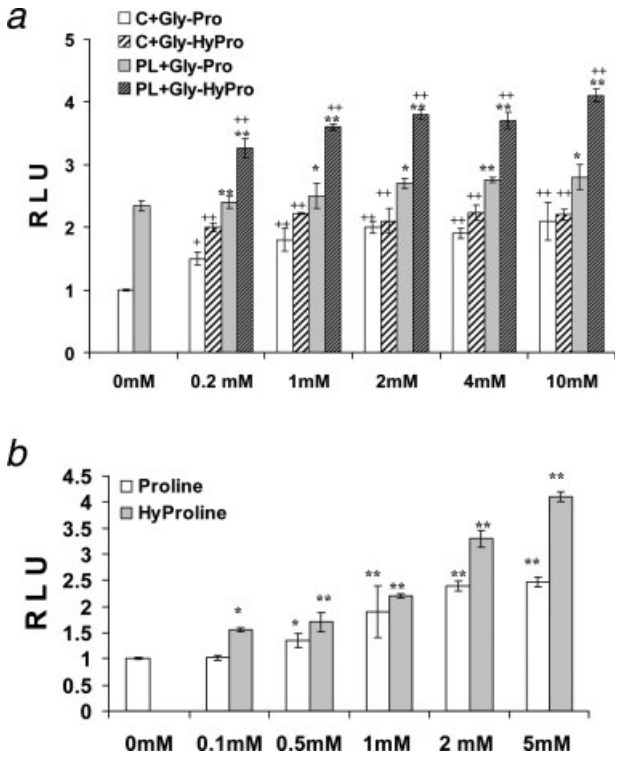

FIGURE 3 – (*a*) Effect of prolidase expression with or without substrate imidodipeptides on the degradation of Luc-ODD (HIF-1α surrogate) in RKO cells stably transfected with prolidase (PL) or with empty vector (C). To determine VHL-dependent degradation, cells were transiently transfected with CMV-Luc-ODD and pRL-tk. They were also incubated with or without various concentrations of Gly-HyPro or Gly-Pro. After 24 hr of treatment, cells were harvested and measured for relative luciferase activity expressed as relative luciferase units (RLU). Data represent the mean ± SEM of at least 3 determinations. Differences compared to the baseline value in C cells without Gly-Pro or Gly-Hypro are indicated by plus signs above the bars; differences between the values in PL cells at each concentration of Gly-Pro or Gly-HyPro, compared to the corresponding value in C cells within each treatment group, are shown with asterisks. Differences are statistically significant by student's "*t*" test: (* or +, $p < 0.05$; ** or ++, $p < 0.01$). (*b*) Effect of various concentrations of hydroxyproline or proline on the degradation of Luc-ODD in RKO cells expressing control levels of prolidase. Methods were as in 3*a*, and data are expressed as relative luciferase units (RLU) and represent mean ± SEM of at least 3 determinations. Differences, based on comparison to the value without proline or HyProline are statistically significant (*, $p < 0.05$; **, $p < 0.01$).

Prolidase in breast cancer cells

Although we showed that overexpression of prolidase decreased HIF-1α degradation and increased HRE-dependent transcription, we sought a correlation with human cancer. It has been shown that prolidase activity and protein is elevated 5-fold in breast tumors compared to normal breast tissue and this is associated with increased degradation of extracellular matrix.[19] Since breast tumors are associated with high levels of HIF-1α,[3] we examined breast cancer cell lines with known differential activities of prolidase. MDA-MB 231 cells, a cell line with highly invasive properties, have prolidase activities nearly 5-fold those of MCF-7 (Fig. 4*a*). Interestingly, the HIF-1α levels under normoxic conditions are markedly higher in MDA-MB-231 cells than in MCF-7 cells (Fig. 4*b*), but the levels in the latter could respond appropriately to

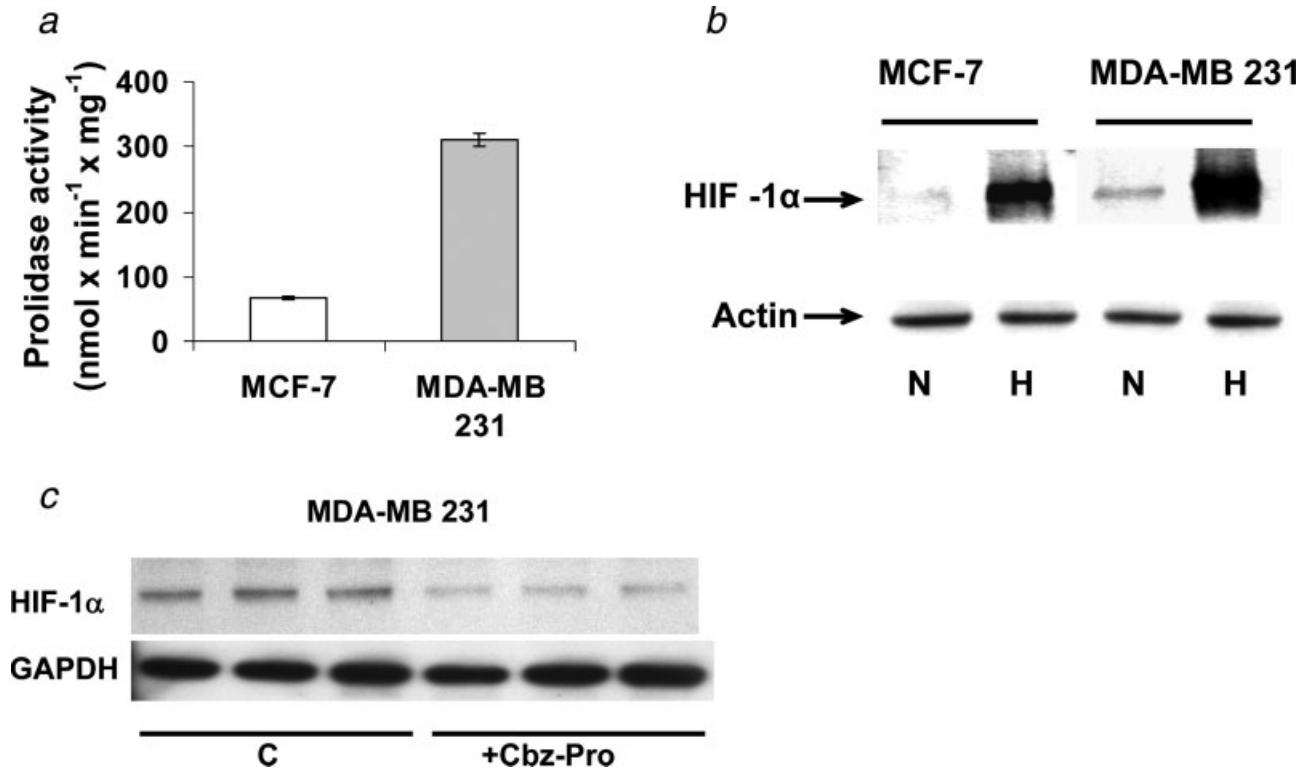

Figure 4 – Prolidase activity in breast cancer cells. Prolidase activities were measured as described and data represent the mean ± SEM of at least 3 determinations. The difference is statistically significant ($p < 0.01$). (*b*) Western immunoblots for HIF-1α in nuclear extracts from MDA-MB-231 and MCF-7 cells. To induce the effects of hypoxia, cells were exposed to $CoCl_2$ (0.25 mM) or none for 24 hr before harvest. N-Normoxia, H-Hypoxia. (*c*) Western immunoblots for HIF-1α in nuclear extracts from MDA-MB-231 cells. Cells were plated at 5×10^5 per 10 cm dish in complete DMEM containing 1 g glucose per liter and incubated for 4 days before the addition of Cbz-Pro (10 mM) for 24 hr before cell harvest. The replicates represent separate cell harvests. An equal amount of DMSO was added to the control preparations.

$CoCl_2$ to mimic hypoxia.[17,18] Importantly, the levels in MDA-MB-231 were markedly attenuated by treatment with Cbz-Pro, the inhibitor of prolidase activity.[20] The direct inhibition of prolidase activity offers strong evidence that the higher levels of HIF-1α in MDA-MB-231 were related to prolidase activity in these cells.

Although hypoxia and inflammation share certain signaling processes and express common endpoints, *i.e.* collagen synthesis, tissue-remodeling and angiogenesis, the initiating mechanisms differ and are not completely understood. In the case of hypoxia, the effects of oxygen on the prolyl hydroxylation of the ODD of HIF-1α determine its nuclear levels by modulating its proteasomal degradation.[1,2] With inflammatory processes, cytokine-mediated activation of matrix metalloproteinases and increased matrix degradation releases proline/hydroxyproline-containing peptides.[21,22] The peptides' final degradation, mediated by prolidase, releases proline and hydroxyproline, which may play an important role in modulating nuclear levels of HIF-1α by directly or indirectly inhibiting its prolylhydroxylation and thereby decreasing its degradation. Defective angiogenic signaling in patients with prolidase deficiency may be responsible for the clinical manifestations seen in this inherited disorder.

On the other hand, inhibitors of MMPs had promising antiangiogenic and antitumor effects in preclinical models, but clinical trials have been disappointing.[23] A potential mechanism contributing to the effects of MMP inhibitors is to reduce substrate for prolidase *i.e.*, reducing the release of free imino acids and hydroxyproline, in particular. Clinical models investigating the potential of MMP inhibitors may benefit by considering endogenous and exogenous sources of proline and hydroxyproline to elicit the full antiangiogenic and antitumor effects.

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