Journal of Cellular Biochemistry 94:1210–1217 (2005)

Inhibition of Prolidase Activity by Nickel Causes Decreased Growth of Proline Auxotrophic CHO Cells

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Abstract Occupational exposure to nickel has been epidemiologically linked to increased cancer risk in the respiratory tract. Nickel-induced cell transformation is associated with both genotoxic and epigenetic mechanisms that are poorly understood. Prolidase [E.C.3.4.13.9] is a cytosolic Mn(II)-activated metalloproteinase that specifically hydrolyzes imidodipeptides with C-terminal proline or hydroxyproline and plays an important role in the recycling of proline for protein synthesis and cell growth. Prolidase also provides free proline as substrate for proline oxidase, whose gene is activated by p53 during apoptosis. The inhibition of prolidase activity by nickel has not yet been studied. We first showed that Ni(II) chloride specifically inhibited prolidase activity in CHO-K1 cells in situ. This interpretation was possible because CHO-K1 cells are proline auxotrophs requiring added free proline or proline released from added Gly-Pro by prolidase. In a dose-dependent fashion, Ni(II) inhibited growth on Gly-Pro but did not inhibit growth on proline, thereby showing inhibition of prolidase in situ in the absence of nonspecific toxicity. Studies using cell-free extracts showed that Ni(II) inhibited prolidase activity when present during prolidase activation with Mn(II) or during incubation with Gly-Pro. In kinetic studies, we found that Ni(II) inhibition of prolidase varied with respect to Mn(II) concentration. Analysis of these data suggested that increasing concentrations of Mn(II) stabilized the enzyme protein against Ni(II) inhibition. Because prolidase is an important enzyme in collagen metabolism, inhibition of the enzyme activity by nickel could alter the metabolism of collagen and other matrix proteins, and thereby alter cell–matrix and cell–cell interactions involved in gene expression, genomic stability, cellular differentiation, and cell proliferation. J. Cell. Biochem. 94: 1210–1217, 2005. Published 2005 Wiley-Liss, Inc.†

Key words: prolidase; nickel; CHO cells; proline

The International Agency for Research on Cancer has classified nickel compounds as human carcinogens [WHO, 1990]. Occupational exposure to nickel has been epidemiologically associated with an increased risk of nasopharyngeal and lung cancer [Doll et al., 1970]. The latter is the leading cause of cancer deaths in the United States [Williams and Sandler, 2001]. Although nickel toxicity has been associated with the generation of reactive oxygen species causing promutagenic DNA damage [Kasprzak, 1995; Bal et al., 2000], inhibition of DNA damage repair [Hartwig and Schwerdtle, 2002], or changes in DNA methylation patterns [Costa et al., 2001], nickel-induced carcinogenesis may also include a variety of other poorly understood mechanisms [Kasprzak et al., 2003].

Prolidase [E.C.3.4.13.9] is a cytosolic enzyme, which specifically hydrolyzes imidodipeptides with C-terminal proline or hydroxyproline [Myara et al., 1984]. This enzyme catalyzes

Grant sponsor: National Cancer Institute, National Institutes of Health; Grant numbers: NO1-CO-12400 and 12401.

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Received 30 August 2004; Accepted 9 November 2004
DOI 10.1002/jcb.20384

the terminal step in the degradation of proline-containing proteins (e.g., collagen by matrix metalloproteinases), providing large amounts of proline for collagen synthesis and thus plays an important role in proline nutrition and in the recycling of proline for protein synthesis and cell growth [Jackson et al., 1975; Myara et al., 1984]. Importantly, prolidase may also provide proline, the substrate for generating the reactive oxygen species by proline oxidase during apoptosis [Donald et al., 2001; Maxwell and Rivera, 2003]. Therefore, mechanisms regulating prolidase expression and enzymatic activity are of considerable interest. Clinical findings in humans with inherited prolidase deficiency include skin ulcerations, immuno-deficiency, and mental retardation [Royce and Steinmann, 1993]. On the biochemical level, prolidase is a manganese-requiring enzyme. Antineoplastic anthracyclines cause poor wound healing in part by inhibiting prolidase activity via a chelation activity towards manganese [Muszynska et al., 2000]. Prolidase interaction with a variety of metals has been reported and its activity modulated by exogenous metals, i.e., nickel, may have clinically relevant implications. The biologically relevant modulation of prolidase activity by nickel and its cellular consequences have not been studied.

We tested the physiological consequence of the inhibition of prolidase in Chinese hamster ovary cells (CHO-K1), which are auxotrophic for proline [Emmerson and Phang, 1993]. Imidodipeptides with C-terminal proline can completely satisfy the proline requirement for these cells. Inhibition of prolidase activity by nickel will inhibit the release of proline from imidodipeptides for important cellular functions. After establishing the physiologic relevance of this inhibition, we explored whether nickel displaces manganese in an interactive, reversible fashion, or causes an alteration of prolidase activity independent of Mn(II). We used a novel mathematical model to characterize this interaction.

MATERIALS AND METHODS

Materials

L-proline, L-glycyl-L-proline and other chemicals were purchased from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium and fetal bovine serum were from Gibco (Rockville, MD).

Cell Culture

The CHO-C9 cell line was subcloned from proline auxotrophic CHO-K1 cells originally obtained from the American Type Culture Collection. CHO-C9 cells lack detectable ornithine-δ-aminotransferase and pyrroline-5-carboxylic acid (P5C) synthase activities whereas the parent CHO-K1 cells retain about 10%–15% of ornithine-δ-aminotransferase activity [Valle et al., 1973]. The cells were maintained at 37°C in 5% CO_2 incubator in 72 cm^2 tissue culture flasks, in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 2 mM glutamine, and 0.5 mM proline. For growth experiments cells were counted in a hemacytometer and 0.5×10^5 cells were inoculated in 5 ml of medium onto a 60 mm Falcon dish. The medium contained addends as described in the legends for specific experiments. Cell number was determined in a hemacytometer in triplicate and the reported data represent the mean of at least three experiments.

Determination of Prolidase Activity

The activity of prolidase was determined according to the method of Myara et al. [1982], which is based on colorimetric determination of proline using Chinard's reagent [Chinard, 1952]. Briefly, the monolayer was washed three times with 0.15 M NaCl. Cells were trypsinized and centrifuged at 200*g* for 15 min and the supernatant was discarded. The cell pellet was suspended in 1 ml of 50 mM HEPES, pH 7.8, and sonicated for 3×10 s at 0°C. Samples were then centrifuged (12,000*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 Mn(II); for routine activation, 100 μl of cell extract supernatant was mixed with 100 μl of 50 mM HEPES, pH 7.8 containing $MnCl_2$ at a final concentration of 1 mM in the mixture. For specific activation procedures at various concentrations of Mn(II) in the presence or absence of inhibitor Ni(II), the methods are described in the legends for the figures. After incubation for 24 h at 37°C, the prolidase reaction was initiated by adding 100 μl of the activated mixture to 100 μl of 94 mM glycyl-proline (Gly-Pro) for a final concentration of 47 mM. Appropriate changes in Gly-Pro concentration were used for certain experiments

as described in legends to the figures. After additional incubation for 1 h at 37°C, the reaction was terminated with the addition of 1 ml of 0.45M trichloroacetic acid. To parallel blank tubes, trichloroacetic acid was added at time "zero." Samples were centrifuged at 10,000*g* for 15 min. The released proline was determined by adding 0.5 ml of the trichloroacetic acid supernatant to 2 ml of a 1:1 mixture of glacial acetic acid: Chinard's reagent (25 g of ninhydrin dissolved at 70°C in 600 ml of glacial acetic acid and 400 ml of 6 M orthophosphoric acid) and incubated for 10 min at 90°C. The amount of proline released was determined colorimetrically by monitoring absorbance at 515 nm and calculated using proline standards. Enzyme activity was reported in nanomoles of proline released per minute per milligram of protein.

RESULTS

Nickel has a wide-spectrum of toxic effects both in cell-free and cell culture systems [Kasprzak et al., 2003]. To establish that prolidase is a specific physiologic target of Ni(II) inhibition, we employed an experimental model using CHO-K1, a cell line auxotrophic for proline. Lacking the pathways for endogenous proline synthesis, these cells grow only if proline or imidodipeptides, e.g., Gly-Pro, are added to the medium. The ability to grow on Gly-Pro, however, depends on the enzymatic cleavage of the imidodipeptide by prolidase [Emmerson and Phang, 1993].

As shown in Figure 1, CHO-C9 (a subclone of CHO-K1) showed no growth without an added source of proline. Growth on Gly-Pro (100 μM) was indistinguishable from that on free proline (500 μM). Thus, Gly-Pro, the most physiologically abundant imidodipeptide, after enzymatic cleavage by prolidase, provided adequate proline to support normal growth of CHO-C9 cells. We then tested the Ni (II) inhibition of prolidase by culturing cells with 500 μM proline or 100 μM Gly-Pro, with or without two concentrations of Ni(II) chloride. Importantly, addition of Ni(II) inhibited growth on Gly-Pro in a concentration-dependent manner. At 24 h, the effects of Ni(II), as compared to Gly-Pro controls, were significant to $P < 0.05$, while at 48 and 72 h, the effects of Ni(II) were significant at $P < 0.01$. At all time points, no effect of Ni(II) on growth with proline was observed. Thus, Ni(II) inhibited prolidase in cultured cells in situ under

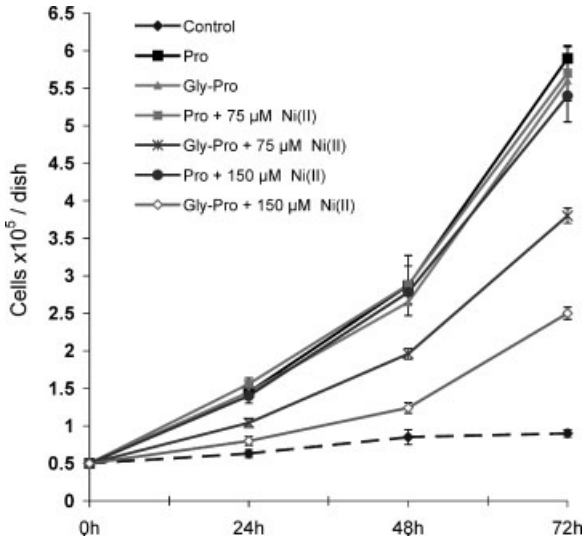

Fig. 1. Growth curves for proline auxotrophic CHO-C9 (a clone of CHO-K1) cells in the presence of 500 μM Pro or 100 μM Gly-Pro, with or without Ni(II) at 75 or 150 μM. Each point represents the mean ± SEM of three experiments, performed individually in triplicate. Cell counts were determined at 24 h intervals. (◆), control without Pro or Gly-Pro; (■), control with 500 μM Pro; (▲), control with 100 μM Gly-Pro; (■), Pro + 75 μM Ni(II); (✱), Gly-Pro + 75 μM Ni(II); (●), Pro + 150 μM Ni(II); (◇), Gly-Pro + 150 μM Ni(II).

conditions which produced no other apparent toxic effects. Although this differential effect was observed at the aforementioned Ni(II) concentrations, at concentrations higher than 150 μM, inhibition of growth on proline was also observed, even though the magnitude of the inhibition on Gly-Pro further increased. The threshold of these general cytotoxic effects are in agreement with previously reported effects in Chinese hamster ovary cells [Shiao et al., 1998].

The demonstration of physiologic inhibition of prolidase by Ni(II) in situ led us to characterize the interaction between prolidase and its putative metal ligand, manganese. Enzyme purified from various tissue sources shows different requirements for manganese to achieve catalytic activity [Hechtman, 2001]. To characterize prolidase from CHO-C9, we incubated extract for 24 h with various concentrations of Mn(II) chloride. As seen in Figure 2, prolidase activity is almost undetectable without added Mn(II). Addition of manganese increased the activity in a concentration-dependent manner which approached saturation at 1 μM, thereby confirming that Mn(II) is critical for prolidase activity in CHO-

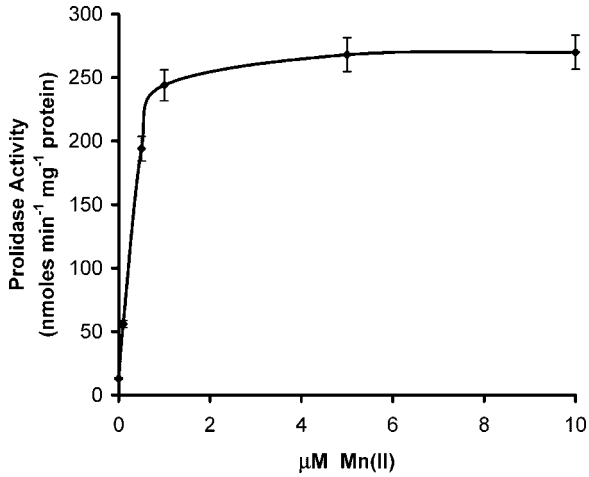

Fig. 2. Effect of manganese (II) chloride on prolidase activity in CHO-C9 extract. The extract was incubated with various concentrations of Mn(II) chloride for 24 h at 37°C. Then substrate Gly-Pro was added, incubation continued for 1 h at 37°C and product proline determined as specified under "Materials and Methods." Data represent mean ± SEM of at least three separate cell harvests.

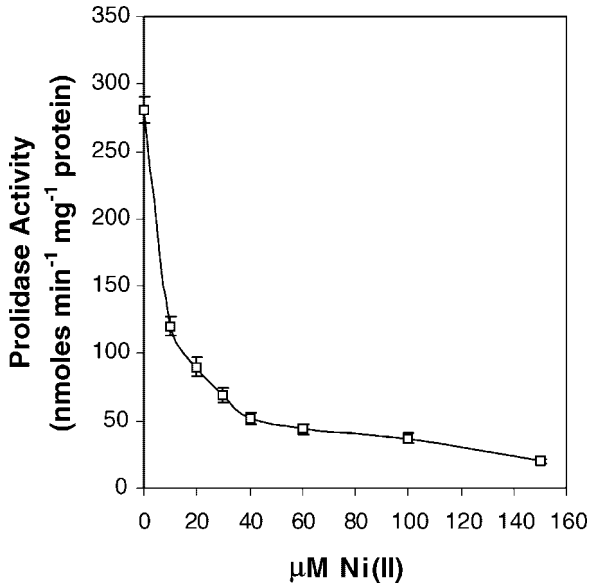

Fig. 3. Inhibition of prolidase activity in CHO-C9 cell extract by Ni(II). The extract was incubated with 1 μM Mn(II) chloride and indicated concentrations of Ni(II) for 24 h at 37°C. Then the homogenate was incubated with substrate Gly-Pro for 1 h at 37°C. Product proline was determined as specified under "Materials and Methods." Data represent mean ± SEM of at least three separate cell harvests.

C9 extract. This requirement for Mn(II) ions was a likely target for Ni(II) interaction. To test this possibility, we incubated extracts of CHO-C9 with 1 μM Mn(II) chloride and various concentrations of Ni(II) for 24 h before prolidase activities were determined. Under these conditions, Ni(II) inhibited prolidase activity in a concentration-dependent manner (Fig. 3). Enzyme activity was inhibited about 50% at 10 μM Ni(II) concentration.

An interaction between Ni(II) and prolidase protein is the likely explanation for this inhibitory effect. However, Ni(II) could also interact with substrate Gly-Pro [Burger, 1990] and alter its availability for enzymatic cleavage. We tested this possibility by using high concentrations of Gly-Pro to assess whether it could reverse the inhibitory effect of Ni(II) on prolidase activity. CHO-C9 homogenate was incubated with 5 μM Mn(II) plus 40 μM Ni(II) and increasing concentrations of Gly-Pro. As shown in Figure 4, high concentrations of Gly-Pro did not reverse the inhibitory effect of nickel on prolidase activity. Thus, it is unlikely that Ni(II) was inhibiting enzyme activity by directly interacting with substrate Gly-Pro.

The nature of the inhibitory effect of Ni(II), whether displacing Mn(II) in a competitive fashion or interacting with prolidase protein in a manner independent of Mn(II), was an important question. We approached this problem kinetically and subjected the data to mathematical analysis. CHO-C9 cell extract was incubated with various concentrations of Mn(II) and Ni(II) for 24 h at 37°C and prolidase activity

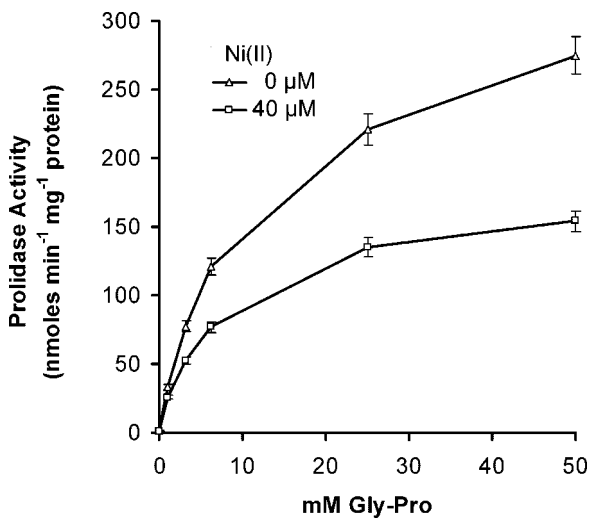

Fig. 4. Effect of increasing concentrations of Gly-Pro on the inhibitory effect of Ni(II) on prolidase activity. CHO-C9 homogenate was incubated with 5 μM Mn(II) with or without 40 μM Ni(II) for 24 h at 37°C. After activation, the homogenate was incubated for 1 h at 37°C with increasing concentrations of substrate Gly-Pro as indicated. Product proline was measured as described under "Materials and Methods." Data represent Mean ± SEM of at least three separate cell harvests.

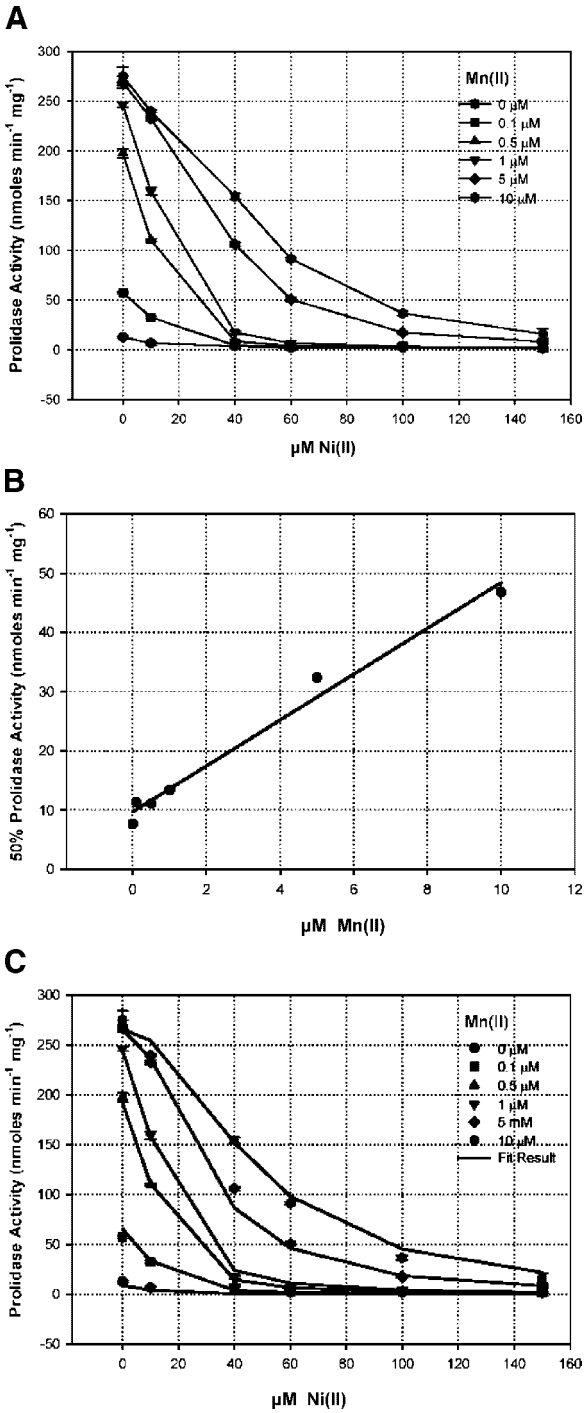

Fig. 5. **A**: Prolidase activity in CHO-C9 extract incubated for 24 h at 37°C with various concentrations of Mn(II) and Ni(II). After activation, substrate Gly-Pro was added at a final concentration of 47 mM and incubation continued for 1 h at 37°C. Product proline was determined as described under ''Materials and Methods.'' Values are the mean ± SEM of three cell harvests. Mn(II) concentrations are: (●), 0 μM; (■), 0.1 μM; (▲), 0.5 μM; (▼), 1 μM; (◆), 5 μM; (●), 10 μM. **B**: Plot of calculated IC_{50}s for Ni(II) against Mn(II) concentrations. **C**: Fit of the data to a logistic function as described under ''Results.'' The line represents the calculated best fit.

was determined. As shown in Figure 5A, the magnitude of the inhibition was affected by the concentration of Mn(II). However, at every Mn(II) concentration tested, maximal concentrations of Ni(II) could completely inhibit prolidase activity. Because these kinetic experiments were performed using a "crude" extract with undefined concentration of enzyme protein and unbound ligand, we used a logistic function to obtain a value at which 50% inhibition occurred.

$$v = \frac{V_{Max} - V_{Min}}{1 + 10^{\log\left(\frac{[Ni(II)]+0.001]}{IC50}\right)B}} + V_{Min}\Bigg|_{\text{given } [Mn(II)]} \quad (1)$$

In this equation, B is a unitless constant describing the slope of the velocity, IC50 is the half velocity concentration, and V_{Max}, V_{Min} describe the maximum and resting production of product. This expression produced an excellent fit of the data (not shown). When calculated values for Ni(II) IC50 were plotted against Mn(II) concentrations (Fig. 5B), a linear relationship was seen. This strongly suggests that there is an interaction between Mn(II) and Ni(II) at the protein level. However, Figure 2 shows that in the absence of Ni(II), the activity of the enzyme is reaching full activation relative to the concentration of Mn(II), a relationship which may be described by the exponential function:

$$\begin{aligned}&\left(V_{Max} - V_{Min}\right)\left([Mn(II)]\right)\\ &\quad = V_{saturation}\left(1 - e^{-c[Mn(II)]}\right) + V_{Resting}\end{aligned} \quad (2)$$

Where c[Mn(II)] shows the rate of [Mn(II)] activity as concentration changes, $V_{Saturation}$ and $V_{Resting}$ are velocities when the concentration of [Mn(II)] is saturating or zero, respectively. Since the IC50 values do not stabilize but actually increase as a function of Mn(II) (Fig. 5B), and this is in contrast to the apparent stabilization by Mn(II) in the absence of Ni(II) (Fig. 1), we may propose that the two metals are interacting in their access to the enzyme. To test this possibility, we formulated the following expression in which enzyme activity is stabilizing while the inhibition is increasing by the ratio of Mn(II) and Ni(II):

$$v = \frac{V_{saturation}\left(1 - e^{-c[Mn(II)]}\right) + V_{Resting}}{1 + 10^{\log\left(\frac{[Ni(II)]+0.001}{IC50}\right)B}} \quad (3)$$

In this expression, The V_{Max} parameter (Eq. 1) is replaced by the exponential function (Eq. 2)

and the IC50 parameter is replaced with a linear function describing the relationship shown in Figure 5B. Using this expression, we calculated and found an excellent fit of the actual data, thereby supporting the hypothesis that there is the aforementioned interaction between Mn(II) and Ni(II) for access to the enzyme (Fig. 5C).

DISCUSSION

To establish that prolidase is a target of nickel toxicity, we took advantage of a cell culture model in which proline auxotrophic CHO-C9 cells can obtain their required proline for growth either from added proline or from added imidodipeptides, e.g., Gly-Pro [Emmerson and Phang, 1993]. Gly-Pro as a source of proline depends on the catalytic activity of prolidase. Under conditions where Gly-Pro supported growth rates indistinguishable from that with proline, nickel at 75 and 150 μM inhibited growth on Gly-Pro by about 30% and 60%, respectively. At these concentrations, growth on added Pro was unaffected. At higher concentrations of nickel, further inhibition of growth on Gly-Pro was seen (data not shown). However, these higher concentrations of nickel also inhibited the growth on Pro such that toxicity was not limited to the effect on prolidase.

Having shown that Ni(II) inhibited prolidase in situ, we performed studies on cell-free extracts of CHO-C9 cells to characterize this inhibitory effect. We first showed that measured activity required the addition of Mn(II) as has been previously observed. However, the concentration required for full activation was much lower than that previously described. We found that prolidase was optimally active when exposed to Mn(II) at 1 μM. Although the conditions for activation were at 37° for 24 h, control experiments included assays of fresh extract without the activation incubation; these assays showed little activity. Additionally, "full activation" was seen in as little as 4 h (data not shown).

The inhibition of Ni(II) in these cell-free extracts of CHO-C9 cells were tested in the presence of 1 μM Mn(II). We found that the IC50 for Ni(II) was about 10 μM, somewhat lower than the apparent IC50 of the growth of CHO-K1 cells on Gly-Pro. Of course, numerous factors could account for this apparent divergence for these two measured inhibitory effects including the intracellular concentration of Mn(II) and Ni(II) as well as other divalent cations and the intracellular localization and interactions of prolidase. On the other hand, the concentrations for inhibitory activity in situ and in vitro are of the same order of magnitude.

To investigate the mechanism for the proposed inhibitory interaction between Ni(II) and Mn(II), we first tested the suggestion that Ni(II) could form a putative complex with substrate Gly-Pro [Burger, 1990]. Such does not appear to be the case as increasing concentrations of substrate did not modulate the inhibitory effect of Ni(II). We then performed kinetics studies in which the effect of increasing concentrations of Ni(II) was determined at Mn(II) concentrations ranging from 0 to 10 μM (Fig. 5A). Using a "crude extract" for our source of prolidase activity, we could not define concentrations of enzyme protein or even the concentration of free metal. Thus, to analyze this kinetics data, we used an IC50 logistics function, which showed that the IC50 for Ni(II) increased as a function of Mn(II) concentration. However, when only Mn(II) was present, the activity rapidly saturated. The divergence of response to Mn(II) in the presence and absence of Ni(II) suggested that their respective interactions were mechanistically different. Mn(II) was necessary for activating enzyme activity, perhaps by contributing to the conformation at the active site. Additionally, Mn(II) stabilized the enzyme from Ni(II) inactivation, but this occurred by a mechanism distinct from that of its contribution to the active site.

Inhibition of prolidase activity by nickel may impair several important cellular functions. Since prolidase catalyzes the final step in the degradation of collagen [Yaron and Naider, 1993], the major component of the extracellular matrix (ECM), inhibition of prolidase by nickel could alter turnover of collagen and modulate its interaction with important cell surface receptors, e.g., integrin receptors. This family of signaling molecules modulates gene expression, genomic stability, cellular differentiation, and cell proliferation [Bissel, 1981; Albeda and Buck, 1990; Carey, 1991]. Research has recently highlighted the possible role of adhesion molecules in the initiation of cancer [Tlsty, 1998], and clearly, the metastatic potential of tumor cells depends on the loss or derangement of cellular interactions with ECM proteins.

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 regulatory functions. A number of these functions involve the oxidation of proline [Phang, 1985] via proline oxidase, a p53-regulated gene with a role in apoptosis [Donald et al., 2001; Hu et al., 2001; Maxwell and Rivera, 2003]. 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 pyrroline-5-carboxylate, which is known to mediate redox-dependent regulatory functions [Phang, 1985; Phang et al., 2001]. Additionally, pyrroline-5-carboxylate can be a source of both glutamate and ornithine. The latter has been emphasized as a source of arginine, the precursor for the cell-signaling molecule, nitric oxide.

Inhibition of prolidase by nickel may cause the accumulation of prolyl dipeptides degraded from both exogenous and endogenous proteins. 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, TNFβ, IGF-1, and bradykinin, which are processed or inactivated by dipeptidyl peptidase IV and/or its structure homologues which recognize the X-Pro N-terminus [Busek et al., 2003]. Interestingly, derivatized imidodipeptide Ala-boroPro is a potent pharmacologic inhibitor of dipeptidyl peptidase IV [Flentke et al., 1991], leading to the hypothesis that these enzymes may be inhibited by other X-Pro imidodipeptides [Hechtman, 2001]. The inhibition of prolidase by nickel in vivo, with the resulting accumulation of prolyl dipeptides, may inhibit the activation or inactivation of these cytokines and alter a variety of cellular responses.

Forlino et al. [2002] reported that fibroblasts from patients with prolidase deficiency undergo necrosis-like cell death. In those cells intracellular concentration of Gly-Pro was at least twice that of control cells. Inhibition of prolidase by Ni(II) could mimic this effect and result in disturbances in cellular metabolism.

Just how these various putative mechanisms contribute to the findings in humans with nickel exposure is not understood. However, the inhibition of prolidase by nickel may be part of the pathophysiologic spectrum resulting in fibrosis and cancer.

ACKNOWLEDGMENTS

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

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